consumption referred to above is the rate per mole of di-(3-methylbutyl) adipate. It has already been indicated that the peroxide concentration during oxidation probably approaches a steady state. It therefore follows that concentration of hydroperoxide in the above kinetic treatment may be assumed constant.

TABLE VI

AIR OXIDATION OF DI-(3-METHYLBUTYL) ADIPATE IN DIETHYL ADIPATE AT 100°. EQUIVALENTS PEROXIDE PER MOLE DI-(3-METHYLBUTYL) ADIPATE

Mole fraction di-(3-methylbutyl) adipate	24 hr.	48 hr.	72 hr.	96 hr.
0.179	2.06	3.02	6.86	..
.367	2.59	6.62	12.1	18.2
.566	..	6.32	11.7	18.1
.778	2.57	6.66	12.5	17.6
1	2.86	7.90	11.9	23.4

The kinetic analysis above does not account for the fate of HO· nor the formation of acid. While it is probable that HO· is associated with the later stages of oxidation, further work is required to clarify this point.

Energy of Activation.—Energies of activation for steady oxidations were determined on di-(3-methylbutyl) adipate, di-(1-ethylpropyl) adipate and di-*n*-pentyl adipate. The log of oxidation rate (moles oxygen consumed per mole ester per second) are plotted against reciprocal of absolute temperature in Fig. 5 and the calculated activation energies are in Table VII. These data provide further evidence for the effect of structure on oxidation rate.

TABLE VII

ENERGY OF ACTIVATION (CAL./MOLE)

Ester	Energy of activation
Di-(3-methylbutyl) adipate	21,900
Di-(1-ethylpropyl) adipate	26,300
Di-*n*-pentyl adipate	26,100

Acknowledgment.—The authors are indebted to the Chemistry Staff of the Pitman–Dunn Laboratories, Frankford Arsenal, where this investigation was conducted in part as an Army Ordnance Project.

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CONTRIBUTION FROM NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH]

Polarographic Analysis of the Serum Albumin–Mercury and Zinc Complexes[1a,b]

BY H. A. SAROFF AND H. J. MARK

RECEIVED OCTOBER 30, 1952

The polarography of protein–metal complex solutions is considered in light of the present limited theory of diffusion currents controlled by rates of reaction and diffusion. An experimental method is described for the use of the polarograph in analyzing protein–metal complexes based on a combination of amperometric titrations and dialysis experiments. Zinc in an acetate buffer at *p*H 6.1 binds bovine albumin at eight similar sites with an intrinsic constant of 1200. Mercuric chloride shows binding to at least two different sets of sites on bovine serum albumin at *p*H 4.9 in an acetate buffer, one, a single (sulfhydryl) site with a constant of 10^7, the other, five sites with a K value of 27000 to 45000.

Proteins have been reported to affect the polarographic wave by lowering the diffusion current[2] and shifting the half-wave potential.[3] Shifts in half-wave potential have been employed for the calculation of some constants of non-protein complexes.[4] Because of the size of the protein molecule (and its small diffusion coefficient) some of the shifts in half-wave potential with proteins are difficult to explain on the basis of complexation of the protein with the substance measured since marked shifts in half-wave potential have been found even under conditions where little change in diffusion current could be detected. It is probable that such shifts in potential are a function of adsorption of the protein on the surface of the mercury drop rather than complexation with the substance electrolyzed. While these shifts are important and must be explained in order to arrive at a complete description of the electrolysis process in studying protein metal complexes with the polarograph, this communication illustrates a method for calculating the binding constants of a protein–metal complex from changes in the diffusion current of metal ion solutions in the presence of proteins.

The problem involved in the electrolysis of a mixture containing free protein, free metal ion and protein–metal complex about a dropping mercury electrode has not yet been solved. The problem may be formulated by considering the diffusion and reaction rate conditions about a dropping mercury electrode in a solution at equilibrium with the reaction defined by

$$M^+ + HP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MP + H^+ \qquad (1)$$

where M^+ is a reducible metal ion, HP a protein, MP the metal protein complex (reducible or non-reducible), and H^+ the hydrogen ion concentration. Solutions to an analogous problem but with the same diffusion coefficient for the reducible substance and the complex (non-reducible) have been published by Koutecky and Brdicka[5] and Delahay.[6] These solutions do not apply to the protein problem mainly because of the difference in the diffusion

(1) (a) Parts of this paper were extracted from Report No. 1 by H. A. Saroff and H. J. Mark, Project NM 000006, Naval Medical Research Institute, Bethesda, Maryland, 1949. (b) Presented before the Division of Biological Chemistry, 116th Meeting, American Chemical Society, September, 1949.

(2) I. M. Kolthoff and J. J. Lingane, "Polarography," Interscience Publishers, Inc., New York, N. Y., 1941, p. 121.

(3) R. H. Coe and L. B. Rogers, THIS JOURNAL, **70**, 3276 (1948).

(4) Reference 2, p. 170.

(5) J. Koutecky and R. Brdicka, *Collection Czechoslov. Chem. Communs.*, **12**, 337 (1947).

