the basis of a simple consideration of electrostatic interactions. According to the Bjerrum formulation,[20] the electrostatic contribution to the free energy of hydrolysis of BTG should differ from that of BTGA by the quantity

$$\Delta(\Delta F_{\text{elect}}) = \frac{-N\epsilon^2}{rD} \qquad (7)$$

where N is Avogadro's number, ϵ is the protonic charge, D is the dielectric constant of the medium and r is the distance between the charges in the glycine zwitterion. Differentiation with respect to temperature gives

$$\Delta(\Delta H_{\text{elect}}) = \frac{-N\epsilon^2}{rD}\left(1 + \frac{\mathrm{d}\ln D}{\mathrm{d}\ln T}\right) \qquad (8)$$

Since d ln D/d ln T for water at 25° is −1.37, the enthalpy difference should be a positive quantity. With $r = 3 \times 10^{-8}$ cm., $\Delta(\Delta H_{\text{elect}}) = 520$ cal. per mole, which is about twice as large as the observed effect.[21]

(20) N. Bjerrum, *Z. physik. Chem.*, **106**, 219 (1923).

(21) It is well known that use of the bulk dielectric constant of the solvent in calculations involving dipolar ions is at best a rough approximation. See for, example, C. Tanford and J. G. Kirkwood, *J. Am. Chem. Soc.*, **79**, 5333 (1957).

[CONTRIBUTION FROM THE CLAYTON FOUNDATION BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS, AUSTIN, TEXAS]

The Inhibition of Urease by Metal Ions at *p*H 8.9

BY WILLIAM H. R. SHAW[1] AND DILIP N. RAVAL

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Urease is inhibited by these various ions arranged in order of decreasing toxicity: Cu(II), Zn(II), Ni(II), Co(II), Fe(II) Mn(II). Metal ion inhibition is non-competitive, and the inhibition index is found to depend on the square of inhibitor concentration. Reaction of the metal ions with sulfhydryl groups removed from the catalytically active sites is postulated. Correlation of toxicity with metal sulfide insolubility and with the sum of the first and second ionization potentials of the metals is reported. Comparison also shows that the ranking of metal ions in the "natural order" coördination compound stability sequence is identical with a ranking according to relative toxicity. The effect of ionic strength on the inhibition is discussed.

Introduction

Results obtained by other investigators on the inhibition of urease by metal ions have been collected from the literature and summarized in a previous paper.[2] On the basis of these data, it was found possible to arrange the common metal ions in a tentative sequence of relative inhibitory effectiveness. From a consideration of this earlier work it became apparent that several points required clarification. The dependence of metal ion inhibition on substrate concentration, for example, had not been studied; consequently, the nature of the inhibition could not be elucidated. The problem of finding a buffer that does not interact strongly with metal ions or with the enzyme was also a difficult one to solve. It had been established,[3] however, that the products of ureolytic activity bring water solutions to a constant *p*H of 8.95 ± 0.1. Thus it is possible to perform experiments at this *p*H without added buffer salts. The present work utilizes this principle.

Experimental

The preparation of urease and general experimental techniques have been described previously.[2-4] Hydrogen sulfide was not employed to stabilize the enzyme containing solutions. Best results were achieved with urease stock preparations that had aged at least one month. The metal ion solutions were made from metal sulfates of the best available grade. J. T. Baker analyzed urea was employed, and both urea and metal salts were used without further purification. All solutions were initially brought to approximately *p*H 8.5 by addition of a dilute NaOH solution. During reaction, the products of ureolytic activity buffered the solutions to a constant *p*H[2] of 8.95 ± 0.1.

