[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH HARVARD UNIVERSITY]

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

BY F. R. N. GURD AND DEWITT S. GOODMAN

The interaction of human serum albumin with zinc ions has been studied in unbuffered solutions of the isoionic protein in 0.15 *M* sodium nitrate to which varying quantities of sodium hydroxide and zinc chloride were added. The results have been interpreted to show that zinc ions bind to the imidazole group of the histidine residues in the albumin, approximately in the proportion of one zinc ion to one imidazole group. Treating the binding of zinc ions and of hydrogen ions as a competition for the imidazole groups, an intrinsic binding constant for zinc ions has been calculated. This constant was found not to vary over a considerable range of values for the number of moles of zinc ions bound per mole of protein. The value of the constant is unchanged by guanidination or diazo-esterification of the albumin, and was identical with the first association constant determined for the interaction of zinc ions with imidazole.

Advantage has recently been taken of the action of divalent metallic ions, notably zinc ion, in selectivity rendering insoluble the proteins contained in human blood plasma[2,3,4] and in extracts of bovine liver.[5] It has been possible in this way either to reduce to a great extent the concentrations of organic solvents employed, or to abolish their use entirely in achieving certain separations.[4] This technique has tended to yield more stable protein preparations, and has simplified the maintenance of temperature control. The use of zinc salts also has rendered the separations less sensitive to ionic strength, which means that it is no longer necessary to lower the ionic strength of body fluids at the expense of extensive dilution.

The object of the present investigation is to prepare a foundation for understanding the effect of zinc ions on the solubility of the plasma proteins by defining the nature of the interaction between zinc ions and human serum albumin. Human serum albumin has been used in this investigation because it has been well characterized, especially in terms of equilibria with hydrogen ions,[6] and because well-defined modifications are available in this Laboratory.[7,8] The characteristics of the interaction of most immediate interest are (a) the maximum number of zinc ions which may be bound to a detectable degree; (b) the strength of the binding as described by one or more intrinsic binding constants; (c) the nature of the group or groups on the albumin molecule which take part in the interaction.

Methods and Materials

The dialysis equilibrium technique was employed to study the interaction of zinc ion and human serum albumin. The apparatus consisted of equilibration tubes prepared from female 24:40 standard taper joints by sealing the ends opposite the joints. Sixteen ml. of solution containing varying quantities of zinc chloride and enough sodium nitrate to give an equilibrium concentration of 0.15 *M* in the total system were placed in each tube, together with a bag of Visking Cellulose Sausage Casing ($^{8}/_{32}$ inches inflated diameter[9]) containing 10 ml. of protein solution to which varying quantities of dilute sodium hydroxide had been added. The tube was then equilibrated, with occasional agitation in a bath maintained at 0.0 ± 0.1°, from 48 to 72 hours. Twenty-five hours was found sufficient to attain equilibrium.

After the equilibration period the dialysis bag was removed from the tube, and the total concentration of zinc in the solutions inside and outside the bag was determined. The concentration of albumin in the solution inside the bag and the *p*H of the solution were also determined.[10]

The concentration of bound zinc ion was determined as the difference in total concentration of zinc inside and outside the bag (considering the Donnan effect to be negligible in the presence of 0.15 *N* $NaNO_3$). The average number of zinc ions bound per albumin molecule, $\bar{\nu}$, is equal to the molar concentration of bound zinc ion divided by the molar concentration of albumin. The molecular weight of the albumin was taken as 69,000.[11]

Human Serum Albumin.—The preparation of human serum albumin used in these experiments was Lot No. 352, prepared according to method 6[12] and containing not less than 97% albumin. Solutions were prepared by dissolving the dry powder in distilled water, electrodialyzing the resultant solution, and diluting the electrodialyzed solution to approximately 2.5 to 3.0% protein with dilute sodium hydroxide. The concentration of the electrodialyzed solution was determined by heating to constant weight at 110°. The concentrations of the solutions after equilibration were determined by comparing their optical densities at 280 mμ as read in a Beckman quartz spectrophotometer with that of an electrodialyzed solution whose concentration was known. Appropriate concentrations of sodium nitrate were used in the blank cuvettes.

