[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, DUQUESNE UNIVERSITY]

Some Metal Complexes of Sulfur-containing Amino Acids[1,2]

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RECEIVED JUNE 6, 1955

The formation constants of the complexes formed by lead(II), cadmium and zinc ions with cysteine, glutathione, cysteine methyl ester and related compounds have been determined and compared. The sites of binding in cysteine to zinc ion are shown to be the amino group and sulfhydryl ion. Cysteine acts as a tridentate ligand toward lead(II) ion. The order of affinity for the sulfhydryl ion in the small molecules is: $Pb^{++} > Cd^{++} > Zn^{++}$. This is in agreement with the order found by Klotz, Urquhart and Fiess of the metal ions for the sulfhydryl group of bovine serum albumin.

Introduction

It has been shown by Klotz, *et al.*,[4] from absorption spectra studies that copper, zinc, cadmium and lead ions form bovine serum albumin mercaptides. They found that the order of affinity for the sulfhydryl group of bovine albumin is $Pb^{++} > Cd^{++} > Zn^{++} > Cu^{++}$, and that the –SH group reacts with the metallic cation before other side chains in the albumin, such as those of histidine, do. In order to get more experimental evidence on this, we have done comparison studies by investigating the complexes of zinc, cadmium and lead ions, respectively, with cysteine, glutathione and histidine. The copper–cysteine complex was not studied because of the well known reaction between copper(II) salt and cysteine hydrochloride to give a precipitate of cysteine and copper(I)–cysteine.[5] The over-all formation constants of zinc complexes of cysteine and histidine have been reported by Albert.[2] However, no individual formation constants have been reported and for some purpose[6] the first individual formation constant is the constant which must be known.

In addition, to obtain further information about the sites at which cysteine binds with various metals, we report here the results obtained for complexes of mercaptoacetic acid, 2-mercaptoethylamine and cysteine methyl ester.

Investigations were carried out to determine whether the –SH group will partake in binding, or if the sulfhydryl ion is necessary to form the complexes mentioned above. Information was obtained by studying the zinc, cadmium and lead ion complexes of methionine and comparing the formation constants of these complexes with the corresponding metal complexes of glycine and cysteine.

Experimental

Materials.—Cysteine hydrochloride and histidine hydrochloride, both reagent grade, were purchased from the Matheson Company and Schwarz Laboratories, Inc., respectively. These were used without further purification, after drying for several days over anhydrous calcium chloride. Mercaptoacetic acid (80% in water, analytical grade) was an Eastman Kodak product and was standardized by *p*H titration with standard potassium hydroxide. 2-Mercaptoethylamine was obtained from the California Foundation for Biochemical Research. The solid material was kept under a nitrogen atmosphere in order to prevent air oxidation.

Cysteine methyl ester hydrochloride was prepared by treatment of cysteine hydrochloride in methanol with dry hydrogen chloride. The product was recrystallized from methanol–ether mixtures. Glutathione in the reduced state was a C.P. Pfanstiehl product. Stock solutions about 0.01 *M* in glutathione were prepared in air-free water and standardized against carbonate-free potassium hydroxide using the *p*H meter. Lead perchlorate was supplied as a 50% aqueous solution by Baker and Adamson, General Chemical Co., New York; perchloric acid solution was prepared by dilution of Fisher 70% reagent acid and standardized by comparison with carbonate-free sodium hydroxide solution. The lead(II), zinc(II) and cadmium(II) solutions were analyzed gravimetrically as $PbSO_4$, $ZnNH_4PO_4$ and $CdSO_4$, respectively.

Aqueous solutions were prepared from oxygen-free water, and all measurements were carried out under an atmosphere of nitrogen. Only freshly prepared stock solutions of the organic acids were used. All the solutions used for *p*H and polarographic measurements were made up to an ionic strength of 0.15 with potassium nitrate (potassium perchlorate was used for lead ion complexes).

Apparatus and Procedure.—The *p*H measurements were made with a Beckman *p*H meter, Model G, equipped with external electrodes. Buffer solutions, *p*H's of 4 and 7, were used to standardize the instrument. In the large majority of the runs, thirty milliliters of a solution containing approximately 4×10^{-3} mole/l. of the organic acid, 1×10^{-3} mole/l. of metal ion and a total ionic strength of 0.15 was titrated with standard potassium hydroxide from a 5-ml. micro-buret. Solutions were maintained at 25 ± 0.1°.

