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Preparation and Properties of Serum and Plasma Proteins. XXXIX. The Interaction of Human Serum Albumin with Plumbous Ions[1a,b,c]

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The interaction of human serum mercaptalbumin with plumbous ion at 0° has been studied, both in terms of the effect of the metal on the solubility of the protein and in terms of binding. Lead ions render the protein insoluble over almost the entire *p*H range 2 to 8. The reaction can be reversed: as many as 70 lead ions have been combined with albumin without obvious effect on the properties of the protein after removal of the metal. Binding was measured, either by the dialysis equilibrium technique or by analysis of the lead–protein precipitate and of the supernatant solution. Studies of the competition between lead and hydrogen ions indicated that lead ions are bound to carboxylate groups in this protein. The failure of lead ions to compete with zinc ions for common binding sites is taken as evidence that lead ions do not bind to the imidazole groups in human serum mercaptalbumin.

Detailed knowledge of the interactions of proteins with metallic ions has two primary applications. First, the information may guide the choice and use of metallic reagents for the separation of individual proteins in a mixture. Second, some idea of the reactivity of the various groups of a protein may be gained by discovering with which groups a given metal reacts and by making some estimate of the number of such groups and of their affinity constants. For both these objectives it is desirable to study a series of metals whose modes of interaction with proteins differ.

Plumbous ion was selected for the present study, partly for its own interest and partly because its interaction with human serum mercaptalbumin appeared to differ markedly from the better known zinc interaction.[2] In preliminary observations, lead and zinc ions were found to have very different effects on the solubility of human serum mercaptalbumin, both qualitatively and quantitatively. Furthermore, the difference was not peculiar to serum albumin; a lead salt had recently been employed by Schmid in the crystallization of the acid glycoprotein of human plasma, a protein which is comparatively difficult to precipitate with zinc.[3]

Materials and Methods

Human Serum Mercaptalbumin.—Two different lots of crystalline mercaptalbumin were used. One preparation was recrystallized three times as the mercury–mercaptalbumin dimer, according to Hughes.[4–6] Most of the results were obtained with a preparation recrystallized five times; this preparation has already been used by Dr. R. H. Maybury for light scattering studies and was of the type denoted as C.[7] The mercury was removed according to the procedure of Dintzis.[8] This procedure removes anionic groups which were not removed by the electrodialysis procedure of earlier studies[2] and raises the isoionic *p*H by an amount corresponding to the removal of about four such groups. We have, accordingly, assumed that such preparations of albumin contain 102 carboxyl groups rather than 106.[9]

The concentration of the deionized solution was determined by heating to constant weight. In some cases, the protein solution was shell frozen in a weighing bottle and placed in a precooled brass block in a vacuum oven connected to a trap cooled by a mixture of Dry Ice and alcohol; after several hours under vacuum, the oven was heated to 75°. Earlier measurements were made by heating to 110° under atmospheric pressure. The method of determining protein concentration after equilibration has been described.[2]

Determination of Lead.—All analyses were performed in duplicate. Three methods were employed. Many of the earlier studies were made by dithizone titration, as used for zinc analysis[2]; in place of the three drops of K_2CO_3 buffer, 0.2 ml. of a buffer composed of 8 *M* NH_3 and 0.13 *M* NH_4NO_3 was used. Equilibration was much more difficult to achieve than with zinc, even when glass-stoppered test-tubes were used to allow more vigorous stirring.

For the analysis of lead in the presence of zinc, a quite different adaptation of the dithizone procedure was used, based, in part, on instructions of Sandell.[10] Two ml. of the unknown solution was placed in a glass-stoppered tube with 4.0 ml. of $CHCl_3$. Two ml. of a buffer composed of 175 ml. of concentrated NH_3 solution, 24.5 g. of NaCN and 1.5 g. of Na_2SO_3 in 500 ml. was added. An excess of standard dithizone contained in 4.0 ml. of $CHCl_3$ was added and the tube was shaken vigorously. One ml. of the $CHCl_3$ layer was then diluted with 6.0 ml. of $CHCl_3$ and read at 510 mμ in a Coleman Universal Spectrophotometer against a similarly prepared blank. A standard curve was prepared in the same way. When the solution to be analyzed contained trichloroacetic acid, four drops of concentrated NH_3 was added. Since the presence of ammonium trichloroacetate appeared to affect the distribution between phases of the excess dithizone, a separate standard curve was required. The method was completely insensitive to the presence of zinc in the quantities employed and gave values for lead accurate to about 3%.

