and 76.39 for K^+, Na^+ and Cl^-, respectively; the values obtained from the extrapolated transference data[10] are 73.50, 50.10 and 76.35.

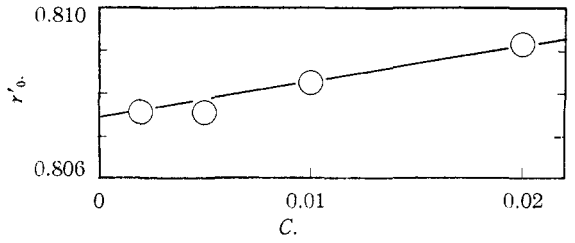


Fig. 1.—The quantity r'_0 as a function of KCl concentration; the radii of the circles correspond to the apparent precision of the measurements.

The results obtained here for the limiting transference numbers and ionic conductances illustrate one restriction on this method. Although the extrapolated r_0 differs from that computed from the limiting transference data by no more than the numerical precision of the latter (a part in 4000) the ionic conductances deviate by a part in 1800 for the faster ions, and by a part in 1000 for the slowest. Inspection of eq. 5 shows that this is due to the relatively small difference between Λ^0 and Λ_i^0, with a resulting limiting Kohlrausch ratio not differing greatly from unity. Thus to attain as high a numerical precision as possible in the ionic quantities, leading and indicator ions should have as large a difference in ionic conductance as possible, and this should be borne in mind in selecting the electrolytes for study in any new solvent.

It should also be noted that while the extrapolation procedure outlined above is probably adequate for strong electrolytes, the extrapolation, if ion pair formation is appreciable, would probably require the use of ionic rather than stoichiometric concentrations in eq. 6 and in the analog of eq. 7. We believe, however, that these results show that limiting ionic conductances can be determined with reasonable precision for any solvent in which precise conductance measurements are possible.

In conclusion, we wish to express our thanks to Mr. R. H. Chappell for constructing the conductance cells, and to the National Research Council of Canada for a grant in aid of this research and for the award to D.R.M. of a studentship and a fellowship.

TORONTO, ONTARIO, CANADA

[CONTRIBUTION FROM THE DEPARTMEMT OF CHEMISTRY, THE UNIVERSITY OF TEXAS]

The Inhibition of Urease by Various Metal Ions

BY WILLIAM H. R. SHAW

RECEIVED JUNE 17, 1953

Data on the relative toxicity of metal ions toward the enzyme urease have been collected from the literature. It has been found possible to arrange the common metal ions in a toxicity sequence. Correlation of toxicity with various properties of the metal ions is discussed and illustrated on the basis of a model mechanism.

Introduction

The enzyme urease is highly sensitive to trace quantities of metal ions. Different metals exhibit quite different behavior in their ability to act as enzyme inhibitors. In the case of urease, for example, the silver ion[1,2] is an extremely efficient inhibitor, while the manganous ion is relatively very weak.[3] A search of the literature has revealed that enough data are now available to order the common metal ions in a tentative sequence of relative inhibitory efficiency. It is the purpose of this investigation: to summarize the available data in such a sequence; to define a quantitative functional measure of inhibitory efficiency; and to correlate this quantity, if possible, with some fundamental property of the metals.

Mathematical Development

It was demonstrated in a previous communication[4] that the inhibition index, for a first-order Michaelis–Menten system, was given by the equation

$$\phi = \frac{V_u - V_i}{V_i} = \frac{K_1 K_m i + K_2 S i}{S + K_m} \qquad (1)$$

S is the substrate concentration, V_u is the uninhibited rate of urea hydrolysis by urease and V_i is the inhibited rate. K_m is Michaelis constant, K_1 the equilibrium constant for the combination of the free enzyme with the inhibitor, and K_2 an analogous constant involving the enzyme-substrate. The concentration of inhibitor not bound to the enzyme[5] is designated by "i." At an experimentally fixed substrate concentration, it has been common practice to determine the concentration of inhibitor (I) necessary to produce some arbitrary inhibition (ϕ_A). Thus for all the types of inhibition listed in Table I, equation 1 may be rearranged to read

$$pI = \text{const.} + \log K_i \qquad (2)$$

where K_i may be K_1, K_2 or K, and p refers to the negative logarithm of the quantity involved. For an enzyme obeying the inhibited Michaelis–Menten mechanism with the above restrictions, a comparison of pI's for various inhibitors corresponds to a comparison of functions that are, at a given temperature, linear functions of the free energy of inhibition

$$-\Delta F_i^0 = RT \ln K_i \qquad (3)$$

If $K_i \approx K$, the total free energy of inhibition will be twice that given by equation 3, since two inhibition reactions are involved. The larger the value of pI the more efficient the inhibitor, since there is a greater loss in the free energy of

(1) J. B. Sumner and K. Myrbäck, *Z. physiol. Chem.*, **189**, 218 (1930).

