the addition of $Cu(hn)^{+2}$, curve B, the titration is nearly identical with the one for copper(II) ion except that no precipitate is formed. The curvature in the vicinity of the end-point of curve B indicates incomplete reaction at the stoichiometric end-point. In this reaction the complex $[Cu(hen\text{-}O^-)(OH)]^0$ is evidently formed in one step. The reaction between $Cu(hn)_2^{+2}$ and hydroxyl ion, curve C, shows no definite end-point and a comparison of this curve with curves A and B indicates a total reaction of only one mole of hydroxyl ion per mole of $Cu(hn)_2^{+2}$. The initial slope, however, indicates that in the large excess of base two equivalents of hydroxyl ion per mole of $Cu(hn)_2^{+2}$ are used. This is additional confirmation that in great excess of strong base, one mole of amine is displaced from the 1:2 complex. The curve E for addition of $Cu(en)_2^{+2}$ indicates negligible reaction of this complex with the hydroxyl ion at this concentration. The curve D for the addition of the mixed complexes is evidently an average of curves C and E.

E. Potentiometric Titrations.—Titrations in which base was added to acidified solutions of the amine, $Cu(hn)^{+2}$ and $Cu(hn)_2^{+2}$ were used to calculate the acid dissociation constants of the amine salt, the stability constants of the complexes and the acid dissociation constants of the complexes. These are being reported in detail in a further publication.[9] For the formation of $Cu(hn)^{+2}$, the value of log K_1 is 10.11. For the formation of $Cu(hn)_2^{+2}$ from $Cu(hn)^{+2}$ and hn, the value of log K_2 is 7.51. For the dissociation of the proton from $Cu(hn)^{+2}$, pK_a is 7.21. These results are all in 0.5 M potassium nitrate.

Summary.—The chart shown in Fig. 6 gives a scheme which is consistent with all of the data presented here. The formulas designated V and VI are drawn to include the assumption of five-coördinate copper(II) ion but are not intended to be indicative of the structure of this ion.

Acknowledgment.—The spectra reported here were determined by Linda S. Gallo.

MORGANTOWN, W. VA.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE AND THE BUREAU OF MEDICAL RESEARCH, EQUITABLE LIFE ASSURANCE SOCIETY OF THE UNITED STATES]

Coördination Complexes and Catalytic Properties of Proteins and Related Substances. I. Effect of Cupric and Zinc Ions on the Hydrolysis of *p*-Nitrophenyl Acetate by Imidazole[1]

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RECEIVED FEBRUARY 8, 1958

The advantages of combining different methods, particularly kinetic and equilibrium techniques, for assessing the reactivity of polar groups in proteins are described. The model system containing cupric and zinc chlorides, imidazole and imidazolium chloride has been studied simultaneously by two independent techniques that measure the concentration of free basic imidazole quantitatively. The first technique depends on the catalysis by imidazole of the hydrolysis of *p*-nitrophenyl acetate (NPA); the second technique involves measurement of the *p*H of the solution. Over a wide range of conditions the two techniques of measurement do not interfere with one another. The *p*-nitrophenolate ion released on hydrolysis of NPA combines with zinc and cupric ions about 100 times less strongly than does imidazole. Previous observations on the rate of decomposition of N-acetylimidazole are confirmed, and zinc ions are shown to have no detectable effect on the rate of decomposition. The method of Scatchard is applied to the computation of the successive association constants in the Cu(II)- and Zn(II)-imidazole systems, with results that differ only slightly from those reported previously.

Detailed knowledge of the reactivity of the polar side-chain and terminal groups of proteins is necessary for an understanding of their structure and function. At least three general approaches may be followed for assessing the reactivity of a group or class of groups: (1) the preparation of stable derivatives under defined conditions; (2) the measurement of equilibria in which the group in question takes part, for example, equilibria with cations; (3) the measurement of the catalytic activity of the group in question.

The third approach has been pursued least vigorously, except for studies specifically dealing with the catalytic properties of the highly specialized "active centers" in enzymes. We have undertaken an extensive investigation of the applicability of the catalytic method for the general assessment of the reactivity of groups in proteins. Knowledge of the catalytic potentialities of a series of model compounds containing these groups may lead also to a better understanding of enzymatic activity.

The catalytic activities of proteins are not necessarily limited to well-recognized enzymic functions. We may expect that the side-chain and terminal groups in proteins may be able to catalyze reactions according to the characteristics of comparable groups in small molecules. Presumably these catalytic effects may be assignable to the action of individual groups or of clusters of groups acting together.

The catalytic approach cannot be expected to circumvent the problem of the overlapping reactivities of the various classes of groups in proteins; for example, many of these types of groups are potentially active in general basic catalysis. Therefore, to be exploited most effectively the catalytic approach should be used in conjunction with the

(1) This investigation was supported by research grant No. H-2739 from the National Heart Institute, U. S. Public Health Service.

(2) Lederle Medical Student Research Fellow, Cornell University Medical College, 1957.