(6) P. Delahay, THIS JOURNAL, **73**, 4944 (1951).

coefficient of the metal ion and that of the protein complex. For the theory already developed, the limiting case, in which the dissociating rate of the complex, k_2, is very large compared to the diffusion rate, will yield a diffusion current for a dissociating non-reducible complex which will be the same as that for the complex reduced at the dropping electrode (Ilkovic limiting case). An implied assumption of such a reducible protein complex at the dropping electrode was made by Stricks and Kolthoff for the calculation of the diffusion coefficient of a serum albumin–methyl orange complex.[7]

While a general theory of diffusion currents controlled by rate of reaction and diffusion has not yet been developed for the protein complex system, the limited available theory may be extended by the proper interpretation of a combination of such types of polarographic analyses as (a) amperometric titrations and (b) dialysis experiments.

Material and Experimental Procedure

Bovine serum albumin, control 4802 four times crystallized, was purchased from Armour and Company, Chicago, Illinois. All albumin concentrations are based on salt-free solutions dried to constant weight at 105–110° and a molecular weight of 69000. Buffers of constant ionic strength were prepared from the data of Green.[8]

Reagents used were Baker C.P. grade chemicals.

pH Measurements were made with a Beckman model G glass electrode assembly at room temperature.

Polarographic Measurements.—The polarograph used was the Sargent model XXI. This instrument has been adequately described by Lingane.[9]

All measurements were made with a saturated calomel reference electrode connected to the cell through a saturated KCl agar bridge. Most of the analyses were made in a cell containing 7 ml. of solution.

Since all of the measurements were carried out in a constant temperature room at 26 ± 0.5°, the cells used were not thermostated but were quite simple, consisting of the end of a test-tube closed with a rubber stopper holding KCl–agar bridge, nitrogen tube and dropping electrode capillary.

Mercury measurements were made both with and without the presence of oxygen. The presence of oxygen presented no special problem although the solution of mercury in the presence of oxygen has been reported.[10]

In order to evaluate this error, a solution containing 1% albumin, total $HgCl_2$, 20×10^{-5} M, pH 4.9 and $\Gamma/2 = 0.1$ sodium acetate, was prepared and readings taken as a function of time at a given voltage. A volume of 3.5 ml. gave a 3 mm. (sens. = 0.03 μ a./mm.) rise in 12 minutes. The small error involved was minimized by corrections for the residual current determined for blank solutions under identical conditions.

It was necessary when working with albumin solutions to take into consideration the effect of the protein on the half-wave potential. This was particularly important in making measurements of mercuric ions in albumin with oxygen present. Under these conditions, the optimum position of the plateau for the measurement shifted with both pH and albumin concentration.

For measurements on oxygen-free albumin solutions, the normal procedure for removing oxygen from an albumin solution was not applicable, due to foaming. At first, the oxygen was removed, when necessary, by evacuation of the cold solutions. Later this procedure was replaced by one in which the protein base solution was separately prepared and kept oxygen free. The oxygen-free solution was then added from a buret to the rest of the solution containing the metal salt and buffer previously freed of oxygen by bubbling nitrogen through the solution.

All of the diffusion current measurements were corrected for the residual current by use of the proper blanks.

Drop times varied from about 3.8 to 4.2 seconds per drop measured at the voltage at which the diffusion current was determined. All of the measurements were recalculated to a 4.0 second drop time by means of the Ilkovic equation. An $m^{2/3}t^{1/6}$ value of 1.82 mg.$^{2/3}$ sec.$^{-1/2}$ was obtained.

In the polarographic evaluation of the standard solutions of mercuric chloride and cupric acetate, methyl red, approximately 1×10^{-5} M was used as a maximum suppressor. It was found important, in the copper analysis, to keep the concentration of methyl red low since the half-wave potential for its reduction is close to that of copper. No maximum suppressor as such was required in the albumin solutions.

Viscosity corrections, when applied, would increase the values of diffusion current by amounts calculated from the effects of the viscosity on the diffusion coefficient.[9,11] Application of these corrections would bring about but minor changes in the albumin complex data. Such a correction to an albumin solution was questioned by McKenzie.[9] In the range of albumin concentrations used, our data were corrected only for the copper ion titration (Fig. 5).

Results and Discussion

General.—A shift of the half-wave potential was observed in most of our experiments as an effect of albumin on the polarographic wave. Oxygen measured in a solution buffered at pH 4.9 ($\Gamma/2$ = 0.1 in sodium acetate) showed on the addition of albumin a shift in half-wave potential to a more negative value with no change in diffusion current. Mercuric, cupric and zinc ions all showed negative shifts in half-wave potentials in the presence of albumin[12] while hydrogen ion showed a positive shift. Not only did the albumin shift the half-wave potential but it also rendered some polarograms asymmetrical even when no change occurred in the diffusion current (zinc and oxygen pH 4.9). Figure 1 illustrates quantitatively the effects of

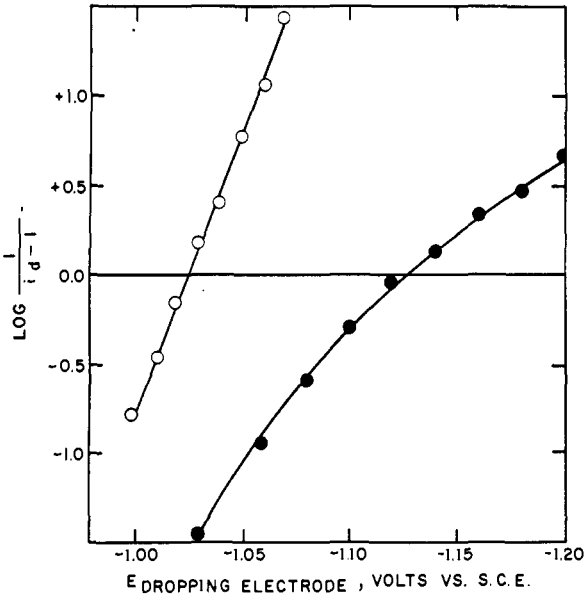

Fig. 1.—Log plot illustrating the effect of albumin on half-wave potential of zinc ions, in NaAc buffer pH 4.9, $\Gamma/2$ = 0.1: O, $ZnSO_4$, 28.6×10^{-5} M; ●, $ZnSO_4$, 28.6×10^{-5} M, albumin 1.9%.