(2) J. F. Ambrose, G. B. Kistiakowsky and A. G. Kridl, THIS JOURNAL, **73**, 1232 (1951).

(3) A. L. Dounce, National Nuclear Energy Series, Div. VI, Vol. 1, McGraw-Hill Book Co., Inc., New York, N. Y., 1949, pp. 839–848.

(4) G. B. Kistiakowsky and W. H. R. Shaw, THIS JOURNAL, **75**, 66 (1953).

(5) In what follows it is assumed that the amount of inhibitor bound to the enzyme is small compared to the total inhibitor concentration. For inhibitor concentrations that greatly exceed the total enzyme concentration, this assumption is entirely justified. For very strong inhibitors, such as silver ion, it should be considered as an approximation.

TABLE I

VALUES OF THE PARAMETERS IN EQUATION 2 FOR THE VARIOUS TYPES OF INHIBITION

Case	Type of inhibition	Condition	Logarithmic term	Constant term
1	Competitive	$K_2 = 0$	$\log K_1$	$p\phi_A + \log \frac{K_m}{S + K_m}$
2	Uncompetitive	$K_1 = 0$	$\log K_2$	$p\phi_A + \log \frac{S}{S + K_m}$
3	Non-competitive	$K_2 = K_1 = K$	$\log K$	$p\phi_A$
4	Others	$K_2S \gg K_1K_m$	Same as case 2	
		$K_mK_1 \gg SK_2$	Same as case 1	

inhibition. The quantity pI is thus a quantitative measure of toxicity based on the thermodynamic consequences of the inhibited Michaelis–Menten model.

Data

Using crude preparations of urease from jack bean meal, Schmidt[6] determined the minimum concentration of metal salt necessary to so reduce the rate that no detectable quantity of ammonia could be found under his experimental conditions. Very dilute solutions of urea were employed (approx. 30 p.p.m.). The reaction mixtures were incubated at 50° for five minutes in unbuffered solutions initially at pH 7. The results obtained have been recalculated to express the inhibitor concentration in moles per liter, and the pI values derived from these calculations are recorded in Table II.

TABLE II

pI VALUES OBTAINED BY VARIOUS INVESTIGATORS

Ion	Salt used	pI Schmidt[6]	pI Dounce[3]
Ag^+	$AgNO_3$	4.73	7.1
Hg^{++}	$HgCl_2$[a]	4.7	6.7 (pH 5)
			5.6 (pH 7)
	$Hg(CN)_2$[a]	4.7	...
Cu^{++}	$Cu(C_2H_3O_2)_2 \cdot H_2O$	3.8	...
	$CuSO_4 \cdot 5H_2O$	..	4.5
Zn^{++}	$Zn(C_2H_3O_2)_2 \cdot 3H_2O$	2.8	...
Cd^{++}	$CdCl_2$	2.6	3.9
Pb^{++}	$Pb(C_2H_3O_2)_2 \cdot 3H_2O$[a]	2.3	...
Co^{++}	$CoCl_2$	0.90	2.4
Ni^{++}	$NiCl_2$	.77	...
Mn^{++}	$MnCl_2$	.04	...
	$MnSO_4$	..	1.5

[a] Not completely dissociated. Unless the enzyme has equal affinity for dissociated and undissociated form, the results are somewhat ambiguous.

The best recent comparative study is that of Dounce.[3] The observations were made at various pH values in several buffers. Crystalline urease was used throughout; measurements were made at 25° with a urea concentration of 3%. To make the data comparable to those of Schmidt, the results have been converted to approximate pI values by calculating the inhibitor concentration in moles/liter necessary to produce 95% inhibition at pH 7.

In view of the semiquantitative character of the data, the pI values reported in Table II should be considered as rough estimates—probably good at best to two significant figures.

Several qualitative statements concerning the relative inhibitory efficiency of metal ions can also be found in the literature. In many cases, however, it is impossible to evaluate critically the significance of these data because the original articles were not available. The results of two such researches[7-9] are presented in Table IIIA.