(3) Holder of a Summer Research Fellowship under a grant from the U. S. Public Health Service to Cornell University Medical College, 1957.

other measures of reactivity that have been mentioned.

In principle the simultaneous application of kinetic and equilibrium measurements should have particular advantages. First, these two techniques are the best suited for avoiding permanent changes in the properties of a protein.[4] Second, the two separate types of measurement may depend on somewhat different facets of the reactivities of the groups in a protein. Consequently, if both kinetic and equilibrium measurements are made on the same series of mixtures of protein and metal ion, then the resulting information about metal binding, *p*H and catalytic activity should be sufficient to define the reactivities of the several classes of groups with much greater certainty than could be expected from either type of measurement alone.

Clearly, any such interpretation of combinations of measurements depends on the lack of interference of one technique with the other. Before attempting to test this combined approach on proteins we have studied a model system containing imidazole, *p*-nitrophenyl acetate (NPA) and zinc chloride or cupric chloride. The catalysis by imidazole of the hydrolysis of NPA has been studied in detail,[5-7] and the formation of complexes of imidazole with Zn(II) and Cu(II) has been studied by the measurement of *p*H values in mixtures at equilibrium.[8] The test of compatibility of the two approaches is to show that (1) the presence of these metal ions does not upset the linear relation between catalytic activity and concentration of the free basic imidazole, and (2) the presence of NPA or its reaction products does not upset the measurement of complex formation by the *p*H method. It will be shown below that these two tests of compatibility are met successfully in this system.

Materials and Methods

Materials.—Imidazole (m.p. 89.5–90.5°; lit. 89.5–90°)[9] obtained from Edcan Laboratories, South Norwalk, Connecticut, was used without further purification, after drying for several days over P_2O_5. Imidazole hydrochloride was prepared by dissolving 1.382 g. of imidazole in 100 ml. of 0.203 *N* HCl. The *p*H was adjusted to 3.86–3.90 by the addition of approximately 0.2 ml. of 1 *N* HCl. The theoretical *p*H of a 0.203 *M* solution of imidazole hydrochloride ($pK' = 7.08$) is 3.89.

p-Nitrophenyl acetate (NPA) was prepared by the method of Spasov[10] as described by Bruice and Schmir.[6] The NPA was dissolved in 95% ethanol and diluted with water to give a final concentration of 1.0×10^{-3} *M* in 1.90% ethanol–water (v./v.). A fresh solution was prepared daily.

Cupric chloride solutions were standardized iodometrically.[11] Zinc chloride solutions were standardized by back-titration according to Biedermann and Schwarzenbach,[12] using ethylenediamine tetraacetate (EDTA).

Kinetic Method.—The following general procedure was used in the kinetic determinations. Two tubes were prepared containing identical mixtures of imidazole, imidazolium chloride, metal salt (as required) and sufficient NaCl to maintain a constant ionic strength of 0.16 during the reaction. The two tubes, along with a solution of NPA were equilibrated in a constant temperature bath at 25.1 ± 0.1°. To one of the tubes was added water (1 part per 9 parts by volume), then the solution was mixed and transferred to a cuvette which was placed in the spectrophotometer as a blank solution. To the second tube was then added the same volume of the NPA solution. This addition was made rapidly from a calibrated syringe and the contents of the reaction tube were thoroughly mixed, transferred to a cuvette and quickly placed in the spectrophotometer. The first reading usually was obtained within 1 minute; during the faster reactions a reading was taken after 30 seconds. Unless specified otherwise, the temperature of the thermostated cell compartment of the Beckman Model DU Spectrophotometer was maintained at 25.5 ± 0.5° by circulating water from a constant temperature bath passing through dual thermospacers. The *p*H values of both the blank solution and reaction mixture were measured at the conclusion of the kinetic run, after the lapse of at least 8 half-lives.

The rate of hydrolysis was determined by following either the rate of appearance of *p*-nitrophenolate ion (NP^-) at 400 mμ, or the rate of disappearance of NPA at 273 mμ. In following the rate of appearance of NP^- it is necessary to maintain a constant concentration of hydrogen ion since the absorption at 400 mμ measures only the NP^- in an equilibrium mixture with *p*-nitrophenol. The imidazole catalyst itself was always in sufficient concentration to maintain a constant *p*H during the reaction. NPA and NP^- were shown to follow Beer's law over the ranges of concentration used.

The first-order rate constants k_{obs} were obtained from the rate of appearance of NP^- using the relation

$$2.303 \log \frac{O.D._\infty - O.D._0}{O.D._\infty - O.D._t} = k_{obs}\, t$$

and from the disappearance of NPA using the relation

$$2.303 \log \frac{O.D._0 - O.D._\infty}{O.D._t - O.D._\infty} = k_{obs}\, t$$

where t = reaction time in minutes; $O.D._0$ is the optical density at $t = 0$, which was obtained by extrapolation; $O.D._\infty$ is the optical density at infinite time, determined after a reaction time of at least 8 half-lives had elapsed; and $O.D._t$ is the optical density at any time after the start of the reaction. Normally the reactions were followed continuously to 50% of completion. For reactions having half-lives greater than 6 hours the $O.D._\infty$ was taken from standard absorption curves of *p*-nitrophenol, and the reaction was followed to 20% of completion.