(7) W. Stricks and I. M. Kolthoff, THIS JOURNAL, **71**, 1519 (1949).

(8) A. A. Green, *ibid.*, **55**, 2331 (1933).

(9) J. J. Lingane, *Anal. Chem.*, **21**, 46 (1949).

(10) E. R. Smith, J. Taylor and R. Smith, *J. Research Natl. Bur. Standards*, **37**, 151 (1946).

(11) D. M. Brasher and F. R. Jones, *Trans. Faraday Soc.*, **42**, 775 (1946).

(12) Similar shifts have been reported for cadmium and lead, C. Tanford, THIS JOURNAL, **74**, 211 (1952).

albumin on the zinc polarogram by means of the familiar log plot.

It may be assumed that for the zinc, mercury and copper ions, the change in diffusion current represents complex formation. However, the proper interpretation of these changes in diffusion current requires, at present, complete amperometric titrations and some additional polarographic data from dialysis or solubility experiments to determine quantitatively the contribution of the protein–metal complex to the measured diffusion current.

Amperometric titrations constitute a sensitive method for making some first order estimates concerning the protein–metal complexes formed. For reactions of the type $nM^+ + H_nP = M_nP + nH^+$, curves were calculated assuming a reducible or very rapidly dissociating complex by use of the equations

$$i_d = \left[c + (c_0 - c) \frac{D^{1/2}_{\text{albumin}}}{D^{1/2}_{\text{metal ion}}} \right] F \qquad (2)$$

$$\bar{\nu} = \frac{c_0 - c}{P} = \frac{Kcn}{1 + Kc} \qquad (3)$$

for a uniform set of n sites where i_d is the measured diffusion current (in microamperes) c_0 and c are the total and free concentrations (in moles per liter), respectively, of the metal ion, D is the diffusion coefficient, F is the diffusion current in microamperes per unit concentration of free metal ion determined from standard solutions corrected to the same value of $m^{2/3}t^{1/6}$, $\bar{\nu}$ is the average number of metal ions bound per mole of protein, P is the total concentration of protein and K is the intrinsic equilibrium constant.[13,14] The sensitivity of such an amperometric titration to the values of n and K is illustrated in Fig. 2. A common situation encountered is that with more than one set of sites. These sets of sites in the protein may all be reducible (or dissociate very rapidly) at the electrode or may be made up of some sets of reducible

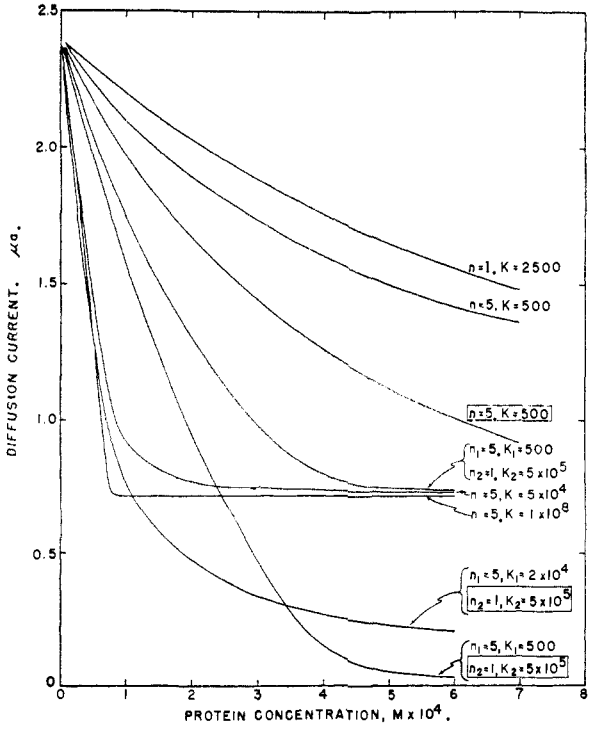

Fig. 2.—Calculated curves, with different n's and K's, for amperometric titrations of a metal ion (total concentration = 4.0×10^{-4} M) with protein. $D^{1/2}$protein/$D^{1/2}$metal ion = 0.30; $F = 6.0 \times 10^3$; K and n values, boxed in the figure, represent sets of sites which do not contribute to the diffusion current.