Polarographic current–voltage curves were obtained manually with the Fisher Electropode. All potentials were measured against the saturated calomel electrode (S.C.E.). The half-wave potentials, after correction for the *IR* drop, were reproducible to ±1 mv. and were determined in the manner previously described.[7]

Calculation of Constants

The Bjerrum method[8] of obtaining formation constants of complexes depends on the *p*H shift produced, in a mixture of the organic acid and metal ion, by the addition of standard alkali solution.

The concentration formation constants for equilibria of the type $MA_{p-1} + A = MA_p$ are designated by the expression $k_p = (MA_p)/(MA_{p-1})(A)$. These constants were calculated by means of the modified development[9] of the Bjerrum method.

Zinc–histidine and cadmium–histidine complexes were studied by means of the polarographic method. The data obtained were used to calculate the number of groups, p, coördinated to each metal ion and the over-all formation constant, k_t, of the complex by means of the equation[7]

(1) This investigation was supported by Grant No. NSF-G510 from the National Science Foundation and Grant No. 1645 from the Penrose Fund of the American Philosophical Society.

(2) Presented before the 127th ACS Meeting, Cincinnati, April, 1955.

(3) Taken from the M.S. thesis of R. A. Manning, Duquesne University, 1955.

(4) I. M. Klotz, J. M. Urquhart and H. A. Fiess, THIS JOURNAL, **74**, 5537 (1952).

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$$(E_{1/2})_0 - (E_{1/2})_s = -(RT/nF) \ln k_f - p(RT/nF) \ln C_{hist} \quad (1)$$

where C_{hist} is the concentration of free histidine in solution.

Results

(A) **Acid Dissociation Constants.**—Table I gives the values of pK's obtained with the various acids. Where available, values from literature are also given as comparison. The pK's used in the calculations of formation constants are those determined in this investigation since only these values were obtained at an ionic strength of 0.15, the same as the ionic strength used in formation constant studies.

TABLE I

ACID DISSOCIATION CONSTANTS, 25°

	pK_1'	pK_2'	pK_3'	pK_4'
Cysteine		8.48	10.55	
		8.60[10]	10.51[10]	
		8.36[5]	10.28[5]	
Histidine		6.05	9.12	
		6.00[11]	9.17[11]	
Mercaptoacetic acid	3.58	9.78		
	3.60[12]	10.0[12]		
2-Mercaptoethylamine	8.35	10.81		
Glutathione			8.75	9.65
			8.75[9]	9.65[9]
Methionine		9.10		
		9.17[11]		
Cysteine methyl ester	6.56	8.99		
Glycine		9.68		
		9.69[7]		

(B) **Formation Constants of Complexes.**—Our pH titration data are so numerous that we are giving only two examples here. The titration data for the zinc complexes of cysteine and mercaptoacetic acid are given in Tables II and III, respectively. The compositions of the solutions used in the two tables are typical of those of many other titrations.

TABLE II

TITRATION OF ZINC NITRATE–CYSTEINE MIXTURES

30 ml. solution containing 0.004420 M cysteine hydrochloride; 0.001668 M $Zn(NO_3)_2$; 0.1400 M KNO_3; titrated with 0.0806 M KOH under nitrogen, 25°.

KOH, ml.	pH	pA	$\bar{n}$
0.00	2.52		
1.83	5.55	10.35	0.25
1.99	5.65	10.18	.39
2.62	6.00	9.61	.93
2.72	6.06	9.51	1.02
2.82	6.12	9.42	1.10
2.94	6.21	9.27	1.20
3.23	6.46	8.85	1.45
3.35	6.58	8.66	1.55
3.43	6.64	8.57	1.62
3.71	7.16	7.65	1.85

Another experiment was done to check the above results.

30 ml. solution containing 0.004413 M cysteine hydrochloride; 0.001667 M $Zn(NO_3)_2$; 0.1407 M KNO_3; titrated with 0.2993 M KOH.

0.51	5.51	10.41	0.21
.62	5.78	9.93	.53
.71	5.93	9.70	.81
1.01	6.80	8.25	1.69

From a plot of $\bar{n}$ *vs.* pA, the following values are obtained: $pA_{\bar{n}=0.5} = 9.95$; $pA_{\bar{n}=1.5} = 8.75$. The preliminary values of log k_1 and log k_2 are therefore 9.95 and 8.75, respectively.