Measurement of *p*H.—A Radiometer Titrator Type TTT1a obtained from Welwyn International, 3355 Edgecliff Terrace, Cleveland 11, Ohio, was used. Some of the earlier measurements were carried out on solutions at room temperature (27–30°); however most solutions were thermostated in a water-jacketed cell at 25.1 ± 0.1°. Unless stated otherwise all measurements reported in the tables were made at 25.1 ± 0.1°. The *p*H measurements were regularly reproducible to within ±0.01 unit. The instrument was calibrated before and after each measurement by using standard Beckman buffer solutions of *p*H 4, 7 and 10. All *p*H readings were corrected by linear interpolation.

Results

Catalysis by Imidazole.—The observed first-order rate constants were taken to be equal to $k_1 + k_w$, where k_1 is the first-order rate constant for catalysis by basic imidazole of the hydrolysis of NPA and k_w the rate constant for hydrolysis in a solution at the same *p*H, temperature and ionic strength in the absence of imidazole. Values of k_w were computed as follows. Rates of hydrolysis were measured in the presence of a series of acetate, phosphate and borate buffers containing sufficient NaCl to maintain the ionic strength at 0.16. For

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the more alkaline buffers the rates were measured by following the appearance of *p*-nitrophenolate (NP^-) at 400 mμ. The observed rates were plotted as a function of *p*H for each ionic strength. Then for each of a series of *p*H values the interpolated rates were plotted *vs.* ionic strength due to buffer and k_w was obtained by extrapolation to zero ionic strength. Near *p*H 8.0 the measurements were made in both phosphate and borate buffers and led to practically identical values of k_w. The results for the *p*H range 4.5 to 9.0 are shown in Fig. 1 in which k_w is shown as a function of *p*H. The inset in Fig. 1 shows the linear relation between k_w and the concentration of hydroxyl ion over the range of the latter between 1×10^{-7} and 60×10^{-7} *M*. In none of the cases reported in this study did k_w exceed 5% of the value of k_1.

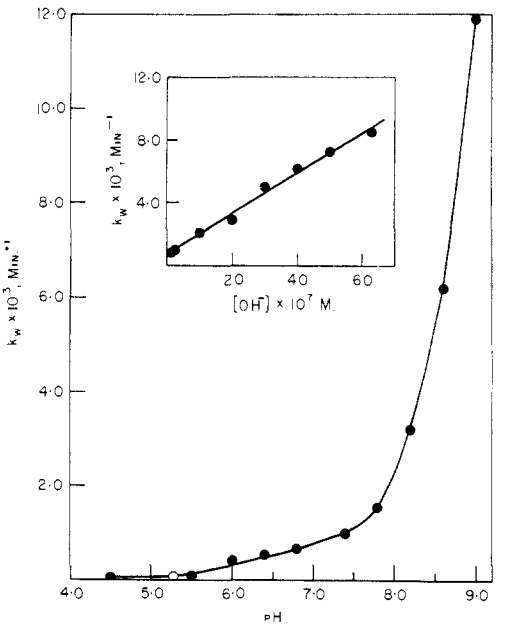

Fig. 1.—Dependence of k_w on *p*H. ●. The open circle shows the rate constant measured in the presence of 0.01 *M* $CuCl_2$ or $ZnCl_2$. Inset: dependence of k_w on (OH^-).

Values of k_1 obtained over a wide range of concentrations of imidazole and of imidazolium chloride are shown in Fig. 2 plotted against the concentration of basic imidazole, (Im). The measurements were made by following the disappearance of NPA at 273 mμ. The concentration of imidazole ranged from 0.000084 to 0.0090 *M*, and of imidazolium chloride from 0.0180 to 0.0900 *M*. By contrast the initial concentration of NPA was 1.0×10^{-4} *M*, so that the quantity of acid released during the hydrolysis was almost always much less than the concentration of basic imidazole, and could not be expected to affect the *p*H of the imidazole buffers by as much as 0.01 unit. This prediction was verified experimentally. One exception was observed at the extreme condition of lowest (Im), 0.000084 *M*. Here the *p*H of the control was 4.84 whereas at the end of the experiment it was 4.75. In a case of this sort the initial rate and initial *p*H must be used for the computations.

