TABLE III

DIELECTRIC CONSTANTS, DENSITIES AND POLARIZATIONS IN BENZENE AT 25°

f_2	ϵ	d	P_2
	Diketene		
0.00000	(2.2725)[a]	0.87222	P_1 = 26.6720
.006407	2.3642	.87355	228.4
.013211	2.4659	.87485	228.4
.022613	2.6109	.87625	226.8
.032564	2.7604	.87793	220.5
.040303	2.8783	.87911	216.3
.060383	3.1746	.88246	203.8
	Methylketene dimer		
0.00542	2.3497	0.87307	239.7
.011488	2.4550	.87396	258.6
.022082	2.6462	.87533	264.4
.032160	2.8227	.87677	258.9
.041811	3.0214	.87811	260.8
.057532	3.3055	.88022	250.1
	Mixed dimer of methyl- and hexylketenes		
0.006361	2.3748	0.87293	292.7
.009736	2.4376	.87330	302.6
.011827	2.4634	.87317	291.0
.014545	2.5165	.87324	298.3
	Hexylketene dimer		
0.004751	2.3555	0.87291	336.4
.009643	2.4398	.87272	335.4
.021604	2.6502	.87369	331.4
.039671	2.9547	.87438	321.3
.047347	3.2051	.87491	346.6
.062258	3.3259	.87472	311.7

[a] Hartshorn and Oliver, *Proc. Roy. Soc.* (*London*), **A123**, 664 (1929).

g. of the mixture of ethyl α-propionylcaprylate and ethyl α-caprylylpropionate was hydrolyzed by refluxing with a carbon dioxide-free solution of sodium hydroxide prepared by dissolving 0.05 g. of clean sodium in 10 ml. of boiled alcohol and diluting with 10 ml. of boiled water. The hydrolysis mixture was acidified with dilute perchloric acid and heated to boiling. The evolved carbon dioxide was swept out with nitrogen and precipitated as barium carbonate which was analyzed in the usual manner.[11]

Ultraviolet Absorption Spectra.—The ultraviolet spectra were determined in purified cyclohexane solution using a Beckman Quartz Spectrophotometer.[16]

Dipole Moments.—The dielectric constants were measured at a frequency of 954 kilocycles using a modification of the heterodyne heat apparatus described by Smyth.[17] The standard condenser was a General Radio Type 722-N. The dielectric cell was similar to the one described previously.[18] The replaceable capacity of the cell at 25° was 123.50 $\mu\mu$ fd. and the volume about 40 ml. The pycnometers were of the graduated type described by Robertson.[19]

The dipole moments were obtained from the dielectric constants and densities of dilute solutions using the customary equations.[17] Values of P_∞ were obtained by least squares extrapolation of P_2 to infinite dilution and the sum of the electronic and atomic polarizations at infinite wave length was assumed to be equal to the experimental value of the molar refraction for the sodium D line, MR_D. The data are given in Tables II and III.

Summary

The isotopic tracer technique has been used to demonstrate that the mixed aldoketene dimer of methyl- and hexylketenes prepared by the Wedekind reaction does not have a cyclobutanedione-1,3 structure.

The physical properties of several aldoketene dimers indicate that these substances probably have structures similar to diketene and are best formulated as vinylaceto-β-lactone or β-crotonolactone derivatives.

(16) Cary and Beckman, *J. Optical Soc. Am.*, **31**, 682 (1941).

(17) Smyth, in Weissberger, "Physical Methods of Organic Chemistry," Vol. 2, Interscience Publishers, Inc., New York, 1946, Chap. XX.

(18) Rogers and Roberts, THIS JOURNAL, **68**, 843 (1946).

(19) Robertson, *Ind. Eng. Chem., Anal. Ed.*, **11**, 464 (1939).

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

The Binding of Organic Ions by Proteins. Effect of Temperature

BY IRVING M. KLOTZ AND JEAN M. URQUHART

The affinity of serum albumin for organic ions has been found to depend on the charge and structure of the anion, as well as on the *p*H and nature of the buffer and on the concentration of the protein.[1-6] From the magnitude of the effects of these different factors, it has been possible to obtain some insight into the molecular nature of the anion–protein complex. Since entropy and enthalpy changes accompanying a chemical transformation are frequently also very illuminating in this respect, it has seemed appropriate to examine also the temperature dependence of the binding process.