In the later studies, the metal indicator method of Biedemann and Schwarzenbach[11] was used. The solution of lead ions was mixed with excess standard disodium ethylenediaminetetraacetate solution in a total volume of 5–10 ml. One-half ml. of buffer composed of 5 *M* NH_3 and 1 *M* NH_4Cl was added and then a few drops of eriochrome black T in alcohol. When trichloroacetic acid was present, 2–4 drops of concentrated NH_3 solution was added. The green solution was back-titrated with standard zinc perchlorate solution to a gray color slightly tinged with pink. The precision of the analysis was about 1%.

Since the presence of protein interferes with all three methods of lead analysis,[12] all protein solutions were in-

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

(3) K. Schmid, *ibid.*, **75**, 60 (1953).

(4) W. L. Hughes, Jr., *ibid.*, **69**, 1836 (1947).

(5) W. L. Hughes, Jr., *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 79 (1949).

(6) W. L. Hughes, Jr., in preparation.

(7) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes, Jr., and J. T. Edsall, THIS JOURNAL, **75**, 5058 (1953).

(8) See reference 7, especially footnote 6.

(9) C. Tanford, THIS JOURNAL, **72**, 441 (1950).

(10) E. B. Sandell, "Colorimetric Determination of Traces of Metals," Interscience Publishers, Inc., New York, N. Y., 1944, p. 286.

(11) W. Biedemann and G. Schwarzenbach, *Chimia*, **2**, 56 (1948).

(12) The metal indicator method may, however, be applied directly to the measurement of zinc in the presence of serum albumin.[13]

(13) F. R. N. Gurd and C. Fitting, in preparation.

itially mixed with 0.25 volume of 2 M trichloroacetic acid, allowed to stand 30 minutes and filtered. When a known amount of lead perchlorate was mixed with serum albumin, the concentration of lead in the filtrate was over 99% of that in a similar aliquot of lead solution treated identically, except that protein had not been added.

Determination of Zinc.—Zinc was determined in solutions containing lead by dithizone titration[2] after removal of the lead by coprecipitation with $SrSO_4$.[14] To 4 ml. of the solution containing zinc and lead was added 1 ml. of 1.8 M H_2SO_4, followed by 5 ml. of 0.01 M $Sr(NO_3)_2$. The mixture was allowed to stand for two to three hours, with occasional stirring, after which it was centrifuged. The supernatant solution was used for zinc analysis. Blanks were set up containing approximately the same quantity of lead as the unknowns. The presence of trichloroacetic acid did not interfere with the coprecipitation procedure.

Standard Solutions and Reagents.—All standard reagents were standardized ultimately against mercuric nitrate solution prepared from a known mass of triple distilled mercury (Eastern Smelting & Refining Corp., Boston, Mass.) dissolved in concentrated nitric acid (duPont C.P. grade). The primary standard was used to standardize dithizone and ethylenediaminetetraacetate (EDTA) solutions: for dithizone, the zinc procedure was followed precisely[2]; for EDTA, the lead method was employed.[11] The EDTA was obtained from Alrose Chemical Co., Providence 1, R. I. The dithizone was an Eastman Kodak product. Lead perchlorate was supplied as a 50% aqueous solution by Baker and Adamson, General Chemical Co., New York. Zinc perchlorate was prepared by dissolving zinc oxide in standard perchloric acid. Solid inorganic reagents were analytical grade. Organic solvents were Merck and Co., Inc., reagents, C.P. grade. The eriochrome black T was obtained from National Aniline Division, Allied Chemical and Dye Corp., New York, and made up as a 1% solution in triethanolamine.[15]

Measurement of *p*H.—A Beckman Model G *p*H Meter was used. The instrument was kept in a cold room at 2.0–2.5°; the temperature in the electrode compartment was 2.5–3.0° under operating conditions. The measurements were standardized as before.[2] A solution of 0.010 N HCl containing 0.08 M $Pb(ClO_4)_2$ gave a *p*H of 2.03; this value was taken to furnish the apparent activity coefficient of 0.03 for computing the hydrogen ion concentration in the most acid solution in which lead binding was studied. A KCl–HCl mixture of the same ionic strength and HCl concentration gave a *p*H of 2.08.