In preparing the experimental solutions for one part of a typical run, two 10 ml. volumetric flasks were employed. To each of these flasks 2 ml. of enzyme stock was added. A suitable aliquot of a solution containing the metal ion under investigation was next introduced into one flask and distilled ion-exchanged water was added to bring the level in both flasks to the fiducial mark. After gentle mixing, the two solutions were allowed to stand for 2 hr. in the refrigerator[5] and 1 hr. in the water bath at a constant temperature of 25.0°. For the kinetic runs, 1 ml. of the metal containing enzyme solution was added rapidly with mixing to a test tube holding 25 ml. of a urea solution. Also 1 ml. of the metal-free enzyme solution was similarly introduced into a second test tube containing an identical urea solution. The concentrations of metal ion and substrate in these reaction mixtures were calculated and employed in reporting results. After a measured time, reaction was stopped by addition of acid, and the concentration of ammonium ion produced was determined by the usual spectrophotometric technique.[4] The rate of urea hydrolysis in the metal containing reaction mixture was recorded as the inhibited rate, V_i. The uninhibited rate, V_u, was calculated from the urea hydrolyzed in the metal-free reaction mixture. All experiments were performed at 25°

Results

The metal ions here listed, arranged in order of decreasing toxicity, were found to inhibit urease: Cu(II), Zn(II), Ni(II), Co(II), Fe(II), Mn(II). Results obtained with Fe(II) are least reliable since some oxidation to Fe(III) during the course of the experiments probably took place. For each ion, the ratio of inhibited rate, V_i, to uninhibited rate, V_n, at a fixed inhibitor concentration was measured at various substrate concentrations (Table I). These results indicate that the inhibi-

(1) Department of Chemistry The University of Georgia, Athens, Georgia.

(2) W. H. R. Shaw, *J. Am. Chem. Soc.*, **76**, 2160 (1954).

(3) G. B. Kistiakowsky and W. H. R. Shaw, *ibid.*, **75**, 2751 (1953).

(4) (a) W. H. R. Shaw and D. N. Raval, *ibid.*, **83**, 2866 (1961); (b) G. B. Kistiakowsky, P. C. Mangelsdorf, Jr., A. J. Rosenberg and W. H. R. Shaw, *ibid.*, **74**, 5015 (1952).

(5) Experiment established that the degree of inhibition reached a constant value after the enzyme had been in contact with the metal ion solution for approximately ten minutes.

TABLE I

RATE RATIOS AT VARIOUS SUBSTRATE CONCENTRATIONS

Ion	Metal ion concn.,[a] M	Urea concn., mM	V_i/V_u[b]
Cu(II)	2×10^{-7}	3.33	0.696
		7.00	.705
		33.3	.700
		333	.702
Zn(II)	4×10^{-6}	3.33	.190
		7.00	.187
		33.3	.188
		333	.189
Ni(II)	5×10^{-5}	3.33	.279
		7.00	.272
		33.3	.272
		333	.280
Co(II)	5×10^{-6}	3.33	.633
		7.00	.641
		33.3	.641
		333	.633
Fe(II)[c]	6×10^{-5}	3.33	.578
		7.00	.580
		33.3	.583
		333	.581
Mn(II)	3×10^{-3}	3.33	.524
		33.3	.534
		333	.520

[a] For each ion at least three metal ion concentrations were studied. These data all show that the rate ratio at a fixed metal ion concentration is independent of urea concentration. For brevity, only data obtained at one metal ion concentration are reported. [b] The ratio of inhibited rate, V_i, to uninhibited rate, V_u, is given in this column. [c] In the basic solutions employed it is probable that some oxidation to Fe(III) has occurred during the run.

tion of urease by metal ions is strictly non-competitive at pH 8.9. The inhibition index

$$\phi = (V_u/V_i) - 1 \qquad (1)$$

can therefore be expressed[3,6] as

$$\phi = KI^n \qquad (2)$$

where I is the concentration of the inhibiting metal ion, n is the order, and K is the inhibition constant. In logarithmic form, eq. 2 becomes

$$\log \phi = \log K - n\,pI \qquad (3)$$

The data obtained at various inhibitor concentrations are displayed on this type of plot in Fig. 1. The x intercept, pI^*, of each line is the negative logarithm of the inhibiting metal ion concentration necessary to produce a 50% inhibition ($\phi = 1$). It is clear from eq. 3 that $\log K = npI^*$. This relationship can be used to evaluate the inhibition constants from the parameters reported in Table II.