The slope of the line in Fig. 2 may be defined as

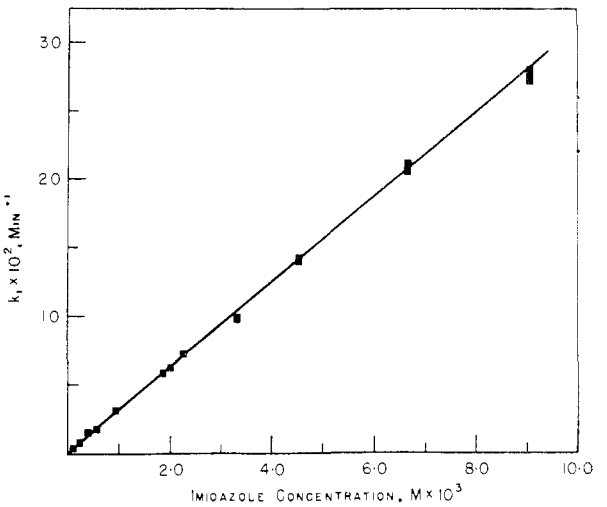

Fig. 2.—Dependence of k_1 on concentration of imidazole. The rectangles indicate the probable errors of the measurements.

the second-order rate constant, k_2.[13] The value of k_2 obtained here at 25.5 ± 0.3° and ionic strength 0.16 in 0.2% ethanol is 31.3 l./mole min. and may be compared with the values obtained by other workers: 12.7 l./mole min. at 25° and ionic strength 0.016 in 28.5% ethanol (v./v.)[6]; and approximately 28.2–29.0 l./mole min. at 26.2° and ionic strength 0.002–0.08 in 5% dioxane–water.[5] Presumably these differences may be ascribed to the differences in solvents used.

The relation between k_1 and (Im) shown in Fig. 2 is used below to estimate values of (Im) from rate measurements in the presence of Cu(II) and Zn(II). During reactions with the longest half-lives, corresponding to (Im) below 0.0004 *M*, the temperature showed a tendency to rise. Temperatures ranged between 26.0 and 26.5° instead of 25.2 and 25.8°. This effect introduced a very slight curvature (corresponding to an increase in k_2 of 8–10%) into the lowest extreme of the standard curve, not shown in Fig. 2. This dependence of k_2 on temperature is in keeping with the observations of Bruice and Schmir.[6]

Measurement of Free Basic Imidazole in the Presence of Cu(II) and Zn(II): (A) Kinetic Measurements.—The finding of a constant value of k_2 at constant temperature and ionic strength in the face of wide variations in the concentration of the imidazolium ion confirms that the latter does not act as a catalyst in this system.[5,6] Accordingly, it is to be expected that imidazole combined in complexes with other cations such as Cu(II) and Zn(II) will not act catalytically, and that kinetic measurements on mixtures containing such metal ions may be correlated solely with the concentration of free basic imidazole, (Im). Table IA shows the results of kinetic measurements performed on Cu(II)–imidazole mixtures in the manner already described. Besides imidazole, imidazolium chloride and NaCl, the solutions contained Cu(II) chloride in the quantities listed. Corresponding results for mix-

(13) Over the *p*H range covered in this study the proportion of imidazole in the anionic form is too small to make a detectable contribution to the catalysis.[7]

TABLE I

CATALYSIS OF HYDROLYSIS OF NPA BY IMIDAZOLE IN THE PRESENCE OF Cu(II)

Composition: Initial total molar concn. of $CuCl_2$	HIm$^+$Cl$^-$	Im	A. Kinetic results: $k_1 \times 10^2$, min.$^{-1}$	−log (Im)	$\bar{\nu}$	B. Equilibrium results: pH	pH of blank soln.	−log (Im)	$\bar{\nu}$
0.01793	0.0180	0.00360	0.103	4.65	0.200	3.99	3.99	4.84	0.202
.00896	.0180	.00360	.162	4.39	.397	4.33	4.33	4.50	.398
.00896	.0180	.00540	.212	4.25	.596	4.61	4.61	4.22	.595
.00896	.0180	.00720	.346	4.02	.793	4.81	4.81	4.00	.792
.00896	.0180	.00900	.497	3.85	.989	4.96	4.96	3.87	.99
.00896	.0180	.01080	.610	3.76	1.19	5.12	5.12	3.71	1.18
.00896	.0180	.01260	.880	3.60	1.38	5.24	5.26	3.59	1.38
.00896	.0180	.01440	1.26	3.44	1.52	5.37	5.37	3.45	1.57
.00896	.0180	.01620	1.76	3.29	1.75	5.53	5.53	3.29	1.75
.00896	.0180	.01800	2.20	3.16	1.93	5.67	5.67	3.15	1.93
.000944	.01923	.000946	0.426	3.93	0.878	4.85	..	3.95	0.884
.000944	.01923	.00237	2.14	3.18	1.80	5.57	5.58	3.23	1.88
.000896	.01827	.00360	4.34	2.86	2.46	5.98	6.00	2.84	2.40
.000896	.01827	.00405	5.83	2.75	2.51	6.05	6.07	2.77	2.62
.000896	.01827	.00450	6.84	2.66	2.60	6.14	6.15	2.68	2.68
.000896	.01827	.00540	9.35	2.53	2.72	6.26	6.27	2.56	2.94
.000896	.01827	.00720	14.5	2.34	2.90	6.47	6.47	2.35	3.03
.000944	.01923	.00142	0.895	3.59	1.28	5.14	5.16	3.66	1.27
.000893	.0180	.01125	25.2	2.09	3.57	6.73	6.73	2.10	3.60