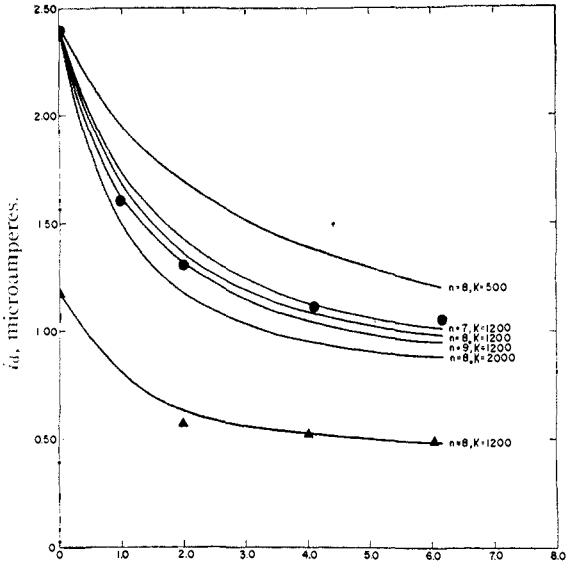

Fig. 3.—Experimental and calculated amperometric titrations of zinc acetate solutions with albumin in NaAc buffer, pH 6.05 ± 0.10, $\Gamma/2 = 0.05$: ●, zinc acetate, 4.0×10^{-4} M; ▲, zinc acetate, 2.0×10^{-4} M.

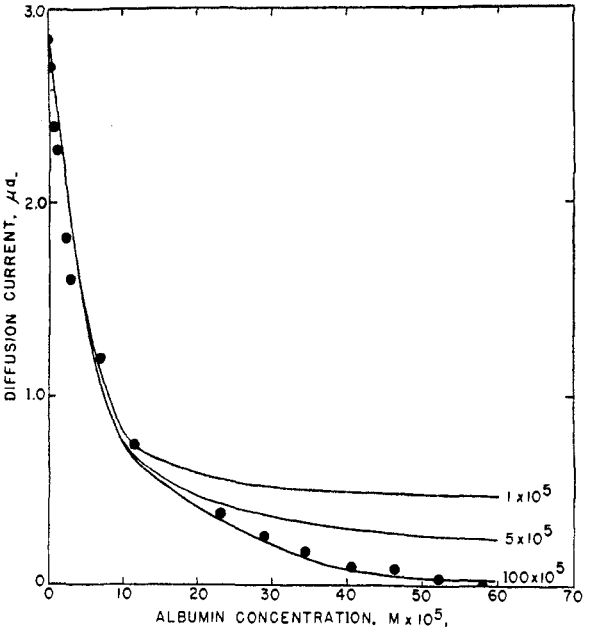

Fig. 4.—Experimental and calculated ($n_1 = 5$, $K_1 = 27000$, $n_2 = 1$, K_2 given in figure) amperometric titrations of mercuric chloride solutions with albumin in a sodium acetate buffer pH 4.9, $\Gamma/2 = 0.1$, total mercuric chloride 40×10^{-5} M.

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

(14) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

sites and other sets which are non-reducible (or dissociate very slowly); Fig. 2 illustrates a calculated curve for different values of n and K for two sets of sites, both reducible. Shown in addition are calculated curves for two sets of sites, one reducible and one non-reducible for which the following equations were used

$$i_d = \left[c + \left(\frac{PK_1cn_1}{1 + K_1c}\right)\frac{D^{1/2}_{\text{albumin}}}{D^{1/2}_{\text{metal ion}}}\right]F \quad (4)$$

$$\bar{\nu} = \frac{c_0 - c}{P} = \frac{K_1cn_1}{1 + K_1c} + \frac{K_2cn_2}{1 + K_2c} \quad (5)$$

where the subscripts 1 and 2 refer to the reducible and non-reducible sets of sites, respectively.

Experimental amperometric titrations made on zinc, mercury and copper ion solutions with bovine serum albumin illustrate three decidedly different titration curves, Figs. 3 and 4 (experimental points) and Fig. 5. Consideration of these data and the calculated curves already described indicates that without further analysis it might be difficult to distinguish between a reaction with a low equilibrium constant value in which the complex is not reduced, and a reaction with a high equilibrium constant value in which the complex is reduced. Moreover, reactions with complexes which contain both non-reducible and reducible sites (as in the mercury–albumin complex) further complicate the interpretation of a titration curve.

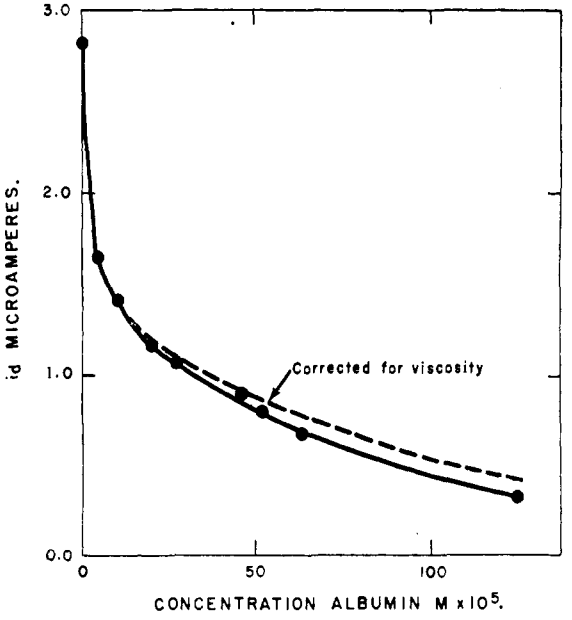

Fig. 5.—Amperometric titration of cupric acetate with albumin in NaAc buffer, pH 4.9, $\Gamma/2$ = 0.1, total $Cu(Ac)_2$ 40 × 10^{-5} M.