The lines in Fig. 1 are all drawn with a slope of two. The fit is acceptable but, in the case of Co(II), the scatter makes assignment of order less certain. Data for Fe(II) are not shown in the figure. As indicated earlier, some oxidation to Fe(III) during the experiments probably occurred. An estimate of pI^* for iron is, however, included in Table II.

The inhibiting metal ions are most likely combining with a negatively charge group; consequently, a salt effect would be anticipated. Since

(6) G. B. Kistiakowsky and W. H. R. Shaw, *J. Am. Chem. Soc.*, **75**, 866 (1953).

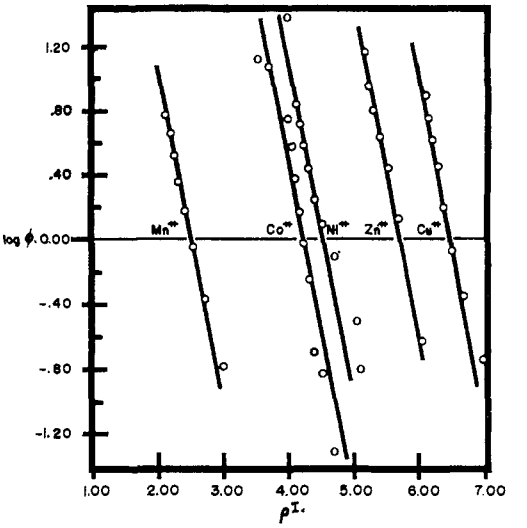

Fig. 1.—The dependence of inhibition on inhibitor concentration. The logarithm of the inhibition index, ϕ (eq. 1-3), is plotted against the negative logarithm of the corresponding molar inhibitor concentration, pI.

previous work[2] already had demonstrated that increasing ionic strength reduces ureolytic activity at high pH, experiments were performed as follows. At a fixed ionic strength obtained by the addition of salts such as $NaNO_3$ and Na_2SO_4, rate measurements were made with and without added inhibitor. In one set of experiments using 6×10^{-5} M Co(II), the extent of inhibition was reduced from 42.9% to zero by the addition of Na_2SO_4. The final ionic strength was 0.96 M.

TABLE II

THE INHIBITION PARAMETERS[a,b]

Ion	Order	pI^*
Cu(II)	2	6.50
Zn(II)	2	5.70
Ni(I)	2	4.52
Co(II)	2[c]	4.23
Fe(II)	?[d]	4.19
Mn(II)	2	2.48

[a] The order, n, and the negative logarithm of the molar inhibitor concentration needed for 50% inhibition, pI^*, are reported. [b] See eq. 3. [c] Or higher, see Fig. 1. [d] Some oxidation to Fe(III) probably occurred.

Results obtained with Cu(II) are plotted in Fig. 2. The apparent inhibition constant at first decreases and then increases as the ionic strength is increased.

Discussion

Metal ions generally are assumed to inactivate urease by reaction with a sulfhydryl group. The reaction is analogous to the formation of a metal sulfide. Application of the principle of linear free energy changes leads to the conclusion that metal ions forming the most insoluble sulfides will also be the most toxic to the enzyme.[1] Further analysis[1] indicates that toxicity, as measured by pI^*, should be a linear function of sulfide insolubility as measured by the negative logarithm of the solubility product constant. A plot of pK_{sp} against pI^* is illustrated in Fig. 3. Correlation of toxicity