(6) E. G. Schmidt, *J. Biol. Chem.*, **78**, 53 (1928).

(7) M. Kitagawa, *J. Biochem. (Japan)*, **10**, 197 (1929); *C. A.*, **23**, 3242 (1929).

(8) A. J. J. Van de Velde, *Meddel. Koninkl. Vlaam. Acad.*, **9**, No. 12, 13 (1947); *C. A.*, **42**, 7803 (1948).

(9) *Ibid.*, **11**, No. 11 (1949); *C. A.*, **45**, 9581 (1951).

TABLE III

A, RELATIVE TOXICITIES OF THE VARIOUS METAL IONS

Investigator	Toxicity sequence
Schmidt[6]	$Ag^+ \sim Hg^{++} > Cu^{++} > Zn^{++} > Cd^{++} > Pb^{++} > Co^{++} > Ni^{++} > Mn^{++}$
Dounce[3]	$Ag^+ > Hg^{++} > Cu^{++} > Cd^{++} > Co^+ > Mn^{++}$
Van de Velde[9]	Ag^+, Hg^{++}, $Cu^{++} > Co^{++} > Ni^{++} > Cd^{++}$, Pb^+, $Zn^{++} > Mn^{++}$
Kitagawa[7]	Hg^{++}, $Cu^{++} > Zn^{++}$
Best sequence	$Ag^+ \sim Hg^{++} > Cu^{++} > Cd^{++} > Co^{++} > Ni^{++} > Mn^{++}$ with Pb^{++} and Zn^{++} unassigned but less than Cu^{++}

B, OTHER PROPERTIES OF THE METAL IONS

Property	Sequence
Insolubility of the sulfide	$Ag^+ \sim Hg^{++} > Cu^{++} > Pb^{++} > Cd^{++} > Zn^{++} > Co^{++} > Ni^{++} > Mn^{++}$
Sum of the first and second ionization potentials of the gaseous atoms	$Hg^{++} > Cu^{++} > Zn^{++} > Cd^{++} > Ni^{++} > Co^{++} > Mn^{++} > Pb^{++}$
Chelate stability series	$Hg^{++} > Cu^{++} > Ni^{++} > Pb^{++} > Co^{++} > Zn^{++} > Cd^{++} > Mn^{++}$
Electromotive series	$Ag < Hg < Cu < Pb < Ni < Co < Cd < Zn < Mn$

Soy bean urease preparations were used in all of these investigations. The over-all agreement of the data collected in Table IIIA is remarkably good, when one considers that enzyme preparation of various degrees of purity[2] from two different sources were employed.

Before turning to the correlation and interpretation of these data, the importance of the buffer in experiments of this type should be considered. Because of its excellent buffering qualities phosphate buffer has unfortunately been widely employed. Phosphate exhibits a strong complexing tendency toward metal ions.[10] Measurements made in this buffer may not reflect the true inhibitory efficiency series of the metals but only a sequence of relative complexing tendencies. As an additional complication it has been well established that phosphate[11,12] buffer interacts quite strongly with the enzyme urease. On the basis of these facts the work of M. Jacoby[13] has been omitted because strong phosphate buffer was employed, and the data so obtained were in conflict with results reported above.[14]

Correlation

Rather extensive experimental data are available supporting the contention that urease contains one or more sulfhydryl groups[15,16] as integral parts of its catalytically active site. In view of this evidence the inhibition of urease by metal ions has been assumed to result from a reaction such as[17]

$$E\begin{matrix}SH\\SH\end{matrix} \rightleftharpoons E\begin{matrix}S(-)\\SH\end{matrix} + H^+ \qquad (4)$$

(10) J. B. Sumner and K. Myrbäck, "The Enzymes," Vol. I, Part 1, Academic Press, Inc., New York, N. Y., 1951, p. 11.

(11) K. M. Harmon and C. Niemann, *J. Biol. Chem.*, **177**, 601 (1949).

(12) G. B. Kistiakowsky, P. C. Mangelsdorf, Jr., A. J. Rosenberg and W. H. R. Shaw, THIS JOURNAL, **74**, 5015 (1952).

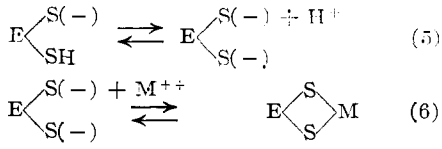
(13) M. Jacoby, *Biochem. Z.*, **259**, 211 (1933).