TABLE II

CATALYSIS OF HYDROLYSIS OF NPA BY IMIDAZOLE IN THE PRESENCE OF Zn(II)

Composition: Initial total molar concn. of $ZnCl_2$	HIm$^+$Cl$^-$	Im	A. Kinetic results: $k_1 \times 10^2$, min.$^{-1}$	−log (Im)	$\bar{\nu}$	B. Equilibrium results: pH	−log (Im)	$\bar{\nu}$
0.0531	0.00203	0.00100	0.184	4.34	0.018	5.45[a]	4.28	0.018
.0496	.01015	.0100	2.00	3.24	.190	5.75[a]	3.28	.191
.0496	.01015	.0200	4.14	2.90	.378	6.01[b]	2.96	.381
.00294	.0203	.00250	3.92	2.93	.449	5.87	2.90	.425
.00196	.0203	.00250	4.72	2.85	.551	5.93	2.84	.541
.0496	.01015	.0300	5.21	2.84	.575	6.17[a]	2.86	.576
.0248	.01015	.0200	6.31	2.72	.729	6.27[a]	2.76	.737
.00294	.0203	.00500	7.62	2.63	.898	6.15	2.62	.891
.00196	.0203	.00500	9.06	2.54	1.06	6.22	2.55	1.12
.00098	.0203	.00500	11.9	2.43	1.33	6.32	2.45	1.49
.00294	.0203	.0100	14.8	2.33	1.80	6.43	2.34	1.85
.00294	.0203	.0100	15.0	2.32	1.77	6.43	2.34	1.85
.00245	.0203	.0100	15.9	2.29	2.00	6.48	2.29	2.00
.00196	.0203	.0100	18.6	2.23	2.07	6.53	2.24	2.18
.00196	.0203	.0100	18.4	2.23	2.10	6.52	2.25	2.24
.00147	.0203	.0100	20.9	2.18	2.26	6.58	2.20	2.51
.00098	.0203	.0100	24.0	2.11	2.36	6.63	2.14	2.86
.00098	.0203	.0100	23.4	2.13	2.56	6.64	2.13	2.68
.00049	.0203	.0100	26.9	2.07	2.84	6.70	2.07	3.14

[a] Measured at 27°; pK' 7.04. [b] Measured at 30°; pK' 6.98.

tures containing Zn(II) chloride are shown in Table IIA. As before, 1×10^{-4} M NPA was initially present during the measurements. The kinetics of the disappearance of NPA were followed at the wave length of 273 mμ.

The values of k_1 listed were obtained after taking appropriate values of k_w from Fig. 1, using the pH values determined as described below. That k_w is unaffected by the presence of Cu(II) or Zn(II) was shown by measurements in acetate buffers similar to those used for the determination of k_w. Zinc chloride or cupric chloride (0.01 M) was substituted for part of the NaCl in the buffers containing 0.015 M sodium acetate, total ionic strength 0.16. The observed rates of hydrolysis of NPA were indistinguishable from those found in the absence of divalent metal ion. The open circle in Fig. 1 shows rates obtained in the presence of Cu(II) or Zn(II).

(B) **Equilibrium Measurements.**—After the kinetic measurements had been followed to completion, the solutions were transferred to thermostated vessels for measurement of the equilibrium pH values at 25.1 ± 0.1°. The results are listed in Tables IB and IIB. These pH values represent a second measure of the concentration of free basic imidazole: taken together with the concentrations of imidazolium chloride used and the measured value of pK' for imidazole under these conditions (7.08), they yield values for (Im) in each solution studied.[8]

The accuracy of the values for the concentrations of free imidazole derived from the pH measure-

ments is not measurably affected by the presence of the hydrolysis products of NPA, as shown by measurements of the *p*H values of the blank solutions that were used in conjunction with the spectrophotometric measurements (Table IB). The *p*H values in the presence and absence of the hydrolysis products were usually identical and did not differ by more than 0.02 unit. The absence of a disturbing effect may be attributed to the low concentration of NPA used, as already pointed out; and also to the fact that, as shown in separate measurements described below, the first association constants for both Cu(II) and Zn(II) with NP^- were of the order of 1/100th that of the corresponding constants with imidazole.

Having computed values of (Im) by the two totally independent methods, we may now compare them. The comparison may be made for each condition shown in Tables I and II by comparing columns 5 and 9, and 5 and 8, respectively. The agreement between the two methods demonstrates the correctness of the hypothesis mentioned earlier that imidazole combined in complexes with Cu(II) or Zn(II) did not act catalytically to a measurable extent. Over the ranges of conditions covered the distribution of the metal ions among the different consecutive complexes, MIm^{++}, MIm_2^{++}, etc., was varied widely,[4,14] and it is unlikely that active catalysis by any of the series of complexes could have gone undetected.