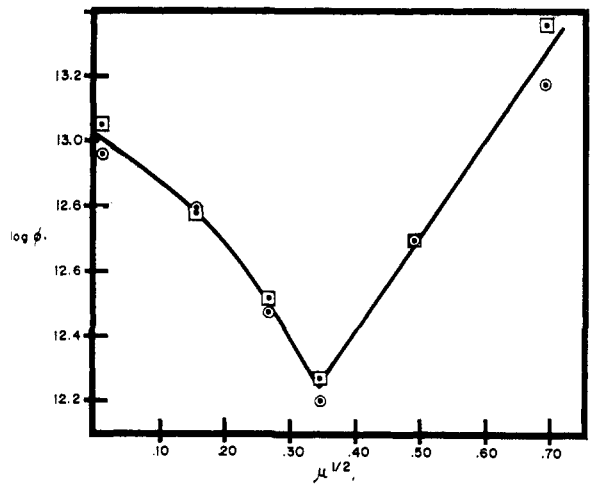

Fig. 2.—Variation of the Cu(II) inhibition constant (eq. 3) with ionic strength: circles, $NaNO_3$; squares, Na_2SO_4.

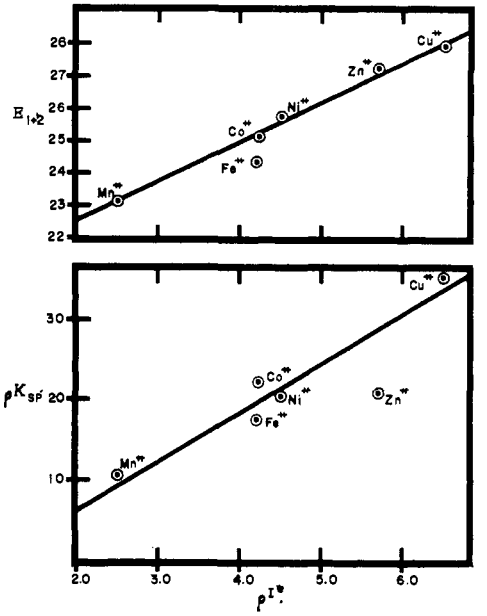

Fig. 3.—Upper plot, correlation of metal ion toxicity, pI^*, with the sum of the first and second ionization potentials, E_{1+2}, of the metals; lower plot, correlation of toxicity with metal sulfide insolubility, pK_{sp}.

with the sum of the first and second ionization potentials, E_{1+2}, of the metals was also previously demonstrated[1] using data obtained by other investigators. Reference to Fig. 3 shows that the present results can be treated similarly. It is also of interest to note that the inhibiting ions can be arranged according to relative toxicity in the order: Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II). This ranking is identical with the "natural order" stability sequence found for coördination compounds.[7-10]

(7) D. P. Mellor and L. Maley, *Nature*, **161**, 436 (1948).
(8) H. Irving and R. J. P. Williams, *J. Chem. Soc.*, 3192 (1953).
(9) R. J. P. Williams, *Biol. Rev.*, **28**, 381 (1953).
(10) R. J. P. Williams in P. D. Boyer, H. Lardy and K. Myrbäck, "The Enzymes," Vol. I, Academic Press, Inc., New York, N. Y., 1959, Chap. 9.

Since our first paper[1] appeared, other pertinent work has been published. Klotz[11] has pointed out that the affinities of metal ions for the sulfhydryl groups on serum albumin closely parallel the corresponding pK_{sp} values. Wallenfels, *et al.*,[12] have collected data on the metal ion inhibition of yeast alcohol dehydrogenase, horse liver dehydrogenase and β-galactosidase. Their findings demonstrate that the negative logarithm of the metal ion concentration necessary for a fixed degree of inhibition is linearly related to pK_{sp}. Schwarzenbach and co-workers[13] have described a similar relationship between pK_{sp} and the stability constants of the complexes formed by metal ions with mercaptoethyliminodiacetic acid. Other work[14,15] has shown that certain metal ions are most probably toxic to aquatic organisms because they combine with the essential sulfhydryl groups of key enzymes. Correlation of toxicity with pK_{sp} was again established. In considering these findings it is important to note that solubility product constants for metal sulfides obtained from different sources show wide variation. A recent paper by Waggoner[16] is devoted to a discussion of these discrepancies. In previous work[1,14,15] the data of Kapustinsky[17] and of Treadwell and Schufelsbergen[18] were utilized. These pK_{sp} values also give good correlation with the pI^* data obtained in the present research. In constructing Fig. 3, however, pK_{sp} values from another source[16] were employed. The divergent information encountered in the literature is illustrated by these examples. For ZnS, pK_{sp} values range from 26[17] to 21.[16] For CuS, values from 35[16] to 41[19] have been reported.