The formation function curve has been extrapolated to a maximum of two cysteine molecules bound per zinc ion. Therefore, the highest order complex is two. By using the successive approximation method of Bjerrum,[8] the values of log k_1 and k_2 become constant at 9.86 and 8.84, respectively, after the second approximation. These are taken to be the final values of log k_1 and log k_2 for the zinc–cysteine complexes.

TABLE III

TITRATION OF ZINC NITRATE–MERCAPTOACETIC ACID MIXTURES

30 ml. solution containing 0.003813 M mercaptoacetic acid; 0.001001 M $Zn(NO_3)_2$; 0.1500 M KNO_3 titrated with 0.0400 M KOH under nitrogen, 25°.

KOH, ml.	pH	pA	$\bar{n}$
0.00	3.05		
2.40	4.17	8.18	0.20
2.80	4.48	7.85	.34
3.00	4.57	7.78	.52
3.20	4.83	7.52	.64
3.80	5.48	6.96	1.29
4.00	5.75	6.73	1.54
4.40	6.85	5.75	2.05

$pA_{\bar{n}=0.5} = 7.65$; $pA_{\bar{n}=1.5} = 6.76$; $pA_{\bar{n}=1.0} = 7.20$

Bjerrum[8] has shown that if the highest order complex is two, the value of pA at $\bar{n} = 1.0$ should be equal to $(1/2)$ log k_1k_2. This is seen to be the case for the zinc–mercaptoacetic acid complexes. After two successive approximations, the final values of log k_1 and log k_2 become 7.44 and 6.97, respectively.

Table IV lists the polarographic results for cadmium–histidine complex. A plot of $-E_{1/2}$ *vs.* $-\log C_{hist}$, according to equation 1, yields a straight line. The value of p is calculated from the slope of the line to be 1.99, so that the highest order complex is $Cd(Hist)_2$. The average value of log k_f ($k_f = k_1k_2$) is calculated by means of equation 1 to be 11.10.

TABLE IV

POLAROGRAPHIC RESULTS FOR CADMIUM–HISTIDINE COMPLEX

Each solution contains 5×10^{-4} M $Cd(NO_3)_2$, histidine hydrochloride half neutralized with KOH, KNO_3 to keep $\mu = 0.15$, 25°.

T_{hist}	$-E_{1/2}$	id, μa	pH	pA	log k_f
0	0.585				
0.0295	.625	3.72	6.03	4.901	11.16
.0591	.639	3.63	6.05	4.600	11.04
.0886	.650	3.49	6.05	4.424	11.07
.1181	.659	3.46	6.05	4.299	11.14
		Av.	6.05		11.10

The zinc complex of histidine was also studied polarographically. A plot of $-E_{1/2}$ *vs.* $-$ log C_{hist} also gives a straight line, from the slope of

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which p is calculated to be 2.03. The average value of log k_f is calculated to be 12.3, as compared to the value log k_1k_2 = 11.78 obtained by the pH method (see Table V). The polarographic value of log k_f however is of doubtful significance because the electrode reaction of zinc–histidine is not strictly reversible ($(E_{3/4} - E_{1/4})$ have the values 0.037 to 0.044).

Table V gives a summary of the formation constants of the metal complexes obtained at an ionic strength of 0.15 and 25°. For the lead(II) complexes, only values of log k_1 are recorded, because in many cases precipitation occurred when $\bar{n} > 1$. In order to determine the formula of the highest order complex of lead–cysteine, as compared to that of the zinc–cysteine complex, we performed potentiometric titrations of cysteinate with zinc and with lead ions. The results are given in Fig. 1.

TABLE V

SUMMARY OF CONCENTRATION FORMATION CONSTANTS

	Zn^{++} log k_1	Zn^{++} log k_2	Cd^{++} log k_1	Cd^{++} log k_2	Pb^{++} log k_1
Cysteine	9.86	8.84	[a]		12.20[b] 12.75[c]
Histidine	6.67	5.11	11.10 (log k_1k_2[d])		6.84[c]
Mercaptoacetic acid	7.44	6.97			[a] (8.5 at μ = 0.002)[c]
2-Mercaptoethylamine	9.90	8.84	10.97	8.78	11.10[b] 11.27[c]
Glutathione	8.30[a]		10.5		10.6[b]
Methionine	4.38	4.09	3.88	3.11	4.40[b]
Cysteine methyl ester	8.42	7.82			9.35[b]
Glycine	5.42	4.52			

[a] Precipitate formed. [b] Using $Pb(NO_3)_2$. [c] Using $Pb(ClO_4)_2$. [d] Obtained from polarographic data.