In his exploratory work on sulfonamide–plasma protein complexes, Davis[1] observed no marked change in degree of binding with rise in temperature. Similarly Putnam and Neurath[7] in their electrophoretic investigations of complexes between albumin and sodium dodecyl sulfate obtained no indication of a temperature dependence.

(1) B. D. Davis, *J. Clin. Invest.*, **22**, 753 (1943).

(2) P. D. Boyer, F. G. Lum, G. A. Ballou, J. M. Luck and R. G. Rice, *J. Biol. Chem.*, **162**, 181 (1946).

(3) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).

(4) J. D. Teresi and J. M. Luck, *J. Biol. Chem.*, **174**, 653 (1948).

(5) I. M. Klotz, H. Triwush and F. M. Walker, THIS JOURNAL, **70**, 2935 (1948).

(6) I. M. Klotz and J. M. Urquhart, *J. Phys. and Colloid Chem.*, **3**, in press (1949).

(7) F. W. Putnam and H. Neurath, *J. Biol. Chem.*, **159**, 195 (1945).

Boyer, Ballou and Luck[8] also have reported a zero temperature coefficient in the binding of caprylate by serum albumin.

The results of the present investigation indicate that the temperature dependence is indeed quite small. At least for methyl orange and azosulfathiazole, however, significant differences in binding by albumin are observed at 0 and 25°, and these can be used to calculate values for the appropriate thermodynamic functions. These quantities, in turn, lead to some pertinent conclusions on the nature of the binding process.

Experimental

The extent of binding of the reference anions by bovine serum albumin was measured by the differential dialysis technique described in detail previously.[3] Experiments were carried out, with mechanical shaking for an eighteen-hour period, in an ice-bath at 0.0 ± 0.1° for the low temperature measurements and at 25.00 ± 0.05° for the upper temperature results.

As in past work, the reference anions used were monovalent methyl orange and divalent azosulfathiazole of high purity.[3] Analyses were made by optical density, as measured by the Beckman spectrophotometer.

Buffers were prepared from reagent grade salts and acid. The acetate buffer of *p*H 5.00 had an ionic strength of 0.1000. The phosphate buffer of *p*H 6.83 had an ionic strength of 0.132.

The bovine serum albumin was a crystallized product of Armour and Company. Its water content was determined by drying a separate portion in an oven at 110°. Binding was measured with solutions containing near 0.2% of the protein.

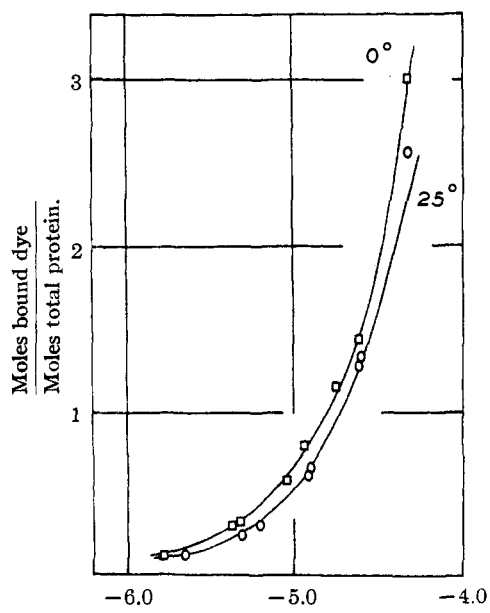

Fig. 1.—Effect of temperature on binding of methyl orange by bovine serum albumin at *p*H 6.8.

(8) P. D. Boyer, G. A. Ballou and J. M. Luck, *ibid.*, **167**, 407 (1947).

Results

The data on the binding of the monovalent anion, methyl orange, by bovine serum albumin in phosphate buffer are presented in Fig. 1. As in the past, the moles of bound ion per mole of protein, r, have been plotted as a function of the concentration of the free ion (A), in equilibrium with the protein complex. Phosphate buffer has been used since previous work[6] has indicated that competition with buffer anions is small in this system.

Corresponding data on the binding of azosulfathiazole, the divalent anion, are assembled in Fig. 2. Acetate buffer at *p*H 5, rather than phosphate at *p*H 6.8, was used in this case, in order to prevent the dissociation of the dye into a trivalent species.