Complexes of *p*-Nitrophenolate with Cu(II) and Zn(II).—The close agreement between the *p*H method and the catalytic method for determining (Im) indicates that the nitrophenolate ion, NP^-, liberated during the reaction does not combine with Zn(II), Cu(II) or their imidazole complexes sufficiently strongly to alter detectably the equilibrium between these cations and imidazole. This conclusion was verified experimentally by titrating 0.0072 *M* *p*-nitrophenol, NP, in the presence of 0.0049 *M* Zn(II), and 0.0504 *M* NP in the presence of 0.0050 *M* Cu(II), using NaOH at 25.1 ± 0.1° and ionic strength 0.16 maintained by the addition of NaCl. It should be noted that the total concentration of NP in these experiments far exceeds the maximum concentration of total NP, 1.0×10^{-4} *M*, liberated in the catalytic experiments. With Zn(II) the binding was so weak that the *p*H of solutions containing Zn(II) was in the extreme case only 0.05 *p*H unit lower than in a comparable solution without the cation. With Cu(II) the binding was somewhat stronger, and resulted in *p*H differences of as much as 0.3 unit. The bound NP^- was computed to be as much as 3% of the total Zn(II) present, and as much as 31% of the total Cu(II) present. Higher degrees of binding could not be studied because of the precipitation of hydroxides.

On the assumption that only the first complex was formed to an important extent, the expression for the first association constant was solved for each experimental point and the value of log $\mathbf{k}_1$ obtained by extrapolation to zero degree of binding. Values of log $\mathbf{k}_1$ of 0.3–0.4 and of 1.7 were obtained for the binding of NP^- to Zn(II) and to Cu(II), respectively. It is apparent that NP^- is bound to these cations less than one-hundredth as strongly as is imidazole.

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Hydrolysis of N-Acetylimidazole.—The results presented thus far indicate that Zn(II) and Cu(II) ions do not interfere with the over-all reaction between NPA and imidazole except to decrease the rate by lowering (Im). However, kinetic measurements of the rate of disappearance of NPA or of appearance of NP^- would not be expected to reflect the rate of hydrolysis of N-acetylimidazole, an intermediate which is thought to occur during the reaction.[5,6,15] Moreover, in δ-chymotrypsin transacetylation involving an imidazole group and the primary alcohol of the seryl residue has been postulated.[16] Thus the intermediate formed in the present reaction should be a potential acetylating agent whose stability merits study, and accordingly we have confirmed previous studies of the effect of *p*H on its hydrolysis and have searched for an effect of Zn(II) ions on the rate of this hydrolysis.

The N-acetylimidazole was formed rapidly by mixing a large concentration of free imidazole, 0.0090 *M*, with 1.0×10^{-4} *M* NPA. The formation and hydrolysis of this intermediate was followed by measuring the optical density at 245 mμ, the wave length of its maximum absorption.[17] Under the conditions used the splitting of NPA was more than 99% complete in 16 minutes, after which time the observed decrease in optical density at 245 mμ may be attributed solely to the decomposition of the intermediate. Figure 3 shows the formation and disappearance of N-acetylimidazole at several *p*H values. The first-order rate constants for the hydrolysis of N-acetylimidazole, computed from the change in optical density at 245 mμ after 16 minutes had elapsed, are shown in Fig. 4 as a function of the *p*H of the solution. Above *p*H 6.0 the *p*H was varied by keeping (Im) constant at 0.0090 *M* and adjusting the concentration of imidazolium ion. Below *p*H 6.0, where imidazole is a poor buffer, the *p*H was varied by diluting 9 parts of the reaction solution with 1 part of an appropriate acetate buffer of *p*H between 4.50 and 5.28, ionic strength 0.20, after the reaction had proceeded 16 minutes. The observed rate constants are in good agreement with those reported by Stadtman.[17]

The rate of hydrolysis of N-acetylimidazole in the presence of Zn(II) was studied by incorporating 0.25 *M* Zn(II) chloride in the acetate buffers used for adjusting the *p*H. The final concentration of Zn(II) ions, 0.025 *M*, was in large excess over the imidazole present, 0.0081 *M*. As shown in Fig. 4, open circles, Zn(II) ions do not affect the stability of N-acetylimidazole under these conditions. Comparable studies with Cu(II) ions could not be carried out because of the high absorption at 245 mμ of Cu(II)–imidazole complexes.

Association Constants for Complexes of Imidazole with Cu(II) and Zn(II) Ions.—The results of the present investigation may be used to make new

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(16) G. H. Dixon and H. Neurath, THIS JOURNAL, **79**, 4558 (1957).

(17) E. R. Stadtman, in "The Mechanism of Enzyme Action," (W. D. McElroy and B. Glass, eds.), The Johns Hopkins Press, Baltimore, Md., 1954, p. 581.