Dialysis.—Similar considerations allow predictions as to expected diffusion current data from dialysis experiments. A typical dialysis experiment is illustrated in Fig. 6 where the presence of an albumin–mercury complex which contributed to the measured diffusion current was demonstrated. For similar conditions, the differences found in these experiments (that is, the diffusion current values in the presence of protein minus the diffusion current values of the equilibrated buffer solution of the ion measured) provide a necessary correction to be applied to amperometric titration data for the conversion of diffusion current measurements to concentrations of free metal ions in protein solutions. Calculations of $\bar{\nu}$ may be made from these data by the usual method[13] and by the application of the Ilkovic equation.[15]

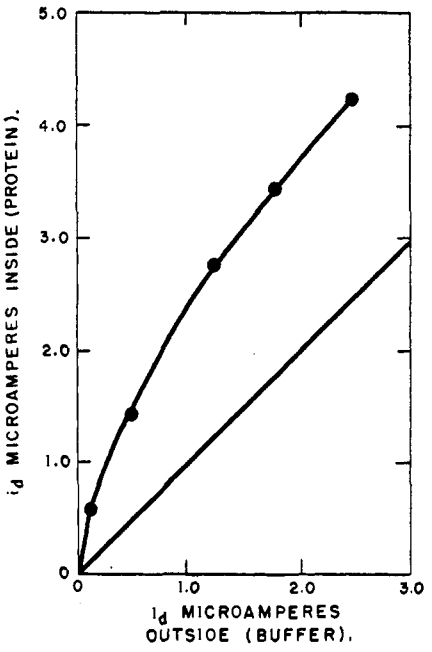

Fig. 6.—Dialysis experiment: concentration $HgCl_2$ varied from 20 to 80 × 10^{-5} M, concentration albumin 14.4 × 10^{-5} M, pH 4.9, $\Gamma/2$ = 0.1 NaAc.

Partial Dissociation.—From the dialysis experiments, corrections will be obtainable for the amperometric titrations regardless of the mechanism by which the protein complex contributes to the apparent diffusion current. Partial dissociation at the mercury drop, as well as a "pure" kinetic current will contribute to the measured diffusion current allowing for correction. An experimental test for either partial dissociation or "pure" kinetic current is possible by correcting, with dialysis data, the amperometric titration with fixed protein concentration, evaluating the data for n and K and calculating, on the basis of complete reduction or rapid dissociation, a curve for the amperometric titration with fixed metal ion concentration. A good fit is an indication of complete reduction or rapid dissociation. A calculated curve yielding higher diffusion current values than those of the experimental one would point to the possibility of the presence of a complex which is partly dissociated at the drop. Another method available for detecting partial dissociation is the application of plots of values of the diffusion current constant as a function of drop time. Such plots[9,16,17] illustrate deviations from the Ilkovic equation. For the dropping electrode, the diffusion

(15) The correctness of such a calculation will depend, in part, on that of the value taken for the diffusion coefficient of the complex. See L. Meites, THIS JOURNAL, **73**, 4257 (1951).

(16) L. Meites and T. Meites, *ibid.*, **72**, 3686 (1950).

(17) J. K. Taylor and R. E. Smith, *J. Research Natl. Bur. Standards*, **48**, 172 (1952).

constant will be independent of the drop time for a pure diffusion current (Ilkovic) while the diffusion current value itself will be independent of drop time for a "pure" kinetic current. These, and the intermediate conditions, are expressed quantitatively by Delahay's theory[6] where a single diffusion coefficient may be used for both the complex and the reducible dissociation product. Although a quantitative interpretation for such an analysis with an albumin–metal complex ($D = 0.067 \times 10^{-5}$) and a reducible metal ion (D = approx. 1×10^{-5}) is not available at present, data were collected for examination. Slopes from diffusion current constant data plotted as a function of drop time (2 to 6 sec.) for solutions containing albumin complexes and the respective free metal ions (mercury and zinc) were found to be nearly the same (3% difference in change of diffusion current constant).

Albumin–Zinc Complex.—An amperometric titration with total protein constant was corrected by means of dialysis experiments with a similar concentration of protein at the same *p*H, 6.1. The corrected titration yielded values of diffusion current which were directly proportional to the concentration of the free zinc in the complex solution. Figure 7 (open circles) shows these data in the form of $\bar{\nu}$ as a function of log c. From these data the values $n = 8$ and $K = 1200$ were determined from a plot of $\bar{\nu}/c$ as a function of ν.[14] With these values[18] found, it became possible to examine more critically the amperometric titration with constant total zinc (experimental points, Fig. 3). Since the lower value for the diffusion current of the amperometric titration was greater than that calculated from the diffusion coefficient of albumin assuming a very large value for K with either complete dissociation or reduction of the complex, three conditions appear most probable: (a) complex not reduced with a relatively low value for K; (b) complex partly dissociated with a relatively larger value for K, or (c) complex reduced or completely dissociated with a K value larger than that for conditions (a) and (b). Condition (a) may be eliminated by the fact that the protein solution containing zinc ions yielded a diffusion current value greater than that for the solution of zinc ions with which it was equilibrated by dialysis across a cellophane membrane. Support for ruling out partial dissociation (condition b) was found in the similarity of the slopes of plots of diffusion current constant as a function of drop time (see above). The remaining condition, that is, reduction or complete dissociation of the complex was given some additional support by comparing values for $\bar{\nu}$ calculated from the dialysis data (two methods of calculation) and from the corrected amperometric titration. Figure 7 illustrates such a comparison showing reasonable agreement.