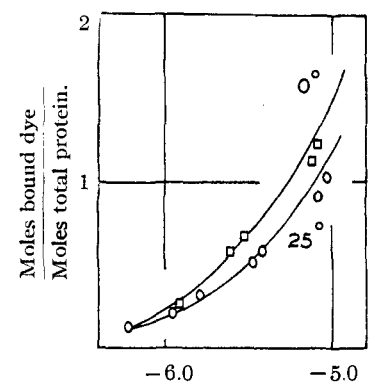

Fig. 2.—Effect of temperature on binding of azosulfathiazole by bovine serum albumin at *p*H 5.0.

Discussion

A cursory glance at the data shows that the extent of binding is not a sensitive function of the temperature. One can conclude immediately, then, that the enthalpy change for the binding process must be small (since $\Delta H = RT^2\, \mathrm{d} \ln K/\mathrm{d}T$) and that the major contribution to the favorable free energy change comes from the entropy term (since $\Delta F = \Delta H - T\Delta S$). To present these considerations in a more precise and quantitative form, however, requires further calculations.

Calculation of Binding Constants for First Complex.—A method of evaluation of free energies of formation of anion–protein complexes, PA_i, from the experimental data on binding has been presented previously.[3] Depending on the application of the law of mass action to the multiple equilibria

$$PA_{i-1} + A = PA \qquad (1)$$

this method enables one to calculate the successive equilibrium constants, k_i, and, in princi-

ple, n, the maximum number of sites on the protein available to the anion.

In practice, however, the determination of n with assurance is a very difficult problem. In previous work, an approach has been made by a plot of $1/r$ *vs.* $1/(A)$. As (A) approaches infinite concentration, r should approach n. Hence the intercept on the ordinate in a graph of $1/r$ *vs.* $1/(A)$ should be $1/n$, since the value (of zero) for $1/(A)$ at that point corresponds to infinite (A). Such extrapolations have been limited largely to situations where electrostatic factors are negligible, and hence where the binding data can be expressed by the linear function[3]

$$\frac{1}{r} = \frac{1}{kn}\frac{1}{(A)} + \frac{1}{n} \qquad (2)$$

In equation (2), k is the intrinsic binding constant and is related to k_i by the expression

$$k_i = \frac{n - (i-1)}{i} k \qquad (3)$$

However, as has been pointed out recently by Scatchard,[9] this type of extrapolation tends to obscure non-statistical, interaction effects, particularly when data at high anion concentrations are lacking, and may lead to incorrect values of n. An alternative method of plotting has been proposed by Scatchard and is based on the use of the function

$$\frac{r}{(A)} = kn - kr \qquad (4)$$

when electrostatic factors are negligible, and of an appropriately modified expression when such factors must be taken into account. As is evident from equation (4), the intercept on the *abscissa* of a graph of $r/(A)$ *vs.* r, must be n, since $r/(A)$ at this point is zero. This type of graph shows one quite clearly the length of the extrapolation from the region of experimental data, and hence gives a good indication of the reliability of the value obtained for n.

An analysis of our present data by the Scatchard method indicates that no reliable value of n can be obtained. Nevertheless, for our present purpose, an investigation of the effect of temperature on binding by albumin, it is unnecessary to know n, for the thermodynamic changes accompanying the formation of PA_i can be evaluated readily *for small values of* i by the following procedure.

From data on the extent of binding at low values of (A), it is possible to evaluate k_1 quite readily. It is evident from equation (3) that for $i = 1$

$$k_1 = nk \qquad (5)$$

Thus k_1 can be obtained from a knowledge of the product nk even if the individual factors are unknown. This product can be obtained in turn from equation (4), for it corresponds to the intercept on the *ordinate* in a graph of $r/(A)$ *vs.* r. This intercept, in contrast to that on the abscissa, can be evaluated readily, for it can be obtained by an extrapolation in the region of low values of r, and hence low concentrations of (A), a region in which adequate experimental data are available.

This procedure has been used to obtain values of k_1 for albumin complexes with each of the two anions, methyl orange and azosulfathiazole, at each of the two temperatures, 0 and 25°. The results obtained are listed in Table I.