Sulfhydryl groups in enzymes may serve as integral parts of the catalytically active sites or as groups involved in maintaining correct structural relationships in the enzyme protein. The –SH groups on urease probably fulfill the latter function, since metal ion inhibition is non-competitive (Table I); but this conclusion must be viewed with caution. If the inhibition is even partially irreversible, non-competitive behavior can result.[20]

The dependence of ϕ on metal ion concentration raised to the second power (see Results) is noteworthy. In terms of the inhibited Michaelis–Menten mechanism,[3,6] it would imply reaction of two ions with the uncombined enzyme and with the enzyme-substrate complex. If it is assumed that each divalent metal ion can combine with two

(11) I. M. Klotz in W. D. McElroy and B. Glass, "A Symposium on Enzyme Action," The Johns Hopkins Press, Baltimore, Md., 1954, p. 267.
(12) K. Wallenfels, H. Sund, M. L. Zarnitz, O. P. Malhotra and J. Fischer in R. Benesch, R. E. Benesch, P. D. Boyer, I. M. Klotz, W. R. Middlebrook, A. G. Szent-Györgyi and D. R. Schwarz, "Sulfur in Proteins," Academic Press, Inc., New York, N. Y., 1959, pp. 218, 227, 237.
(13) G. Schwarzenbach, G. Anderegg, W. Schneider and H. Senn, *Helv. Chim. Acta*, **38**, 1147 (1955).
(14) W. H. R. Shaw, *Science*, **120**, 361 (1954).
(15) W. H. R. Shaw and B. Grushkin, *Arch. Biochem. Biophys.*, **67**, 477 (1957).
(16) W. H. Waggoner, *J. Chem. Ed.*, **35**, 339 (1958).
(17) A. F. Kapustinsky, *Doklady Akad. Nauk, S.S.S.R.*, **28**, 144 (1940); *C. A.*, **35**, 3144 (1941).
(18) W. D. Treadwell and F. Schufelsbergen, *Helv. Chim. Acta*, **29**, 1935 (1946).
(19) I. M. Kolthoff, *J. Phys. Chem.*, **35**, 2711 (1931).
(20) F. L. Hoch and B. L. Vallee in ref. 12, p. 261.

ionized sulfhydryl groups, then four –SH groups must be involved. In studies of urease inhibition by thiourea[3] and methylurea[6] a similar dependence of inhibition index on the square of inhibitor concentration was noted.

Although it seems quite reasonable to assume that metal ions inhibit urease by combination with an essential –SH group, the possibility that other important negative groups also act as ligands can not be disregarded. The excellent correspondence between the toxicity sequence and the "natural order" stability sequence noted in the first paragraph of this section is certainly not in conflict with this possibility.

The influence of ionic strength on the inhibition by Cu(II) shown in Fig. 2 is qualitatively in accord with expectations based on the assumption that Cu(II) is combining with a negatively charged group. Quantitative treatment of the data does not seem to be justified. Because of the salt effect, however, pI^* values reported in Table II would be expected to differ from pI^* values obtained at infinite dilution. The difference, in general, should be small, with the greatest deviation expected in the case of Mn(II).

Although the inhibition of urease by metal ions is non-competitive in alkaline solution, it is by no means certain that similar behavior would be observed below *p*H 7. In the case of thiourea[6] and methylurea[3] a change in the character of inhibition occurs with changing *p*H. Both of these inhibitions become competitive at *p*H 6.

Acknowledgment.—It is a pleasure to express our gratitude to the Administration of The University of Texas for the special assignment given to W. H. R. S. that provided time for research and the preparation of this paper.

[CONTRIBUTION FROM THE INSTITUTE OF MICROBIOLOGY RUTGERS, THE STATE UNIVERSITY, NEW BRUNSWICK, N. J.]