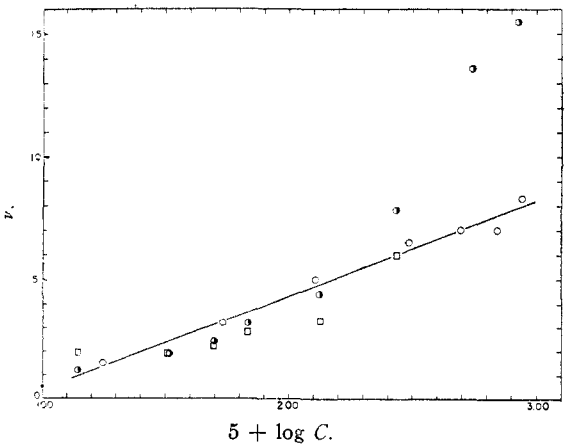

Fig. 7.—Comparison of binding data for zinc and 0.99% albumin at *p*H 6.1, $\Gamma/2 = 0.05$ in NaAc; O, corrected amperometric titration; □, Ilkovic equation; ◑, distribution data (two values at high free zinc concentrations were large due to the fact that a small amount of the albumin–zinc complex precipitated under the conditions of the dialysis experiment).

(18) The values $n = 8$ and $K = 1200$ are those for the buffer conditions, *p*H 6.1, $\Gamma/2 = 0.05$ NaAc, in which competition with both the hydrogen ion and the buffer affect n and K.

Given the assumption of either reduction or very rapid dissociation of the complex, equations (2) and (3) were employed to describe the measured diffusion current for the amperometric titration of zinc acetate with albumin with the diffusion coefficient values 0.067×10^{-5} cm.^{2} sec.$^{-1}$ for serum albumin, (average of reported values) and, 0.77×10^{-5} cm.^{2} sec.$^{-1}$ for zinc ions (from the polarographic measurements) and the value 6.01×10^3 microamperes mole^{-1} for the constant, F. A series of curves calculated from these equations for different values of n and K are shown with some experimental amperometric titration data in Fig. 3. The agreement of the $n = 8$, $K = 1200$ plot with the experimental points supports the use of equation (2) for a reduced or rapidly dissociating complex with the diffusion coefficient of the albumin controlling the contribution of the zinc–albumin complex to the total diffusion current.

The albumin–zinc complex represents an example where a single set of sites, with a single K, appears sufficient to describe these data at *p*H 6.1. Gurd and Goodman[19] reported a value of from 510 to 810 for K using dialysis equilibrium data and attributed the binding to the 16 imidazole groups of serum albumin. Tanford[12] reported a value of from 670 to 1000 for K using polarographic data with an entirely different approach.[20] These data are in good agreement with our result for the value of K.

Albumin–Mercury Complex.—Several amperometric titrations were made with both fixed concentrations of mercuric chloride and fixed concentrations of albumin, Figs. 8 and 9. Mercury was bound by albumin in increasing amounts with increasing *p*H. At *p*H values above 6, a precipitate of the mercury albumin complex[21] formed at

(19) F. R. N. Gurd and D. S. Goodman, THIS JOURNAL, **74**, 670 (1952).

(20) The method of Tanford will give reliable results for the albumin–zinc complex where there is a single set of sites in spite of the errors involved in the choice of the value of α (a constant representing the contribution of the complex to the measured diffusion current). However, this method may give misleading results when more than one K is necessary to describe the complex, as in the albumin–mercury complex (see below), since the concentration ratio of metal ions to albumin at which the measurements are made then becomes a critical factor.

(21) This property was used by W. Haarman, *Biochem. Z.*, **314**, 1 (1943), to determine mercury binding by bovine serum albumin.

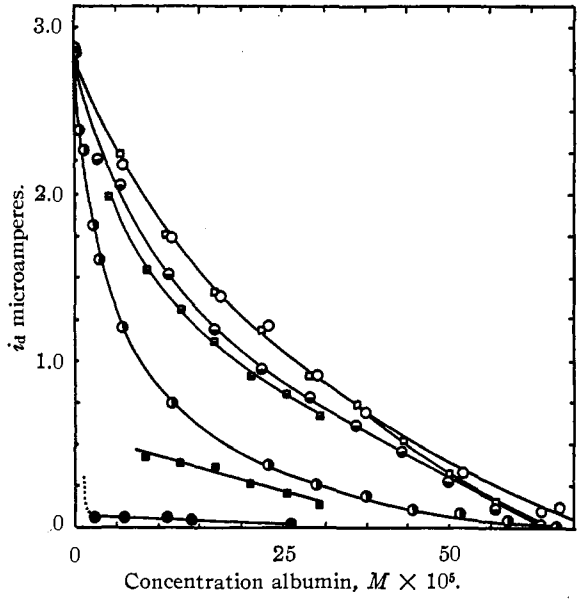

Fig. 8.—Amperometric titrations of mercuric chloride solutions, concentration, 40×10^{-1} M, with albumin; $\Gamma/2 = 0.1$, sodium acetate from pH 3.5 to 4.9 inclusive, sodium phosphate for pH 6.2 and 7.5: ○, pH, 3.5; □, pH 4.0; ◒, pH 4.4; ◨, pH 4.6; ◑, pH 4.9; ■, pH 6.2; ●, pH 7.5.