TABLE I

BINDING CONSTANTS FOR COMPLEXES WITH BOVINE ALBUMIN

Anion	Constant	Temperature 0°	Temperature 25°
	k_1	6.9×10^4	5.0×10^4
Methyl orange	k_2	3.4×10^4	2.5×10^4
	k_3	2.2×10^4	1.6×10^4
	k_1	2.4×10^5	1.75×10^5
Azosulfathiazole	k_2	0.88×10^5	0.64×10^5
	k_3	0.43×10^5	0.31×10^5

Calculation of Binding Constants for Higher Complexes.—Since the binding data in the present series cover complexes with an average value of i as high as nearly 3, it has seemed pertinent to attempt to calculate binding constants up to k_3. Strictly speaking this cannot be done without a knowledge of n since an evaluation of the statistical contribution from equation (3), or its equivalent, depends on the value of n. However, if we assume that n is large, for example 20 or more, it can be shown readily that the magnitude of the statistical contribution is not sensitive to the specific value chosen for n so long as only small values of i are considered.

Starting from known values of k_1, one can obtain succeeding equilibrium constants by means of the equation[3]

$$RT \ln (k_{i-1}/k_i) = RT \ln \left[\frac{n - (i-2)}{n - (i-1)}\frac{i}{i-1}\right] - \Delta F_{elect.} \qquad (6)$$

where $\Delta F_{elect.}$ is the change in electrostatic free energy in the reaction

$$PA_i + PA_{i-2} = 2PA_{i-1} \qquad (7)$$

The term in the brackets on the right-hand side of equation (6) is the statistical factor. That it is not sensitive to the value of n, so long as n is large, is shown by the comparison of numerical values listed in Table II. In a more general fashion, this characteristic of the statistical contribution is evident also from the fact that the first factor,

TABLE II

COMPARISON OF STATISTICAL FACTOR FOR DIFFERENT VALUES OF n

	$\frac{n-(i-2)}{n-(i-1)}\frac{i}{i-1}$	
	$n = 22$	$n = 50$
2	2.10	2.04
3	1.58	1.53
4	1.40	1.36
5	1.32	1.28
6	1.27	1.23

(9) G. Scatchard, *Annals N. Y. Acad. Sci.*, in press.

$n(i-2)/n-(i-1)$, will be close to unity for different values of i, so long as n is large, and i is kept to the first few integers. Thus the numerical value of the bracket in equation (6) is determined essentially by the second factor $i/(i-1)$, which is obviously independent of n.

For the data on the binding of methyl orange, $\Delta F_{\text{elect.}}$ was assumed to be zero, as in previous work, because the results did fit a linear presentation in terms of equation (2). The constants k_2 and k_3 can then be evaluated readily at each temperature by application of equation (6). The results obtained have been included in Table I. The corresponding free energy changes can be expressed in the form of the following equations (in units of calories/mole)

$$\text{At } 0°: \quad \Delta F_i^0 = -6049 + \sum_{j=2}^{j=i} RT \ln \frac{n-(j-2)}{n-(j-1)} \frac{j}{j-1} \tag{8}$$

$$\text{At } 25°: \quad \Delta F_i^0 = -6411 + \sum_{j=2}^{j=i} RT \ln \frac{n-(j-2)}{n-(j-1)} \frac{j}{j-1} \tag{9}$$

In complexes between serum albumin and azosulfathiazole, on the other hand, electrostatic interactions between successively bound anions do not seem to be negligible. The term $\Delta F_{\text{elect.}}$ in equation (6) was calculated, as previously,[3] on the assumption of a spherical protein molecule of radius 30 Å., and a value of 4 Å. for the radius of the dye anion. At 0°, $\Delta F_{\text{elect.}}$ turns out to be 160 cal./mole, and at 25°, 177 cal./mole. With these values, k_2 and k_3 can be calculated readily from equation (6), and the results obtained have also been placed in Table I. The corresponding free energy changes may be expressed by the following equations (in units of calories/mole)

$$\text{At } 0°: \quad \Delta F_i^0 = -6725 + \sum_{j=2}^{j=i} RT \ln \frac{n-(j-2)}{n-(j-1)} \frac{j}{j-1} + 160(i-1) \tag{10}$$

$$\text{At } 25°: \quad \Delta F_i^0 = -7151 + \sum_{j=2}^{j=i} RT \ln \frac{n-(j-2)}{n-(j-1)} \frac{j}{j-1} + 177(i-1) \tag{11}$$

The additional term in each of the equations (10) and (11), in comparison with (8) and (9), represents the contribution of the electrostatic interaction factor.