Discussion

It is seen from Table V that the log of the first formation constant of lead cysteine is 2.34 units higher than that of zinc–cysteine. It is also seen that log k_1 of lead–glutathione complex is 2.3 units higher than that of zinc–glutathione and 0.1 unit higher than that of cadmium–glutathione complex. This order of stability is in agreement with the findings of Klotz, *et al.*,[4] that lead ion has a greater affinity than cadmium which in turn has a greater affinity than zinc for the sulfhydryl group of bovine albumin. The nearly equal formation constants of cadmium glutathione and lead glutathione complexes may be attributed to the greater affinity of cadmium than lead for the amino group.

Cadmium forms a precipitate upon the addition of cysteine and, therefore, it was not possible to determine the formation constants of cadmium cysteine complexes.

The log k_1 values of the metal ion complexes of cysteine, glutathione and 2-mercaptoethylamine are higher than those of the corresponding metal ion complexes of histidine. This is also in agreement with the conclusion of Klotz, *et al.*,[4] that these metal ions react more readily with the sulfhydryl group than with histidine side chains.

In the binding of cysteine with zinc ion, there are three possible pairs of actual binding sites: (1) $-NH_2$ and $-COO^-$, (2) $-S^-$ and $-COO^-$ and (3) $-S^-$ and $-NH_2$. The possibility of the first pair as the binding sites is ruled out because the formation constants of the zinc–cysteine complexes are much higher than those of the zinc–glycine complexes. The possibility of the second pair as the binding sites is also ruled out because the formation constants of the zinc–cysteine complexes are again much higher than those of the zinc–mercaptoacetic acid complexes, in which the binding sites in the ligand for attachment of a metal ion are the $-S^-$ and $-COO^-$ groups. The elimination of the first two pairs as the binding sites in cysteine leaves only the third pair. The correctness of the choice of $-S^-$ and $-NH_2$ groups as the actual binding sites is shown by the equal stability of the zinc complexes of cysteine and of 2-mercaptoethylamine. For 2-mercaptoethylamine it is obvious that only $-S^-$ and $-NH_2$ can be the binding sites for attachment of metal ions.

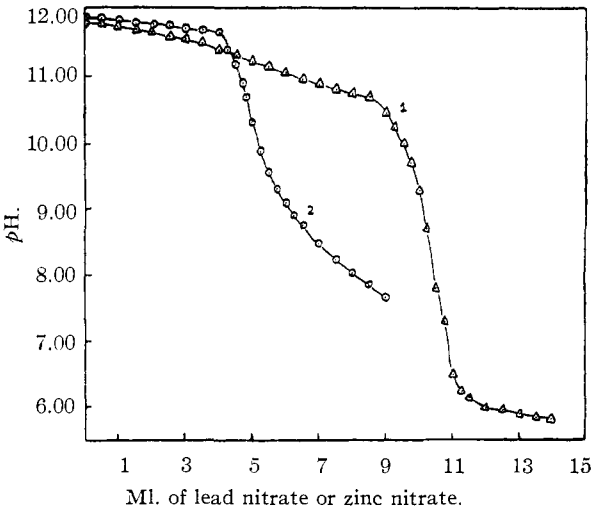

Fig. 1.—Results of titrations of 1.005 equivalents cysteinate with: curve 1, 0.09753 M lead nitrate; curve 2, 0.09753 M zinc nitrate.

For the lead(II) complexes the same conclusion about the binding sites in cysteine may be reached as with zinc. The log k_1 of lead cysteine complex, however, is 1 unit higher than that of lead 2-mercaptoethylamine complex and this may be accounted for as follows: the potentiometric results of Fig. 1 show that the highest order zinc complex is 1:2 whereas the highest order lead complex is 1:1. Cysteine then must act as a tridentate ligand toward lead ion (binding sites: $-S^-$, $-NH_2$ and $-COO^-$), and this complex should be more stable, therefore, than the 2-mercaptoethylamine complex, in which the ligand is bidentate. This is in agreement with the findings of Gurd and Murray[13] that lead ions and not zinc ions are bound to carboxylate groups in human serum mercaptalbumin.