(14) In the work of Dounce,[4] measurements with the Hg^{++} were conducted in phosphate, but other ions were studied in maleate or unbuffered solutions. This author recognized and emphasized the complexing tendencies of phosphate, and observed that Hg^{++} is probably a stronger inhibitor than he reported.

(15) L. Hellerman, "Cold Spring Harbor Symposium Quant. Biol.," VII, 165 (1939).

(16) C. V. Smythe, *J. Biol. Chem.*, **114**, 601 (1936).

(17) L. Massart, ref. 10, p. 328.



(5)

(6)

From a consideration of the last reaction in this sequence, it becomes evident that those metal ions that have the greatest affinity for the negatively charged sulfur in the active site will be the most toxic. As a first approximation the insolubility of the corresponding sulfide may be taken as a rough estimate of this affinity. The metals that form the most insoluble sulfides should consequently be the best inhibitors of sulfhydryl enzymes. Table III shows that, in the case of urease, this conclusion appears to be qualitatively quite satisfactory. In hope of obtaining semiquantitative correlation, the solubility product constants for the various metal sulfides were obtained from the literature.[18-21]

The loss in free energy ($-\Delta F_s$) associated with the formation of the metal sulfide is given by

$$-\Delta F_s^0 = RT \ln (1/K_{sp}) = 2.303RT(pK_{sp}) \quad (7)$$

A comparison of pK_{sp} values at a given temperature corresponds to a comparison of functions that are proportional to the standard free energy changes. The pK_{sp} values from various sources are listed in Table IV. The most recent data, those of Kapustinsky,[20] were obtained from thermal measurements. His values have been used for the plot in Fig. 1. The other data in Table IV demonstrate the rather wide range of values reported by various investigators.

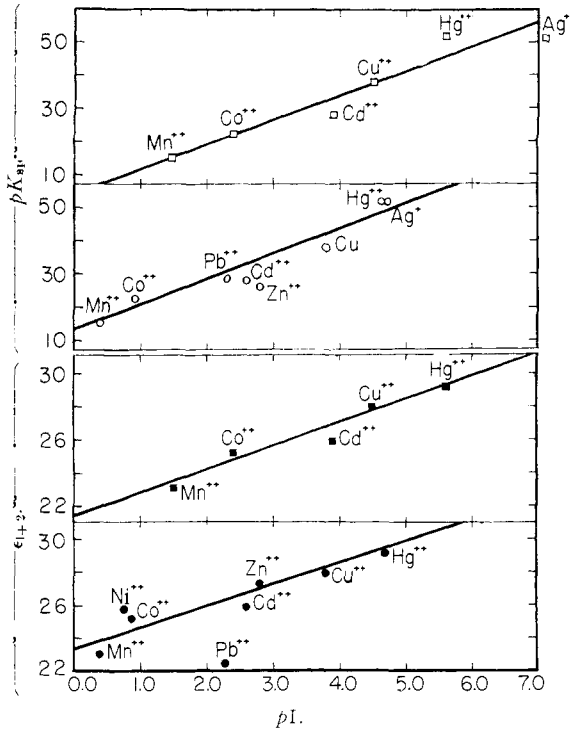


Fig. 1.—Upper plot: correlation of the toxicity (pI) of various metal ions toward urease with the insolubility of the corresponding sulfide (pK_{sp}). Lower plot: correlation of toxicity with the sum of the first and second ionization potentials (ϵ_{1+2}) of the corresponding gaseous atoms. Circles, data of E. G. Schmidt; squares, data of A. L. Dounce.

(18) I. M. Kolthoff, *J. Phys. Chem.*, **35**, 2711 (1931).

(19) S. F. Ravitz, *ibid.*, **40**, 61 (1936).

(20) A. F. Kapustinsky, *Doklady Akad. Nauk. S.S.S.R.*, **28**, 144 (1940); *C. A.*, **35**, 3144 (1941).

(21) M. A. Lange, "Handbook of Chemistry," 7th Edition, Handbook Publishers, Inc., Sandusky, Ohio.

TABLE IV

pK_{sp} VALUES FOR THE METAL SULFIDES

Metal sulfide	Kapustinsky[20]	Ravitz[19]	Kolthoff[18]	Lange[21]
Ag_2S	51.23	51.48	50	48.8[b]
HgS	51.52[a]	...	53.5	48.7–52.4
CuS	37.50	37.46	41.46	44.1
ZnS	26.13	25.4	25.3–23.96	22.9
CdS	27.92	27.94	27.15–28.29	28.4
PbS	28.17	29.16	27.47	27.5
CoS	22.50	...	26.72	25.5
NiS	...	...	26.96	23.9
MnS	14.96	...	15.16	14.9

[a] W. D. Treadwell and F. Schufelsbergen, *Helv. Chim. Acta*, 29, 1935 (1946). [b] These values are for 18°, the others are for 25°.