The *p*H values obtained were probably subject to some error, however, since almost all were necessarily measured in solutions free of protein (*vide infra*). Nevertheless, all measurements below *p*H 5 were free of drift, and the instrument checked back with standard buffer to within ±0.02 *p*H unit after the measurements on the lead solutions had been made.

Equilibration Procedures.—Many of the earlier experiments were made by the dialysis equilibrium method.[2] Since under a wide range of conditions the protein was rendered completely insoluble by the lead, it was often possible to estimate directly the quantity of lead bound without using the dialysis technique. As a first approximation, it was assumed that the protein precipitate could be considered as a mixture of insoluble protein and occluded supernatant solution. The quantity of lead present in dissolved form in the occluded solution (and not bound to the insoluble protein) was calculated from the weight of the precipitate (less the dry weight of the protein) and the quantity of lead found in a known weight of supernatant solution.

The protein solution, in a tared centrifuge tube, was first adjusted to the desired *p*H by addition of dilute sodium hydroxide or perchloric acid at 0°. Lead perchlorate was added, and the tube was rotated in a bath at 0°. After equilibration, the precipitate was removed by centrifugation at 0°. A volume aliquot of the separated supernatant solution was weighed and analyzed for lead. The tube containing the precipitate was warmed to room temperature and weighed to the nearest milligram. The precipitate was then redissolved at 0° by the addition of a buffer 1 M in acetic acid and 1 M in sodium acetate, brought to volume and analyzed for lead and for protein. If the supernatant solution was free of protein by trichloroacetic acid test, the quantity of protein in the precipitate was taken as equal to the quantity added. Dividing the number of moles of bound lead by the number of moles of protein gave $\bar{\nu}$, the average number of moles of lead bound per mole of albumin.

Results

Constancy and Reversibility of Binding.—Membrane distribution experiments in which no precipitation occurred approached equilibrium in about 40 hours. The equilibrium point was the same, whether the lead solution was placed inside or outside the bag, and whether or not the system had previously been brought into equilibrium at another concentration of unbound lead ion. When complete precipitation of the protein occurred in the dialysis bags, constant values of $\bar{\nu}$ were observed within 66 hours. Dialysis studies were carried on routinely for 180 hours. Control experiments showed that exposing the protein solutions to the stirring procedure for 146 hours before introducing the lead did not alter the results. When the lead perchlorate was added directly to the protein, the latter precipitated instantly, and constant values of $\bar{\nu}$ were obtained much more quickly. After one hour, the values were nearly the same as after 24 hours (Table III).

The reaction of serum albumin with lead at 0° does not appear to damage the protein irreversibly. A solution of mercaptalbumin was mixed in a dialysis bag with a more concentrated lead solution than that which was found to give $\bar{\nu} > 70$ (Table I). The bag was dialyzed for a total of 12 days against four changes of 1000 times its volume of 0.01 M ethylenediaminetetraacetate (EDTA) of *p*H 6.4–6.8. Within two days, the protein had completely dissolved to give a clear solution. A sample of untreated albumin of approximately the same concentration was dialyzed against the last change of buffer. Samples of each solution and of a 1:1 mixture of each were then partially precipitated by being brought to *p*H 5.2 ± 0.1, 0.005 M EDTA, −6° and 23% ethanol. The solubility of each, measured after centrifugation at −6°, was 8.7, 9.7 and 9.1 g./l. for the lead-treated and untreated albumin preparation and their mixture, respectively.

Solubility.—The addition of less than eight moles of lead perchlorate per mole of mercaptalbumin was sufficient to render the protein completely insoluble in the neighborhood of *p*H 6 to 7. Under these conditions, even four moles of lead perchlorate caused considerable precipitation. Raising the ionic strength increased solubility but, even in molar sodium perchlorate, concentrations of lead ion above about 2×10^{-3} M caused precipitation in a 1% solution of albumin.

Mercaptalbumin was precipitated by lead over a very broad *p*H range. In 0.05 M lead perchlorate, complete precipitation occurred between *p*H 3 and 7, and solubility was still low at *p*H 2 and 8. By contrast, the solubility of human serum albumin at 0° is affected by zinc salts only between *p*H values of about 5 to 8[16] and organic solvents must be added to precipitate the protein completely.[17,18]

Binding of Lead Ion.—As indicated above, at low ionic strength, *p*H near 6, the onset of precipitation came with values of $\bar{\nu}$ (the average number of moles of lead ion bound per mole of albumin) near 4. In the presence of 1.25 M sodium perchlorate, the albumin remained in solution up to a value of $\bar{\nu}$ of about 15. Addition of more lead perchlorate to either system rendered the protein insoluble, but in neither case was this transition marked by any discontinuity in the steady increase of $\bar{\nu}$.