Guanidinated Albumin.—Preparation U of Hughes, Saroff and Carney,[7] kindly provided by Dr. W. L. Hughes, Jr., was used. The maximum number of free amino groups are guanidinated in this preparation. A stock solution of

(1) (a) This work has been supported by funds of Harvard University and the Eugene Higgins Trust, by grants from the Rockefeller Foundation and the National Institutes of Health, and by contributions from industry. (b) This paper is No. 94 in the series "Studies on the Plasma Proteins" from blood collected by the American Red Cross, on products developed by the University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University. (c) Abstracted from a thesis by DeWitt S. Goodman in partial fulfillment of the requirements for the A.B. degree with Honors, Harvard College, 1951. (d) Presented to the XIIth International Congress of Pure and Applied Chemistry, New York, N. Y., September 10–13, 1951.

(2) 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).

(3) W. F. Lever, F. R. N. Gurd, E. Uroma, R. K. Brown, B. A. Barnes, K. Schmid and E. L. Schultz, *J. Clin. Invest.*, **30**, 99 (1951).

(4) E. J. Cohn, D. M. Surgenor, W. B. Batchelor, R. K. Brown, M. J. Hunter, H. Isliker, K. Schmid and P. E. Wilcox, in preparation.

(5) E. J. Cohn, D. M. Surgenor and M. J. Hunter, in "Enzymes and Enzyme Systems," ed. J. T. Edsall, Harvard University Press, Cambridge, Mass., 1951, pp. 105–143.

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

(7) W. L. Hughes, Jr., H. A. Saroff and A. L. Carney, *ibid.*, **71**, 2476 (1949).

(8) P. E. Wilcox, unpublished studies on diazo-esterified human serum albumin.

(9) The cellulose tubing was washed thoroughly before use by soaking in several changes of distilled water over a period of about an hour. Prolonged equilibration thereafter with water yielded a solution of negligibly low optical density at 280 mμ.

(10) The outside solution was useless for *p*H determination because of the absence of buffers.

(11) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947).

(12) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

this protein could not be electrodialyzed because it is insoluble at its isoionic point. It was instead dialyzed against 0.15 N $NaNO_3$, and the protein concentration determined as the difference between the dry weights of the protein solution and the dialyzing solution. The molecular weight of this material, calculated from the extent of guanidination, is 71,500.

Diazo-esterified Albumin.—Diazo-esterified human serum albumin was kindly provided by Dr. P. E. Wilcox. This material differs from ordinary albumin in that several of the carboxyl groups have been replaced by the grouping $-CO-CH_2CONH_2$, through treatment with diazoacetamide. The number of groups so altered was estimated from the vertical displacement of the titration curve of the modified albumin from that of the unmodified albumin, in the region slightly acid to the isoelectric point of the unmodified albumin.[8] The result of this estimate was that twelve carboxyl groups had been modified. The material was used directly after electrodialysis, without addition of base. Its molecular weight was taken to be 70,000.

Preparation of Zinc Solutions.—Zinc chloride solutions were prepared by dilution of a stock solution, prepared gravimetrically by neutralizing zinc oxide with the equivalent quantity of constant boiling HCl.

Determination of Zinc.—Total zinc concentrations were determined by titration with dithizone (diphenylthiocarbazone) according to a method developed by Hughes.[13] For protein-free solutions, 5 ml. of the zinc solution of unknown concentration is added to 5 ml. of CCl_4 and 3 drops of a 5 M K_2CO_3–0.1 M $KHCO_3$ buffer in a test-tube, and then titrated with dithizone in chloroform. Thorough mixing at the interface is accomplished by vertical motion of a glass plunger made by flanging the end of a glass rod. At the start of the titration the aqueous phase becomes pink as the dithizone solution falls through it, and, as the end-point is approached, the color changes to yellow, which fades upon agitation with the plunger. The best end-point is the persistence of a definite yellow hue in the aqueous phase after agitation. The amount of zinc present is calculated from the stoichiometry of the reaction[14] and the concentration of the dithizone used.