Considering equations 2, 3 and 7 it becomes apparent that if a quantitative or semiquantitative relationship between sulfide insolubility and toxicity exists, a plot of pK_{sp} *vs.* pI will be nearly linear (Fig. 1, upper plot).[22]

Several other correlations were attempted with varying degrees of success. From a somewhat more generalized point of view the inhibition reaction may be regarded as the combination of a positive metal ion with a negative group. Thus the bivalent metal ion that has the greatest affinity for the two electrons on the sulfur atoms in the active site will be the strongest inhibitor. If the sum of the first and second ionization potentials[23] of the gaseous atoms, ϵ_{1+2}, is chosen as a measure of the affinity of the metal ion for these two electrons, a linear relationship between ϵ_{1+2} and pI may be expected (Fig. 1, lower plot).

Attempted correlations with the standard electrode potentials, with the stability constants of metal chelates,[24] with the ionic radii, and with the reciprocal ionic radii were less successful (see Table III).

Discussion

It appears from the arguments presented above that it is possible to draw several rather general conclusions concerning the relative toxicity of metal ions toward the enzyme urease. The metal ions that form the most insoluble sulfides are also the strongest inhibitors. Whether this conclusion is applicable to all sulfhydryl enzymes or is limited to urease cannot be determined on the basis of the data presented here.

According to the reaction scheme presented above (equations 4–6), an intensified inhibition would be expected with increasing pH. In qualitative analysis certain metal ions may only be precipitated as sulfides from basic solutions of hydrogen sulfide, whereas others (such as the silver ion) are insoluble enough to be precipitated from both acidic and basic solution. An exactly analogous situation would be expected in the case of metal ion inhibition. The data of Kitagawa[8] are in excellent agreement with this contention. The findings of Dounce[3] for Cu^{++} and Ag^{+} are also in accord with this hypothesis. For Hg^{++}, Dounce observed exactly the opposite behavior.[25]

The correlation of inhibitory efficiency with the

(22) The inclusion of the data of Schmidt is open to criticism on the grounds that the solubility product constants were determined at 25° and Schmidt incubated his reaction mixtures at 50°. A rigorous comparison would involve a knowledge of the various enthalpy changes.

(23) G. Herzberg, "Atomic Spectra and Atomic Structure," 2nd Ed., Dover Publications, New York, N. Y., 1944, p. 200.

(24) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice-Hall, Inc., New York, N. Y., 1952.

(25) Whether this is due to the phosphate buffer, the partial dissociation of the $HgCl_2$, or to the formation of mercuric hydroxide in basic solution is difficult to say.

sum of the first and second ionization potentials is also quite suggestive. Such a correlation may be applicable in general to metal ion inhibition that involves reaction with negative groups.

Further investigation of the above points is being undertaken in this Laboratory.

Previous work[26] has demonstrated the existence of a linear relationship between pK_{sp} and the lattice energy of the crystal. Consequently, the correlations of pI with pK_{sp} and with ϵ_{1+2} cannot be considered as independent relationships.

Although good correlation with the general chelate stability sequence was not obtained, it is still quite possible that this process is involved in metal ion inhibition. In a complex protein molecule like urease, with the large number of electron donor groups available, it would be rather surprising if chelation were not involved.

Since more detailed knowledge of true inhibition mechanism was not available, the mathematical development of the pI concept was based on the inhibited Michaelis–Menten mechanism. Within this framework the conclusions presented above appear to constitute an acceptable tentative explanation of the relative toxicity of metal ions toward the enzyme urease.

(26) See, for example, O. K. Rice, "Electronic Structure and Chemical Binding," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 417.