(14) I. T. Rosenqvist, *Am. J. Sci.*, **240**, 356 (1942).

(15) E. M. Diskant, *Anal. Chem.*, **24**, 1857 (1952).

(16) Initial observations in this Laboratory were made by J. M. Gillespie in 1948.

(17) E. J. Cohn, F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid and E. Uroma, THIS JOURNAL, **72**, 465 (1950).

(18) Zinc ions combine with many other proteins more tightly than with human serum mercaptalbumin and have a stronger precipitating action. One such protein is human serum β-lipoprotein[19]; solubility is particularly affected when the very sensitive complexes with γ-globulins are present.[20] More recently, influenza virus and *Rous sarcoma* virus have been found to interact very strongly with zinc ions.[21]

(19) F. R. N. Gurd, unpublished observations.

(20) J. L. Oncley, E. Ellenbogen, D. Gitlin and F. R. N. Gurd, *J. Phys. Chem.*, **56**, 85 (1952).

(21) M. D. Eaton and S. S. Chapman, in preparation.

Table I shows the values of $\bar{\nu}$ corresponding to increasing concentrations of unbound lead perchlorate. The protein solutions in this series were mixed with 18.5 moles of NaOH, before equilibration with the lead solutions for 24 hours at 0°. Sodium perchlorate was not added. Complete precipitation of the protein occurred in every case. Since sufficiently accurate measurements of $\bar{\nu}$ could not be made in the presence of higher concentrations of lead ion, the maximum lead binding capacity could not be measured directly.

TABLE I

VARIATION OF $\bar{\nu}$ WITH CONCENTRATION OF UNBOUND LEAD ION

Concn. of unbound Pb^{++}, $M \times 10^3$	*p*H	$\bar{\nu}$
2.51	6.04	26.9
12.4	5.45	40.5
35.9	5.74	59.0
86.4	5.11	62.1
385	4.60	73.2

The quantity of NaOH added in this series was the largest employed in the experiments in which lead binding was measured. The purpose of adding the base was to make sure that lead binding would not be depressed by competition of hydrogen ions. The values of $\bar{\nu}$ for the first three points in Table I may be somewhat too high, due to formation of lead hydroxides.

Smaller but still sizable values of $\bar{\nu}$ were obtained in solutions of mercaptalbumin equilibrated with very low concentrations of lead ion. For example, albumin combined with 10.2 moles of perchloric acid per mole of protein showed a $\bar{\nu}$ of 7.5, even when in equilibrium with only 6.75×10^{-5} *M* lead ion (sodium perchlorate 1.25 *M*, *p*H 5.4).[22] Indeed, the binding was sufficiently strong that accurate measurements of unbound lead could not be made at very low values of $\bar{\nu}$.

Table II shows the variation of $\bar{\nu}$ with the addition of increasing quantities of perchloric acid. The average numbers of moles of hydrogen ion bound were calculated from the quantities of acid added and the *p*H values. The acid was added to the protein solution before a fixed quantity of lead perchlorate. The ionic strength in this series was between 0.12 and 0.16. Equilibration continued for 37 hours at 0°. With the exception of the experiment at *p*H 2.20, the protein precipitated as the lead solution was added and precipitation was ultimately complete. In the tube containing the most acid, the precipitate formed gradually and could be centrifuged only with difficulty. At the end of the experiment, however, precipitation was 94% complete. The redissolved precipitate gave a slightly turbid solution.[23] In an excess of trichloroacetic acid (final *p*H < 1), the mercaptalbumin did not bind lead ions (see Materials and Methods).

TABLE II

VARIATION OF $\bar{\nu}$ WITH *p*H

*p*H	Moles H^+ bound/mole albumin[a]	Concn. of unbound Pb^{++} $M \times 10^3$	$\bar{\nu}$
2.20	95	54.4	3.6
3.28	58	48.0	18.8
3.37	50	50.4	25.5
3.41	50	49.9	23.5
5.10	0	41.7	45.8

[a] Measured from the isoionic state.