When protein is present, it is first precipitated with 2 M trichloroacetic acid, a procedure which has been found to remove the zinc ion quantitatively from its combination with the protein.[13] The suspension is then centrifuged and the titration carried out on the supernatant solution as described, sufficient carbonate buffer being added to neutralize the excess acid before beginning the titration.

The precision of the analysis is between 1.0 and 1.5%. All analyses were carried out in duplicate, and in the very few instances where agreement was not better than 1.5%, two more titrations were performed and the total average used as the final result.

Dithizone solutions were prepared by dissolving the solid reagent in chloroform. Their concentrations were determined by titration against a standard zinc solution.

The CCl_4 and $CHCl_3$ reagents were treated with a solution of ethylenediaminetetraacetic acid before use, in order to extract any traces of heavy metals. The buffer solution (5 M K_2CO_3–0.1 M $KHCO_3$) was prepared by dissolving the solid reagents in distilled water and repeatedly extracting the resultant solution with dithizone reagent until no further pink color was produced, the excess dithizone being then removed by extraction with toluene.

Measurement of pH.—Hydrogen ion activities were determined using a pH meter manufactured by the Cambridge Instrument Company of Ossining, N. Y. The temperature scale of the instrument was extended to 0° by adjustment of the temperature-compensation resistance to give the theoretical values of dE/dpH over the temperature range in question.[15] The instrument was standardized against a pH 7 buffer supplied by the National Technical Laboratories of Pasadena, California, which had been standardized by them against a Bureau of Standards buffer. The buffer was described by the suppliers as being accurate to 0.01 pH unit. The probable error in the measurements is 0.02 pH unit.

This meter was situated in a cold room, at temperatures close to 0°. Temperatures were read from a thermometer in the electrode compartment. The measurements were made at 1.5 ± 1.5°, this amount of temperature fluctuation being unavoidable in the cold room at this temperature.

Reagents.—The solid inorganic reagents were Mallinckrodt Analytical Reagents. The acid and organic solvents were Merck and Co., Inc., reagents, C.P. grade. The dithizone was an Eastman Kodak Co. product.

Reversibility of the Binding

The reaction has been demonstrated to be reversible by the following procedure.[16] A tube identical with that described by the highest point on the full circle curve in Fig. 1 was permitted to reach equilibrium. Most of the zinc solution outside the dialysis bag was next removed and replaced by 0.15 M sodium nitrate. The system was then allowed to reach a new equilibrium, after which it was analyzed in the usual manner, and $\bar{\nu}$, the average number of moles of zinc ion bound per mole of albumin, was calculated. The observed value, 4.8, is within experimental error of 5.0, the value expected for the observed free zinc ion concentration (3.00×10^{-3}) as read from the curve relating $\bar{\nu}$ to the free zinc ion concentration under these conditions (Fig. 1, curve in full circles).

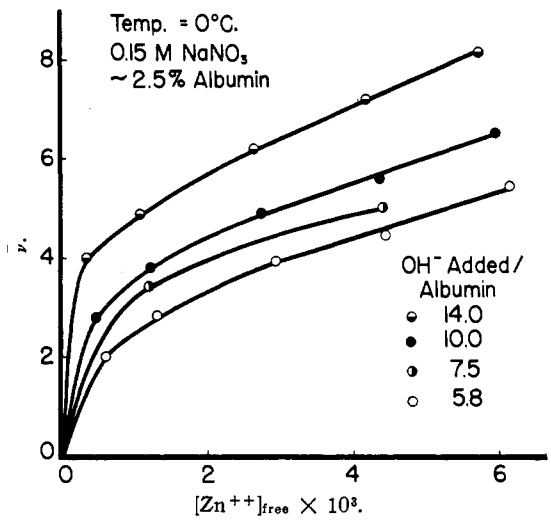

Fig. 1.—Effect of zinc ion concentration on zinc binding to human serum albumin.