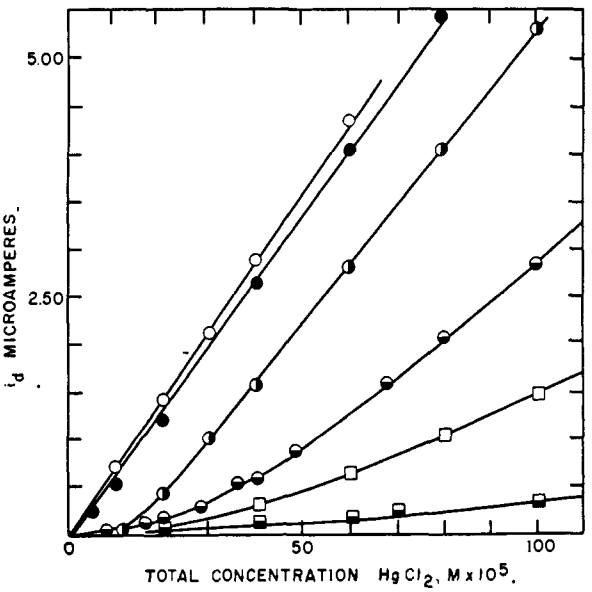

Fig. 9.—Amperometric titrations of albumin with mercuric chloride: $\Gamma/2 = 0.1$, sodium acetate, pH 3.5 to 5.6, and sodium phosphate pH 7.5: ○, mercuric chloride solutions, pH 4.9; ●, mercuric chloride solutions, pH 3.5; ◑, albumin concentration 14.8×10^{-5} M, pH 3.5; ◒, albumin concentration 15.7×10^{-5} M, pH 4.9; □, albumin concentration 13.7×10^{-5} M, pH 5.6; ◨, albumin concentration 13.7×10^{-5} M, pH 7.5. Mercuric chloride values at pH 5.6 and pH 7.5 were identical with those at pH 4.9.

albumin concentrations of less than approximately 0.3% at an ionic strength of 0.1. With an increase in concentration of protein or a decrease in pH the complex was redissolved. All of our measurements, unless otherwise noted, were made on solutions in which the complex was soluble. The titrations in which total mercuric chloride concentrations were held constant indicated the formation of an albumin mercury complex which was not reduced and did not dissociate at the dropping electrode. However, dialysis experiments gave data (at pH 4.9, Fig. 6) indicating that an albumin-mercury complex was formed which contributed to the diffusion current by reduction or dissociation. Some evidence excluding partial dissociation of the contributing complex at pH 4.9 was found in the data on the diffusion current constant as a function of drop time. With the assumption of either a reducible or completely dissociating contributing complex, calculations were made on the binding of mercury by albumin both from the dialysis data and from the amperometric titration (constant albumin) at pH 4.9, corrected by the dialysis data. The dialysis data values from the Ilkovic equation represent the number of mercury ions per mole of albumin bound in the reducible (or contributing complex) while the other values represent the total number of mercury ions bound. The results of the calculations are given in Table I.

These data show reasonable agreement, for this type of analysis, for the number of ions bound. Here, and with the analysis for the albumin–zinc complex, the corrected amperometric titration was considered the most reliable data due to the fact that first-order errors in the dialysis experiments either do not affect the correction or appear as second-order errors when the data are applied as a correction to the amperometric titration. Thus any ion that is bound by the cellophane will not affect the correction value but will alter the values calculated from the dialysis distribution data.

TABLE I

CALCULATED VALUES OF NUMBER OF MERCURY IONS BOUND PER MOLE OF ALBUMIN ($\bar{\nu}$) FROM AMPEROMETRIC TITRATION AND DIALYSIS DATA FOR ALBUMIN SOLUTIONS AT pH 4.9, $\Gamma/2$ = 0.1 NaAc

Concn., free $HgCl_2$, $M \times 10^5$	Distribution[a] (dialysis) data	Ilkovic[b] equation (reducible complex)	Amperometric[c] titration corrected
0.7			1.7
1.1	3.8	1.9	
1.5			2.2
1.8			2.4
3.0			2.9
6.9	6.4	4.1	
7.0			3.8
11.5			4.3
17.6	7.6	6.0	
19.8			5.1
25	9.7[d]	6.2	
35	12.1[d]	7.1	

[a] Uncorrected for adsorption of mercuric chloride by cellophane. [b] Concentration albumin 0.99%. [c] Concentration albumin 1.08%. [d] These values were large because some of the albumin–mercury complex precipitated under the conditions of the experiment.