An indication of the reversibility of the binding of lead and hydrogen ions is given by the third and fourth experiments summarized in Table II. In both these experiments, the same quantity of perchloric acid, as was used to reach *p*H 2.20, was first added; in the third experiment, the acid was half-neutralized with NaOH before the addition of lead perchlorate; in the fourth experiment, the lead was added before the base. Twenty minutes intervened between each addition. The results show that the final equilibrium was essentially independent of the order of addition of reagents. Furthermore, these experiments accord with that at *p*H 3.28 in which the protein was never exposed to such high concentrations of hydrogen ion.

The results in Table II show that, by the addition of hydrogen ions, plumbous ions, like zinc ions,[2] are dislodged from their combination with binding sites in human serum mercaptalbumin. Rather high hydrogen ion concentrations are required to remove lead; whereas zinc binding becomes negligible between *p*H 5 and 4.[13]

Binding of Lead and Zinc Ions.—To determine whether lead and zinc ions combine with the same or different sites in the albumin molecule, the binding of each in the presence of the other was measured. One micromole (69 mg.) of human serum mercaptalbumin was mixed with 10 micromoles of NaOH followed by 52 and 78 micromoles of zinc perchlorate and plumbous perchlorate, respectively, in a total volume of 8 ml. The albumin was equilibrated for one hour at 0° with either the zinc or lead salt, after which the other metal perchlorate was added and equilibration continued for 1 or 24 hours. Complete precipitation of the protein occurred when the lead was added. After centrifugation at 0°, analyses were performed for both metals in the supernatant solution and in the redissolved precipitates. The *p*H values of the supernatant solutions were between 5.6 and 5.8.[24]

Experiments 1 to 4 in Table III show that $\bar{\nu}_{Zn^{++}}$ and $\bar{\nu}_{Pb^{++}}$ were little affected by the order of addition of the metals or by the time of equilibration beyond one hour. Comparison of experiments 2 and 4 with experiment 5, in which zinc was omitted, shows that $\bar{\nu}_{Pb^{++}}$ was practically unaffected by the presence of bound zinc ions. The value in the last line for $\bar{\nu}_{Zn^{++}}$, obtained by interpolation from dialysis equilibrium studies on the same protein preparation,[13] indicates that the presence of bound lead ions made little difference to the affinity for zinc.[26]

TABLE III

BINDING OF ZINC AND LEAD IONS TO HUMAN SERUM MERCAPTALBUMIN

Expt. number	Order of addition	Hours of final equilibration	Concn. in supernatant soln. $M \times 10^3$ Zn	Pb	Moles metal bound per mole protein $\bar{\nu}_{Zn^{++}}$	$\bar{\nu}_{Pb^{++}}$
1	Zn, Pb	1	5.76	6.50	5.5	20.7
2	Zn, Pb	24	5.64	6.35	6.5	25.6
3	Pb, Zn	1	5.75	6.36	5.6	21.1
4	Pb, Zn	24	[a]	6.50	[a]	23.5
5	Pb	24		7.07		25.9
	Zn		5.5		5.0	

[a] Not measured.

Discussion

The experiments summarized in Table II indicate that carboxylate groups are necessary for the binding of lead ions. The large values of $\bar{\nu}$ shown in Table I are in keeping with such a conclusion.

(22) A comparable $\bar{\nu}$ for zinc ions would have required a concentration of unbound metal ion of the order of 10^{-2} *M*.[2]

(23) When the precipitate was brought to room temperature for weighing, it cleared completely, partly as a liquid and partly as a gel which flowed extremely slowly, when the tube was inverted. After it had been returned to 0°, the liquid layer became turbid again but did not regain its original opacity.

(24) The *p*H range about 5.7 was chosen to assure a convenient degree of zinc binding without risking the formation of lead hydroxides. The *p*H of the zinc–protein mixtures before addition of lead was below 6.0. We have confirmed Britton's observations[25] that the onset of precipitation of lead hydroxides is near *p*H 6, and we have found that raising a lead perchlorate solution of about the concentration used in these experiments to *p*H 5.7 required the addition of less than 5% as many equivalents of NaOH. Note that if the protein solution had initially had a higher *p*H, the hydrolysis of part of the added lead would have lowered the *p*H to about 5.5–6.0; in itself, a *p*H below 6.0 does not prove that some hydrolysis of the lead did not occur.

(25) H. T. S. Britton, *J. Chem. Soc.*, **127**, 2148 (1925).

(26) In contrast, similar experiments have indicated that cadmium ions compete effectively with zinc ions for binding sites in human serum mercaptalbumin.[27]

(27) F. R. N. Gurd, unpublished results.