Studies of Human Serum Albumin. Demonstration of Imidazole Groups as Sites of Binding

Preliminary experiments using zinc chloride and isoionic mercaptalbumin to which no sodium hydroxide or sodium nitrate was added showed the bound zinc to be too small, relative to the quantity of free zinc, to be very accurately measured. Large changes of pH, however, between −0.05 and −0.49 unit, were observed. The pH changes expected from electrostatic effects alone, calculated according to Scatchard and Black,[17] all fell between −0.02

(13) W. L. Hughes, Jr., personal communication.

(14) E. B. Sandell, "Colorimetric Determination of Traces of Metals," Interscience Publishers, Inc., New York, N. Y., 1944, p. 78.

(15) This adjustment was kindly made by Mr. H. Diatzis.

(16) Detailed tabulations of experimental data and calculations may be obtained from the American Documentation Institute, 1719 N Street, N. W., Washington 6, D. C., by ordering Document 3364, remitting $1.00 for microfilm (images 1 inch high on standard 35 mm. motion picture film) or $1.00 for photo copies (6 × 8 inches) readable without optical aid.

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

and −0.12 unit.[18] It was therefore postulated that the large increase in the activity of hydrogen ion might be due to competition between zinc and hydrogen ions for some common site.

By competition it is meant that the zinc and hydrogen ions cannot occupy any particular single site at the same time. If ions of both species are in equilibrium with a class of sites on the protein molecule, the number of either species bound to these sites depends upon the affinities and activities of both. To study such competition quantitatively one should determine: (a) the activities of both hydrogen and zinc ions in the solution in equilibrium with the unbuffered protein; (b) the average number of zinc ions bound to the protein molecule; (c) the average number of hydrogen ions bound to the class (or classes) of sites on the protein molecule involved in the binding of zinc ions. From the experimental data (a) and (b) may be obtained directly. The last value, (c) may be obtained from the experimental results together with the available detailed information about hydrogen ion equilibria with human serum albumin. For rigorous work it is also necessary to know the characteristics of the binding of the small anions to the protein, so that appropriate electrostatic corrections may be made.

If the postulate is correct that the binding of zinc ions to isoionic serum albumin involves competition with hydrogen ion, then the groups which may most reasonably be assumed to be involved in the binding of zinc ions are the 16 imidazole groups of the histidine residues. At isoionic *p*H the 106 carboxyl groups in the molecule are almost entirely ionized.[6] If the binding sites were the carboxyl groups, therefore, no competition with hydrogen ion would be involved. Furthermore, for significant binding to occur at the amino groups in competition with the hydrogen ion in this *p*H range the association constant would have to be of the order of 10^6, whereas in the interaction of ammonia with zinc ion[21,22] the first constant is only about $10^{2.2}$. The groups most likely to be responsible for the binding, therefore, seemed to be the imidazole groups of histidine residues.

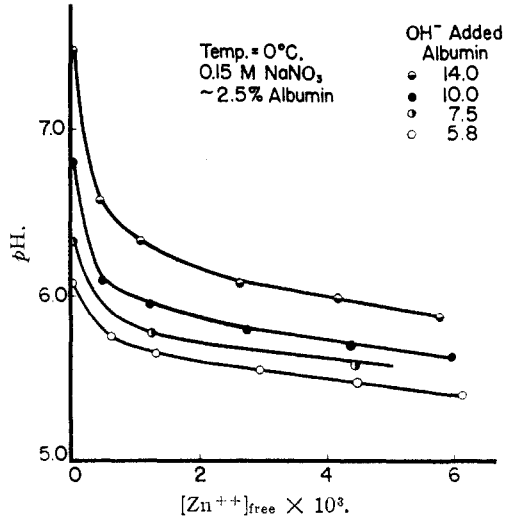

Fig. 2.—Effect of zinc ion concentration on equilibrium *p*H values.

In order to test this hypothesis the experiments summarized in Figs. 1 and 2 were performed.[16] Varying quantities of zinc chloride were equilibrated across a membrane with isoionic human serum albumin in 0.15 *M* sodium nitrate to which varying quantities of sodium hydroxide had been added. Analysis of the solutions inside and outside the membrane gave the *p*H, the concentration of free zinc ion and $\bar{\nu}$ (see Methods and Materials). The variation of $\bar{\nu}$ with the concentration of free zinc ion is shown in Fig. 1. Not only does $\bar{\nu}$ increase with increasing concentrations of unbound zinc, but also with increasing equivalents of sodium hydroxide added to the albumin solutions. In Fig. 2 the corresponding equilibrium *p*H values are shown.