Calculation of Enthalpies and Entropies of Binding.—From the equations for the free energy changes it is possible to deduce expressions for the enthalpy and entropy changes accompanying the formation of the respective protein–anion complexes by standard thermodynamic procedures. These functions can be summarized as follows (the units for ΔH being calories/mole, those for ΔS being calories/mole degree)

Methyl orange–albumin complexes

$$\Delta H^0 = -2100 \tag{12}$$

$$\Delta S_i^0 = 14.5 - \sum_{j=2}^{j=i} R \ln \frac{n-(j-2)}{n-(j-1)} \frac{j}{j-1} \tag{13}$$

Azosulfathiazole–albumin complexes

$$\Delta H^0 = -2000 \tag{14}$$

$$\Delta S_i^0 = 17.1 - \sum_{j=2}^{j=i} R \ln \frac{n-(j-2)}{n-(j-1)} \frac{j}{j-1} \tag{15}$$

Strictly speaking, the ΔH^0 for azosulfathiazole complexes should have two additional terms, one containing the factor $\Delta F_{\text{elect.}}$ and the second its temperature coefficient. In practice, however, it turns out that these terms balance each other approximately, so that ΔH^0 does not vary significantly for different values of i.

Significance of the Thermodynamic Changes in Binding.—In considering the magnitudes of the thermodynamic changes associated with the binding process, one is struck particularly by the positive entropy values. Despite the fact that the formation of the complex, as represented by the simple expression in equation (1), is an association reaction, the entropy of the system does not drop, but increases. Thus the formation of albumin–anion complexes stands in direct contrast with that of cellulose–anion complexes, where the association reactions are accompanied by negative entropy changes,[10] as one would expect from a simple molecular picture. The difference between these two systems may be, however, in the use of fibers, rather than soluble macromolecules, in the cellulose investigations; for the values of the equilibrium constants and temperature coefficients for anion–wool fiber combinations reported by Steinhardt[11] indicate a decrease in entropy for the uptake of organic ions.

The positive entropies associated with many reactions involving proteins are attributed frequently to disorientation and unfolding of the protein molecule, and a similar explanation might be offered here. Such a picture in connection with anion–protein complexes seems unlikely, however, since the enthalpy changes involved are negative and small, whereas a process of unfolding presumably requires the breaking of several bonds and hence should be an endothermic reaction of appreciable magnitude.

An alternative explanation, which is consistent with both the observed entropy and enthalpy changes, would focus attention on the electrostatic nature of the species involved in the formation of these anion–protein complexes. Though the anion may be indicated merely by the symbol A^-, it is recognized that this ion has several polarized water molecules "frozen" to it in the aqueous solution. Similarly the protein molecule is highly hydrated, particularly, perhaps, around the charged loci of the free carboxyl groups and the quaternary nitrogen atoms. Recent evidence from measurements of the decrease in binding with increasing pH,[12] as well as from observations of

(10) W. J. Marshall and R. H. Peters, *J. Soc. Dyers Colourists*, **63**, 446 (1947).

(11) J. Steinhardt, *Annals N. Y. Acad. Sci.*, **41**, 287 (1941).

(12) I. M. Klotz and F. M. Walker, THIS JOURNAL, **69**, 1609 (1947).

loss in binding ability on acetylation of the amino groups of albumin,[13] points to the participation of the charged nitrogen loci in the bond with the anion. The formation of such an electrostatic bond would release some of the "frozen" water molecules from the constituent ions. Thus there would be an *increase* in the number of molecular species upon formation of the anion–protein complex, rather than a decrease, as indicated by equation (1), and hence one would reasonably expect an increase in entropy for the process. It is of interest in connection with this interpretation that Lundgren[14] has observed an increase in the volume of the solution when an alkylsulfonate anion is bound by egg albumin.