At least at the moderate values of $\bar{\nu}_{Pb^{++}}$ studied in the experiments of Table III, the binding of lead ions does not interfere noticeably with the binding of zinc ions. This suggests that the lead ions do not combine with the imidazole groups to which the zinc is believed to bind.[2] Tanford has concluded that both imidazole and carboxylate groups are involved in the binding of lead ions by bovine serum albumin.[28]

The calculation of an intrinsic constant for the combination of lead ions with the carboxylate groups of human serum mercaptalbumin is made difficult by the problem of the large charge contributed to the protein by the hydrogen and lead ions. Instead of attempting to estimate the counterbalancing effect of the binding of perchlorate ions,[29] or to speculate about the nature of the electrostatic corrections to be applied to the measurements on the insoluble protein, we have chosen for comparison a group of experiments in which the formal charge due to H^+ and Pb^{++} was nearly constant. The values of $(\bar{\nu}_{H^+} + 2\bar{\nu}_{Pb^{++}})$ in the series of experiments of Table II are, respectively, 102, 96, 101, 97 and 92. An apparent constant was calculated, according to equation 1

$$k'_{Pb} = \frac{\bar{\nu}_{Pb^{++}} [H^+] k_{COOH}}{[Pb^{++}] \bar{\nu}_{H^+}}$$

by using the values in Table II and taking log k_{COOH} as 4.0.[9] The values of k'_{Pb} calculated for the experiments in Table II are 47, 34, 42, 35 and 44.[30] The constancy of these values gives some further support to the assumption that the carboxylate groups are the sites of binding of the lead ions. Obviously, agreement of this sort does not establish that every carboxylate group is capable of binding a lead ion with the same intrinsic affinity or that no lead ions may be bound to more than one carboxyl group. But it is likely that most of the carboxylate groups which are capable of binding hydrogen ions are also able to bind lead ions, and that most of the lead ions are bound to only one carboxylate group at a time. We have made no attempt to allow for the possible binding of lead to the single sulfhydryl group in human mercaptalbumin.[31]

The results do not establish that k'_{Pb} remains constant as $\bar{\nu}_{Pb^{++}}$ changes, but they show that k'_{Pb}/k_{COOH} is approximately constant. If k_{COOH} is not itself single-valued, it would be possible for it to vary in such a way that k'_{Pb}/k_{COOH} appeared constant within the accuracy of our measurements.[32]

The value of the association constant, k'_{Pb}, is of a reasonable order of magnitude when compared with that of the first association constant of lead ion with acetate ion, reported to be near 100 at an ionic strength of 1.0.[33] Considering the appreciable positive charge contributed by the bound lead and hydrogen ions, it is to be expected that k'_{Pb} should be somewhat lower than 100. Too much should not be made of the numerical similarity. It seems very likely that some of the bound lead ions are involved in cross-linkages connecting a carboxyl group of one protein molecule with a similar group on another protein molecule. Such arrangements should distort the simple interpretation of the affinity constants. However, only a few such cross-linkages appear to be necessary to render the protein insoluble.[34]

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(28) C. Tanford, THIS JOURNAL, **74**, 211 (1952).

(29) G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949).

(30) For these calculations ν_{H^+} has been taken equal to the values in the second column of Table II increased by 2, the difference between the total number of carboxyl groups and basic groups in the protein (see Materials and Methods). The reasoning is similar to that of reference 2, footnote 25. It is assumed that a negligible number of imidazole groups are un-ionized.

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

(32) Owing to the large changes in net charge borne by the protein molecules during the course of the titration, it is difficult to demonstrate that k_{COOH} is truly constant throughout a titration with an acid alone.[9] In the present study, a reasonably constant net charge was achieved by varying both $\bar{\nu}_{Pb^{++}}$ and $\bar{\nu}_{H^+}$. A satisfactory solution to this problem might be approached by searching for an ion which would not compete with hydrogen ion for carboxylate and whose binding could be adjusted to counterbalance changes in $\bar{\nu}_{H^+}$.

(33) S. M. Edmonds and N. Birnbaum, THIS JOURNAL, **62**, 2367 (1940).

(34) Internal chelation involving, for example, hydroxyl groups, in addition to carboxylate groups, may possibly occur.[35]

(35) J. C. Abels, THIS JOURNAL, **58**, 2609 (1936).