Photochemical Cleavage of Water by Riboflavin. II. Role of Activators

BY GEORGE STRAUSS AND WALTER J. NICKERSON

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The role of ethylene diamine tetraacetate and methionine in promoting the photoreduction of riboflavin and in suppressing other, irreversible, photochemical changes in the flavin was studied by spectrophotometric and chromatographic methods and by measuring the oxidation-reduction potential of riboflavin during photochemical reduction. A special absorption cell, permitting simultaneous measurements of light transmission and potential in deoxygenated solutions, was developed for this purpose. The reoxidation of dihydroriboflavin by oxygen is accompanied by conversion of part of the flavin into lumiflavin and lumichrome. These irreversible changes are inhibited by substances possessing an electronegative nitrogen or sulfur atom. In the presence of such "activators" the oxidation-reduction potentials obtained during photoreduction agree with published values obtained by reductive titration with dithionite throughout the *p*H range from 4 to 10; without activator, this agreement is limited to *p*H 4 to 5. It is concluded that, both in the absence and presence of activator, the hydrogen donor is not the ribityl side chain but water which is hydrogen-bonded to the riboflavin. Activators form hydrated complexes with riboflavin. On illumination the complex dissociates, resulting in the formation of dihydroriboflavin and the oxide of the activator. Due to its complexing action, the activator: (1) weakens the O—H bond of complexed water, thus raising the quantum yield of its photodecomposition, (2) serves as acceptor for the oxygen moiety of the water and (3) protects the ribityl group of riboflavin from irreversible degradation by reactive radicals arising during the reoxidation of dihydroriboflavin by oxygen.

Introduction

It has been shown[1] that illumination of an air-free riboflavin solution, in the presence of an "activator," such as methionine, results in the formation of leucoriboflavin and methionine sulfoxide. In addition to its reversible photochemical conversion to the leuco-form, riboflavin may undergo irreversible light-induced changes, *i. e.*, conversion to lumichrome and lumiflavin.[2,3] Thus, photoreduction of riboflavin is accompanied by photodegradation, except in the presence of a relatively high concentration of activator. The results obtained with methionine as activator show this substance to act, not as a hydrogen donor, but as an acceptor for the oxygen moiety derived from water. Other activators, including ethylenediamine tetraacetate (EDTA), appear to function in a similar manner. The role of such activators now has been further characterized by a study of their protective action against irreversible degradation of the flavin and of their stoichiometry in systems containing riboflavin, activator and a third (reducible) substance.

The course of the photoreduction, as distinct from the accompanying photodegradation, was followed by making simultaneous measurements of the potential and of the flavin concentration and by spectral and chromatographic studies before and after the photoreaction.

Experimental

Materials.—Riboflavin [6,7-dimethyl-9-(D-1'-ribityl)-isoalloxazine] was obtained from Hoffman-LaRoche, Inc. Lumiflavin and lumichrome were formed in solution by exposing a 10^{-4} *M* solution of riboflavin in water to sunlight for several days. Chromatographically pure solutions of the above three compounds were obtained by column chromatography as described by Whitby[4]; in this procedure, specially purified Whatman paper pulp and water saturated with isoamyl alcohol were used. Recrystallized disodium-EDTA was obtained from the Geigy Chemical Co. DL-Methionine was obtained from the Sigma Chemical Co. and was recrystallized from hot water until chromatographically pure. Lyophilized catalase was obtained from the Worthington Biochemical Corp. Doubly

(1) W. J. Nickerson and G. Strauss, *J. Am. Chem. Soc.*, **83**, 5007 (1960). See this reference for a bibliography of related work.

(2) R. Kuhn, H. Rudy and T. Wagner-Jauregg, *Ber.*, **66**, 1950 (1933); R. Kuhn and H. Rudy, *ibid.*, **67**, 1125 (1934).

(3) B. Holmstrom and G. Oster, Am. Chem. Soc. Meeting, New York, N. Y., September, 1960, p. 72-S.

(4) L. G. Whitby, *Biochem. J.*, **50**, 433 (1952).