In the following treatment of these results it is assumed that zinc ions do not bind to amino groups under the conditions studied. This assumption will be verified experimentally.

Since the binding of zinc ions to any sites on the albumin molecule may be expected to exclude hydrogen ions from those sites, the question of whether or not the imidazole groups are responsible for the binding of zinc ions was attacked by determining n, the total number of imidazole groups available to hydrogen ions, *i.e.*, which can take on or give off protons. In the event that zinc ions are bound to imidazole groups to the exclusion of hydrogen ions n will be less than 16, the total number of imidazole groups in the molecule, and will decrease with increasing $\bar{\nu}$.

The value of n is determined from the mass action relation

$$k_{\mathrm{HIm}} = \frac{(\mathrm{HIm^+})}{(\mathrm{H^+})(n - \mathrm{HIm^+})} \qquad (1)$$

in which k_{HIm} is the acid association constant of the imidazole groups, $(\mathrm{HIm^+})$ is the number of imidazole groups bearing protons, and $(n - \mathrm{HIm^+})$ is the number of free imidazole groups in the molecule.

The value of $(\mathrm{H^+})$ was obtained from direct measurement of *p*H; $(\mathrm{HIm^+})$ and k_{HIm} were estimated from $\mathrm{H^+}$ titration data using appropriate electrostatic corrections as follows.

$$(\mathrm{HIm^+}) = 16 - B - (\mathrm{HC} - 6) \qquad (2)$$

where (HC) is the number of un-ionized carboxyl groups in the albumin molecule at equilibrium, 16 is the number of imidazole groups, 6 is the excess of total carboxyl groups over the total number

(18) For this calculation[19] the equation $\Delta p\mathrm{H} = -2w\Delta z_p/2.303$ of Scatchard and Black[17] was employed. The charge borne by the albumin molecule, Δz_p, which equals $2\bar{\nu}_{\mathrm{Zn^{++}}} - \bar{\nu}_{\mathrm{Cl^-}}$, was obtained from the measured zinc concentrations by solution of the Donnan equations and the equation of Scatchard, Scheinberg and Armstrong[20] for chloride binding, using a method of successive approximations.

(19) D. S. Goodman, Thesis for the A.B. degree with Honors, Harvard College, 1951.

(20) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 535 (1950).

(21) J. Bjerrum, "Metal Formation in Aqueous Solution," P. Haase and Son, Copenhagen, 1941.

(22) Even if the binding to amino groups were fortified by chelation to neighboring carboxyl groups, a constant greater than 10^5, the first association constant for zinc ion and glycine[23,24] would not be expected.

(23) H. Flood and V. Loras, *Tidsskr. Kjemi Bergvesen Met.*, **6**, 83 (1945).

(24) D. S. Goodman and F. R. N. Gurd, unpublished studies.

of cationic groups,[6] and B is the number of equivalents of sodium hydroxide added per mole albumin.[25,26] In applying this equation (HC) was calculated according to the relation (6) employed by Tanford,[6] restated as

$$\log k_{HC} = \log k'_{HC} - 2\,w\Delta z_p/2.303 = \log \frac{(HC)}{(106 - HC)} + p\mathrm{H} \quad (3)$$

where k'_{HC} is the hydrogen ion association constant at 0° for the charge on the albumin molecule when the isoionic protein is placed in 0.15 M sodium nitrate[27]; w was taken as 0.030[28]; and Δz_p, the difference in charge on the protein molecule from that on the isoionic albumin in 0.15 M sodium nitrate, has been taken as

$$\Delta z_p = 0.6\,(2\nu - B) \quad (4)$$

The coefficient 0.6, introduced to allow for the change in nitrate binding accompanying the changes in charge due to reaction with added hydroxyl and zinc ion[29] was estimated by interpolation of the data of Scatchard, Scheinberg and Armstrong[30] for the variation of the binding to albumin of chloride and thiocyanate ions as a function of protein charge. It was assumed that nitrate ion binds in a similar way with an intermediate affinity.[17] The assumptions about nitrate binding are not critical in their effects on the calculated value of (HIm^+).