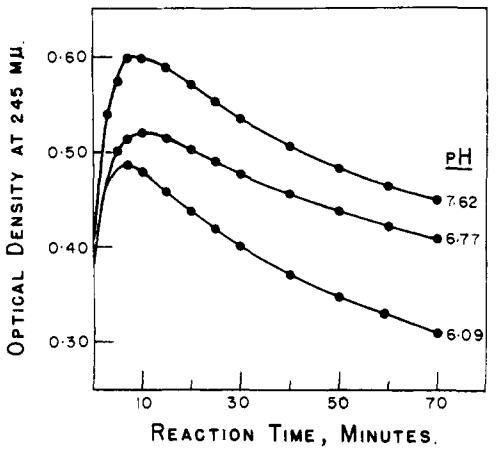

Fig. 3.—Course of formation and decomposition of N-acetylimidazole at *p*H 6.09, 6.77 and 7.62 as shown by a plot of optical density at 245 mμ *vs.* reaction time in minutes.

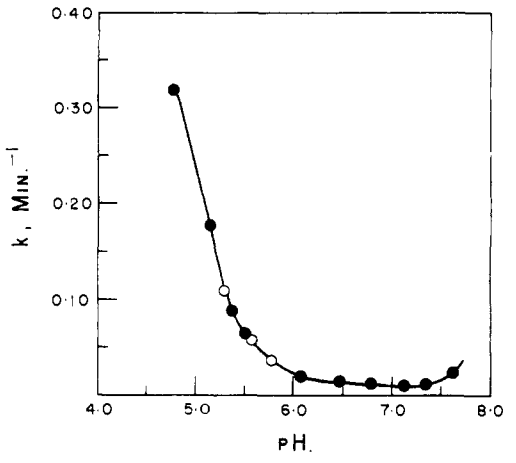

Fig. 4.—Dependence of rate constant for the decomposition of N-acetylimidazole on *p*H. The open circles represent measurements made in the presence of 0.025 *M* $ZnCl_2$.

computations of the values of the successive association constants in the Cu(II)- and Zn(II)-imidazole systems by the method of Scatchard used previously.[8,14] In Tables I and II are listed the computed values of $\bar{\nu}$, the average number of imidazole molecules bound per mole of metal ion present. Values derived from each method of measurement are listed in the appropriate parts of the tables.

The data in Tables I and II have been analyzed by the method of Scatchard from plots of log Q *vs.* $\bar{\nu}$ where

$$Q = \bar{\nu}/(4 - \bar{\nu})(\mathrm{Im})$$

These plots are shown in Fig. 5. Points derived from the equilibrium measurements are shown in full circles, those from kinetic measurements in open circles. In Fig. 5a are shown the points for the system containing Cu(II), and in Fig. 5b those for the system containing Zn(II). The curves were constructed using the log κ values shown in Table III. These values were chosen to fit the points derived from *p*H measurements because the latter were made under conditions of better control of temperature than proved possible with the kinetic measurements. For example, the kinetic measurements on the Zn(II)-system were made at 26.0–26.5° instead of at 25.2–25.8°, and the slightly low values of log Q in Fig. 5b are the expected consequence. The effect of temperature on the kinetic measurements *per se* was taken into account by using the standard curve made under comparable conditions, but of course the association constants for the Zn(II)-imidazole complexes are slightly lower at the higher temperature.[8] Using

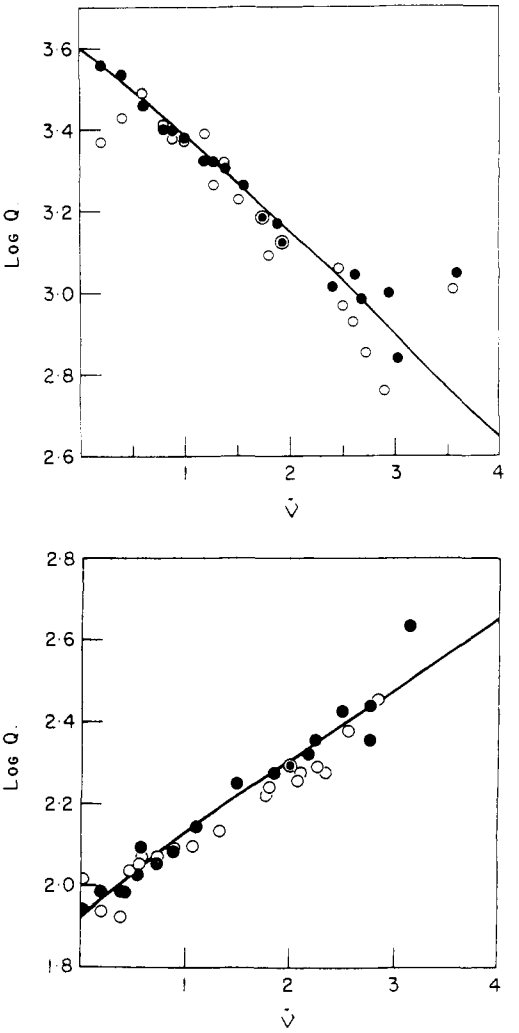

Fig. 5.—Plots of log Q *vs.* $\bar{\nu}$; the curves are computed using the values of κ listed in Table III and are intended to fit most closely the points derived from *p*H measurements at 25.1° and ionic strength 0.16, shown in closed circles, ●. Points derived from kinetic measurements are shown in open circles, ○: (a) Cu(II)-imidazole, (b) Zn(II)-imidazole.