AUSTIN, TEXAS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, STATE UNIVERSITY OF IOWA]

The Physical Chemistry of Insulin. I. Hydrogen Ion Titration Curve of Zinc-free Insulin[1-3]

BY CHARLES TANFORD AND JACK EPSTEIN

RECEIVED AUGUST 21, 1953

The hydrogen ion titration curve of zinc-free insulin indicates the presence of 4 α-carboxyl groups, 8.5 β- and γ-carboxyl groups, 4 imidazole groups, 4 α-amino groups, 10 phenolic plus ϵ-amino groups (which could not be separated in the analysis) and 2 guanidine groups per insulin monomer, in complete agreement with the best available amino acid analyses. The intrinsic pK's of these groups are, respectively, 3.6, 4.73, 6.40 (or 6.0), 7.45 (or 7.2), 9.60 and 11.9. These values suggest that all of the acidic and basic groups freely into the solvent and do not participate in intramolecular bonding. Empirical values for the electrostatic interaction energy are used to estimate rough values of the degree of association of insulin at various pH values. The results are in fairly good agreement with the molecular weight determinations of Doty and others, and difficult to reconcile with a molecular weight of 6000 in aqueous solution below pH 10. About one chloride ion is bound per insulin monomer at pH 2. and some potassium binding may occur above pH 10.

The work described in this paper is part of a long-range program to investigate the reactive groups of protein molecules, with emphasis on their interaction with other molecules or ions, and on changes in chemical structure accompanying such interaction. In the case of insulin, the primary objective has been to gain an understanding of its interaction with zinc, since insulin, as isolated from living tissue, always contains combined zinc. However, prerequisite to an investigation of this interaction is a knowledge of the behavior of the reactive groups of the protein in the absence of zinc and of other ions with which they may combine. The present study was therefore undertaken, using a preparation from which all or most of the zinc had been removed.

Experimental

Insulin.—The insulin used was lot no. 190-4B-213A, and was donated by Eli Lilly and Co. It contained 0.027% of zinc or less, and had an activity of 27 u./mg., indicative of a high order of purity. The sample was completely amorphous, and dissolved in water to give a pH of 3.31, and in 0.075 M KCl to give a pH of 3.48. From the final assignment of the end-points of the titration curve, the latter pH value was found to correspond to 7.5 bound hydrogen ions per insulin monomer.[4] From e.m.f. measurements with silver–silver chloride electrodes (see below) a water solution was found to contain 8.0 ± 1.0 chloride ions per insulin monomer, *i.e.*, a number equal to the number of bound hydrogen ions. It was therefore concluded that the sample supplied was an insulin hydrochloride (Insulin, 7.5HCl). The insulin was stored in a stoppered bottle below 0°. Samples were allowed to come to equilibrium in a room at constant temperature and humidity before being weighed. Under the conditions used the moisture content, determined by heating to constant weight at 105°, was found to be 5.7%. Correction for this, and for an ash content of 0.37%, was made in all calculations.

Other Reagents.—Hydrochloric acid solutions were prepared by weight from a stock solution of constant boiling HCl.[6] Potassium hydroxide solutions were prepared by the method of Kolthoff,[7] and standardized against HCl. Potassium chloride solutions were prepared by weight from reagent grade KCl of low iodide and bromide content (J. T. Baker Chemical Co., Phillipsburg, N. J.). Conductivity water was used throughout.

Solutions for Measurement.—Solutions for all measurements were prepared by weight. About 0.05 g. of insulin was used for each determination, and the other reagents and water were added so as to attain a final ionic strength of 0.075 in each case.

Determination of pH.—All pH measurements were made with a Beckman model G pH meter, using an external glass electrode of type 1190-80. The instrument was standard-

(1) Presented at the 124th meeting of the American Chemical Society, Chicago, Ill., Sept. 6–11, 1953.

(2) This investigation has received its principal support from a research grant by Eli Lilly and Co. The general program, of which this investigation is a part, has been supported by a research grant (RG-2350) from the National Institutes of Health, Public Health Service, and by a grant from the National Science Foundation.

(3) Abstracted from the dissertation submitted by Jack Epstein in partial fulfillment of the requirements for the Ph.D. degree, State University of Iowa, August, 1953.

(4) Throughout this paper the term *insulin monomer* will be used to refer to the molecular unit of molecular weight about 11,500, consisting of the four polypeptide chains described by Sanger (ref. 5). It appears to be fairly well established that this is the lowest molecular weight unit of insulin which exists in aqueous solution, at least below pH 8.

(5) F. Sanger and E. O. P. Thompson, *Biochem. J.*, **53**, 353, 366 (1953); F. Sanger and H. Tuppy, *ibid.*, **49**, 463, 481 (1951).

(6) C. W. Foulk and M. Hollingsworth, THIS JOURNAL, **45**, 1220 (1923).

(7) I. M. Kolthoff, *Z. anal. Chem.*, **61**, 48 (1922).