This view of the mechanism of the binding process makes it analogous to the ionization process of organic carboxylic acids. Written as an association reaction, the ionic changes in these acids may be represented by the equation

$$H^+ + A^- \rightleftharpoons HA \qquad (16)$$

For the aliphatic organic acids the entropy change accompanying the reaction shown is around 20 calories/mole degree and the enthalpy change of the order of several hundred calories/mole.[15] Here too, then, there is an increase in entropy in a reaction which superficially involves a reduction in the number of molecular species. When one recognizes, however, that the disappearance of the free ions is accompanied by the release of fixed water molecules, the positive entropy for reaction (16) becomes reasonable.

The volume changes accompanying the ionization processes of organic acids also parallel those for anion–protein complexes. Thus it has been observed[16] in several different ionic systems that reaction (16) is associated with an increase in volume of the order of 20 cc./mole. While corresponding quantitative data for the binding process by albumin are not available, the qualitative observations of Lundgren[14] are of the correct sign. In both cases, an increase in volume fits the assumption that water molecules are released in the association reaction.

The analogy between these two processes may be pursued even further by a comparison of the changes in the thermodynamic quantities for the association process with variation in the structure of the anion. It is of interest to note that the change of 1369 calories/mole[15] in ΔF^0 for reaction (16) in going from formic to acetic acid is due almost entirely to the 4.5 calories/mole degree difference in ΔS^0 for the reaction with hydrogen ion, a difference which may be attributed to the greater degree of hydration of the neutral molecule with the single carbon atom. Correspondingly, in the present investigation with a protein, a comparison of the thermodynamic changes in the formation of the first[17] complex, PA_1, with methyl orange and azosulfathiazole, respectively, reveals that the more favorable free energy of binding of the latter ion is due to the greater change in entropy in the formation of the protein complex. On the molecular level, then, these characteristics of the thermodynamic quantities associated with the binding process indicate that the higher affinity for some of the larger anions is not due so much to increased van der Waals interaction, but rather to a greater degree of dehydration of the complex.

Conclusion.—While the present investigations have been carried out with a single type of molecule, sulfonated azo compounds, the qualitative observations on other anions[1,7,8] of very different atomic structure indicate that here too the heat of binding is quite small and that the favorable free energy for association is due primarily to the positive entropy term. It is of interest to note also that substantially the same conclusions have been reached in a study of metallic cation–albumin complexes.[18] It becomes apparent, then, that in comparisons of changes in binding energy with variations in structure of the small ion, greater attention should be paid to the ability of the ion to force the release of frozen water molecules on the ion–protein complex.

Acknowledgments.—This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service. The authors are indebted also to Dr. Fred Karush of New York University Medical School, and to Dr. Ralph Pearson of Northwestern University for numerous profitable discussions.

Summary

The binding of methyl orange and of azosulfathiazole, respectively, by bovine serum albumin has been examined quantitatively at 0 and at 25°. For the formation of the first anion-protein complex, the following thermodynamic changes have been calculated: Methyl orange; $\Delta F_0^0 = -6049$ cal./mole, $\Delta F_{25}^0 = -6411$ cal./mole, $\Delta H^0 = -2100$ cal./mole, $\Delta S^0 = 14.5$ cal./mole deg.; azosulfathiazole: $\Delta F_0^0 = -6725$ cal./mole, $\Delta F_{25}^0 = -7151$ cal./mole, $\Delta H^0 = -2000$ cal./mole, $\Delta S^0 = 17.1$ cal./mole deg.

The large positive entropy of binding, coupled with the small enthalpy of binding, have been interpreted to mean that the major contribution to the free energy of binding comes from the release of solvent molecules from the anion–protein complex.

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(13) Unpublished work in this Laboratory.

(14) H. P. Lundgren, *Textile Research J.*, **15**, 335 (1945).

(15) D. H. Everett and W. F. K. Wynne-Jones, *Trans. Faraday Soc.*, **35**, 1380 (1939); H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publishing Corp., New York, N. Y., 1943, p. 514.

(16) H. H. Weber, *Biochem. Z.*, **218**, 1 (1930); I. M. Klotz and C. F. Eckert, THIS JOURNAL, **64**, 1878 (1942).

(17) The first complex only has been chosen for comparison since electrostatic interactions between successively bound ions would obscure the picture in considerations of higher complexes.

(18) I. M. Klotz and H. G. Curme, THIS JOURNAL, **70**, 939 (1948)