The amperometric titration with constant total mercuric ion and other data[22] indicate a single sulfhydryl site on the albumin molecule binding mercury with a very large association constant. The mercuric ion at this single site apparently was

(22) W. L. Hughes, *Cold Spr. Harb. Symposium Quant. Biol.*, **14**, 79 (1950).

not reduced and did not dissociate at the dropping electrode. The other bound mercuric ions were either reduced or dissociated very rapidly at the dropping electrode. The n and K values for the mercury complex contributing to the diffusion current were obtained by a modified plot, that is, $(\bar{\nu} - 1)/c$ as a function of $(\bar{\nu} - 1)$.[23]

The data for this plot were taken from the amperometric titration with constant total albumin, at pH 4.9, corrected with the dialysis data. Taking the values $n_1 = 5$, $K_1 = 27{,}000$,[24] for the contributing complex and $n_2 = 1$ for the non-reducible complex, a series of titration curves for constant total mercuric chloride concentration were calculated for different values of K_2 for the non-reducible complex from equations (4) and (5) with the assumption that the contributing complex ($n_1 = 5$, $K_1 = 27{,}000$) was either reduced or dissociated very rapidly at the dropping electrode. Employed in these calculations was the value $F = 7.10 \times 10^3$ microamperes mole^{-1} determined by measuring a series of mercuric chloride standards at the same pH corrected to the same value of $m^{2/3}t^{1/6}$. The value used for D for mercuric chloride was 1.04×10^{-5} cm.^{2} sec.$^{-1}$ calculated from the polarographic data. These calculated curves are shown with the experimental amperometric titration at pH 4.9 in Fig. 4. From these plots, a value of 10^7 was chosen for the equilibrium constant for the single, non-reducible site for mercury, at pH 4.9.

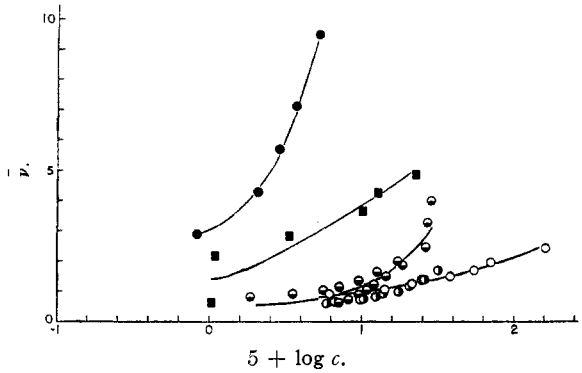

Fig. 10—Mercuric chloride binding by bovine serum albumin: ○, pH 3.5; ◑, pH 4.0; ◒, pH 4.4; ■, pH 4.9; ●, pH 7.5.

(23) The use of the value, 1, to be subtracted from $\bar{\nu}$ for all values of c introduced a small error since this value actually varied, due to partition with the other sites, from 1.0 in the region of excess mercury to 0.89 in the region of one mole mercury to one mole albumin for the values $K_1 = 27{,}000$, and $K_2 = 10^7$ arrived at below. The use of 2/3 [G. Scatchard, *American Scientist*, **40**, 76 (1952)] instead of 1 will give values $n_1 = 5$ and $K_1 = 45{,}000$ compared to the values $n_1 = 5$ and $K_1 = 27{,}000$. A more precise value of K_1 between 27,000 and 45,000 could be found from these data by successive approximation. However, this was not done since it was felt that the over-all accuracy of the method did not warrant a more precise value.

(24) These values, as with those for zinc, reflect the competition with the buffer environment.

From these data, it is evident that the sites involved in zinc binding are not the same ones at which all the mercury binds to the albumin molecule. While the zinc ions may be located at the imidazole groups[12,19] the data thus far presented elsewhere are insufficient to locate any of the other metals at this site. Examination of the curve in Fig. 5 for copper, the series of curves in Fig. 8 for mercury and those in Fig. 2, will make evident the fact that the previous interpretation of polarographic data[12] may be misleading for the metals other than zinc. The data herein presented show that albumin, even in a pH 3.5 buffer, has at least one site in addition to the sulfhydryl which binds mercury. Calculations[25] from the data in Fig. 9 gave the data shown in Fig. 10 illustrating the effect of pH on the binding of mercury. At pH 7.5 albumin binds at least two mercuric ions with a very large K (Fig. 9). The total number of mercuric ions bound at this pH also appears large. An exact value cannot be given from these equilibrium data because of the formation of a precipitate when the molar ratio of mercuric ion to albumin is greater than 14. If the data from the precipitate formed (which, of course, includes adsorbed ions) may be used as a measure of the number of mercuric ions bound, the value is about 45.[10] It is thus necessary, for an explanation of the mercury data, to include other sites in addition to the imidazole groups. Studies are at present being carried out in this Laboratory on the preparation and characterization of suitable modified albumins aimed at evaluating the role of the carboxyl groups in mercury binding.

While only an amperometric titration is presented in this paper on copper binding, polarographic measurements on competitive binding indicated that this metal competed with mercury for some sites. Thus, in an albumin–mercuric chloride solution at pH 4.9 the diffusion current for mercury (and the contributing albumin–mercury complex) was elevated on the addition of an equal concentration of total copper acetate, indicating the release of bound mercuric ions. A quantitative interpretation of this kind of competitive data must, however, await the completion of a copper analysis similar to that for mercury.

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(25) Since calculations on the partition of mercury between the single non-reducible site and the contributing sites indicated that $\bar{\nu}_2$ at pH 4.9 varied from 1.0 to 0.89 for the values of K_1 and K_2 found, the equation

$$i_d = \left[c + (c_0 - c - P) \frac{D^{1/2}_{\text{albumin}}}{D^{1/2}_{\text{mercuric ion}}} \right] 7.10 \times 10^3$$

was used for all values of diffusion current to solve for c for the amperometric titrations from pH 3.5 to 4.9. For the amperometric titration at pH 7.5 it is evident that only a small error was introduced with the assumption that the measured diffusion current was directly proportional to the concentration of mercuric chloride.