The zinc complexes of cysteine methyl ester are less stable than the zinc–cysteine complexes. This is easily explained by the smaller pK's of cysteine methyl ester as compared to those of cysteine (see Table I). The basic strength, with its tendency to share its electron pair with a metal ion in the

(13) F. R. N. Gurd and G. R. Murray, Jr., THIS JOURNAL, **76**, 187 (1954).

formation of a chelate, of the amino group in the cysteine methyl ester complex is therefore decreased, with a consequent decrease in the stability of the complex. In the case of lead ion complexes, an additional factor comes into play: it will be recalled in our previous discussion that the carboxylate ion becomes a binding site in the lead–cysteine complex. In going from cysteine to cysteine methyl ester, the negatively charged carboxylate ion is replaced by an uncharged $-COOCH_3$ group, thus causing a further decrease in stability of the lead complex, in addition to that caused by the decrease in pK's discussed above.

We have not identified the pK's of cysteine methyl ester with the dissociation of any particular group. Although most workers would assign the higher pK value to the –SH group and the lower pK value to the $-NH_3^+$ group, Calvin[14] has expressed the opinion that the assignment should be reversed. We are however in agreement with Edsall's interpretation that, since the intrinsic affinity of the amino and the sulfhydryl ion for proton is so similar, the two dissociation constants are really of a composite nature.

It is seen in Table V that for lead complexes of cysteine and 2-mercaptoethylamine, the formation constants depend on the anion of the lead salt. The order $ClO_4^- > NO_3^-$ is in agreement with the well known fact that lead forms the least possible amount of complex with perchlorate ion and a firmer complex with nitrate ion.

A comparison of the formation constants of glycine and methionine complexes of zinc leads to the conclusion that the sites of binding in methionine are the same as in glycine, namely, $-NH_2$ and $-COO^-$. Gonnick, Fernelius and Douglas[15] have given the following values

$$Cu^{++}\text{–}CH_3SCH_2CH_2NH_2,\ \log k_1 = 5.58\ (30°)$$
$$Cu^{++}\text{–}NH_2CH_2CH_2NH_2,\ \log k_1 = 10.55\ (30°)$$

which show that the amino group binds far more strongly than a $-SCH_3$ group. Inasmuch as the highest order zinc complex is two for both glycine and methionine as ligands and the characteristic coördination number of zinc ion is four, we may state that the $-SCH_3$ group in methionine is not involved in binding. Our interpretation is that since no sulfhydryl ion can be produced by the reaction of methionine with metal ion, no chelation with zinc ion can take place which would involve sulfur. In the case of the cysteine complex, therefore, the sulfhydryl ion rather than the –SH group is taken as the actual binding site.

Acknowledgment.—The authors wish to thank the National Science Foundation and the American Philosophical Society for the research grants which made this work possible.

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Ultracentrifuge Studies with a Synthetic Boundary Cell.[1] II. Differential Sedimentation

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RECEIVED MARCH 22, 1955

Through the use of the recently developed synthetic boundary ultracentrifuge cells it is possible to form concentration boundaries between two solutions of the same sedimenting substance. The movement of such boundaries gives differential sedimentation coefficients. A theoretical treatment of such experiments is presented and it is shown that the theory based on the conservation of mass accounts satisfactorily for the results observed for different types of systems commonly studied in the ultracentrifuge. Not only are the observed sedimentation coefficients in agreement with values calculated from the theory but the areas of the boundaries also demonstrate the validity of the theory. Also there is presented a practical application of the differential sedimentation method to the determination of both the dependence of sedimentation coefficient on concentration and the value of the sedimentation coefficient at infinite dilution, and it is shown that the differential method gives reliable results with less effort than is required by conventional ultracentrifuge experiment.

Introduction

Ultracentrifugal studies by the sedimentation velocity method[3] have heretofore involved analyses of the movement and shape of the boundaries originally formed by the migration (either sedimentation or flotation) of solute molecules from a region in the centrifuge cell in which they were originally present into another region in which there were already molecules of the same solute. By this process there is created a region devoid of a particular solute. If the system is composed of only two components, then the resulting boundary represents the transition zone between a pure solvent and a solution of uniform concentration of the solute. For three component systems,[4] or multicomponent systems in general, each boundary developed as a result of the sedimentation process is really a compound boundary across which there is, in addition to the disappearance of one of the components, a change in concentration of the other components present in the solution.

There are different types of ultracentrifuge bound-

(1) These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of California, NR-121-175; and by grants from Lederle Laboratories and the Rockefeller Foundation.

(2) National Science Foundation Predoctoral Fellow, 1954–1955. Some of this work is submitted in partial fulfillment of the requirement for the Ph.D. degree in Biophysics at the University of California.

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