TABLE III

COMPUTED INTRINSIC ASSOCIATION CONSTANTS FOR Cu(II) AND Zn(II) WITH IMIDAZOLE

Log κ values for ionic strength 0.16 and 25°.

	Cu(II) complexes	Zn(II) complexes
log κ_1	3.60	1.92
log κ_2	3.24	2.14
log κ_3	3.06	2.50
log κ_4	2.65	2.65

the usual statistical relations[4,8]

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

the log κ values have been converted to the log **k** values listed in Table IV. Included for comparison are log **k** values interpolated from those computed previously.[14]

TABLE IV

SUCCESSIVE ASSOCIATION CONSTANTS FOR Cu(II) AND Zn(II) WITH IMIDAZOLE

Log k values for ionic strength 0.16 and 25°.

	Cu(II) complexes		Zn(II) complexes	
	Former[a]	Present	Former[a]	Present
log k_1	4.33	4.20	2.57	2.52
log k_2	3.54	3.42	2.36	2.32
log k_3	2.82	2.88	2.22	2.32
log k_4	2.03	2.05	2.01	2.05

[a] Y. Nozaki, F. R. N. Gurd, R. F. Chen and J. T. Edsall, THIS JOURNAL, 79, 2123 (1957).

The method of Scatchard for estimating successive constants was chosen in preference to that of Bjerrum because it yields a value of k_1 that is probably more precise. If this type of study is to be useful as a model for the reactivity of imidazole groups in proteins it is usually the value of k_1 that must be estimated most accurately.[4] The present values for k_1 are probably more accurate than those previously reported, but the differences are not marked. Comment has been made previously on the errors involved in arriving at the other successive constants.[8,14] In the present study the higher values of $\bar{\nu}$ have not been explored because the technique that we have used does not allow accurate measurements of the kinetics when the half-life approaches one or two minutes. The value of k_4 is therefore rather uncertain. Nevertheless, the logarithm of the product of the constants for the Cu(II)-system agrees well with the value of 12.6 determined polarographically by Li, White and Doody.[18]

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Discussion

The foregoing results show that the kinetic and equilibrium methods of measuring the concentration of basic imidazole are mutually compatible. The presence of Cu(II) or Zn(II) ions does not affect the kinetic measurements, and the presence of NPA or its hydrolysis products does not affect the equilibrium measurements detectably. Studies to be reported later have shown a similar compatibility of the measurements when the imidazole radical is incorporated in the histidyl residue of a peptide.

In terms of their potential role in assessing the reactivity of imidazole groups in peptides and proteins, the two methods show an interesting contrast in characteristics. The kinetic method depends for its usefulness on the fact that it does not alter a condition of equilibrium, whereas the equilibrium method depends on the alteration of such a condition of equilibrium and on our ability to describe the new multiple equilibrium quantitatively in terms of the mass law.

The nucleophilic properties of the basic imidazole group resemble those of classes of groups in proteins, such as amino and phenoxyl groups,[19,20] and probably others. It is to be anticipated, therefore, that other classes of groups in proteins in addition to imidazole groups may react with NPA. Whether or not the products of the reaction are stable or unstable, the combined approach described here should be directly applicable whenever relatively minute quantities of NPA are employed.

Acknowledgments.—The authors are grateful to Dr. Thomas C. Bruice for a gift of *p*-nitrophenyl acetate and for making available unpublished results. Dr. George Scatchard was kind enough to offer suggestions concerning the manuscript. The technical assistance of Miss Reta Roth is gratefully acknowledged.

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Spectrophotometric Investigation in the Near Ultraviolet of the Cobalt(II) Monothiocyanato Complex

BY PASCHOAL SENISE AND MADELEINE PERRIER

RECEIVED MARCH 19, 1958

Use has been made of the absorption band in the near ultraviolet of the $CoSCN^+$ ion to determine the formation constant of this ion in acid aqueous solutions of unit ionic strength, by two independent methods. At *p*H 3.0 and 25 ± 1 the average value of that constant obtained by the method of McConnell and Davidson was found to be 10.09 ± 0.12 and by the method of corresponding solutions of Bjerrum 10.38 ± 0.07.

Introduction

The nature of aqueous solutions containing cobaltous and thiocyanate ions has been investigated spectrophotometrically by several authors.[1-4] Consecutive formation of complexes was found to occur, the first species formed being the cation $CoSCN^+$. The value of the formation constant of this ion reported by Lehné[2] is very uncertain—as stated by the author—since its calculation was based on the rather small difference of the molar extinction coefficients of the complex and cobalt(II) ions in the visible range of the spectrum. A dif-

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(3) A. K. Babko and O. F. Drako, *Zhur. Obshchei Khim.*, **20**, 228 (1950); *C. A.*, **44**, 5684 (1950).

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