Owing to the variations from one experiment to another in the net charge borne by the protein molecules, different values of k_{HIm} are required in equation (1) for each experiment. The hydrogen ion titration curve of the albumin in 0.15 M sodium nitrate at 0° offered a ready means of determining k_{HIm}.[16] This was done by evaluating the charge on the protein molecule as given by $(2\bar{\nu} - B)$, and choosing the point on the titration curve at which the corresponding number of hydrogen or hydroxyl ions had been added to the isoionic protein dissolved in 0.15 M sodium nitrate at 0°. Taking note of the corresponding pH, together with the fact that here $n = 16$, and evaluating (HIm^+) as described, k_{HIm} was calculated by the use of equation (1). This value of k_{HIm}, derived from measurements made in the absence of zinc ion, was then taken as identical with the value of k_{HIm} for the corresponding zinc binding experiment. This involved the assumptions that substituting charges due to zinc ions for those due to hydrogen ions does not change the binding of nitrate ions, and that at a given temperature and ionic strength the only factor affecting k_{HIm} is the charge on the protein molecule. On the other hand, this method of determining k_{HIm} had the advantage of requiring no estimate of the magnitude of the nitrate binding, which would have had to be quite accurate if a calculation according to an equation of the form of equation (3) had been attempted.

The observed pH and the calculated values of (HIm^+) and k_{HIm} were introduced into equation (1) to yield n.[16] The relation between n and $\bar{\nu}$ is shown in Fig. 3. These results show that every zinc ion, or nearly every zinc ion, was bound to one imidazole group. If the binding site were not the imidazole groups, n would have been 16 at all times, and the graph of n *vs.* $\bar{\nu}$ would have been a vertical straight line through $n = 16$. If each zinc ion had been bound to two imidazole groups the points in Fig. 3 would have scattered about a line with intercepts $n = 16$, $\bar{\nu} = 8$. For the association of each zinc ion with one imidazole group, however, a line with intercepts $n = 16$, $\bar{\nu} = 16$ would be expected. The solid straight line through the experimental points in Fig. 3 and with intercept $n = 16$ has its other intercept about $\bar{\nu} = 14$. This indicates that the binding of zinc is primarily a one-to-one coördination of a zinc ion with an

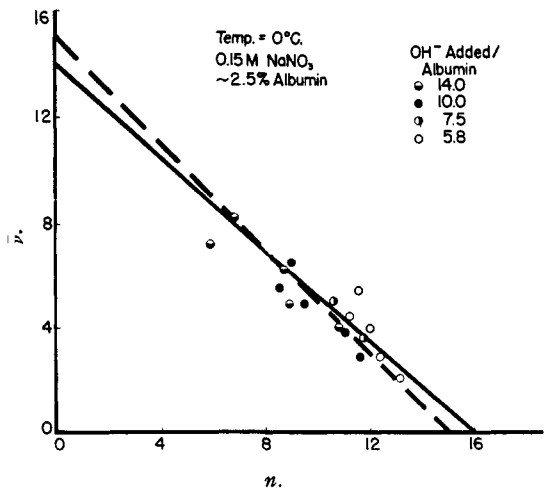

Fig. 3.—Dependence of n on $\bar{\nu}$.

(25) Under the conditions of the experiments any hydroxyl ion added to the protein solution can either remove a proton from an un-ionized carboxyl group or from a charged (ionized) imidazole group. Let q be the number of un-ionized imidazole groups in isoionic albumin. Since there are a total of 100 cationic nitrogen atoms in the protein, and since none of the cationic groups other than the imidazoles will be un-ionized at this pH, electrical neutrality requires that $6 + q$ of the 106 carboxyl groups be un-ionized (uncharged). The total number of ionized imidazole groups at equilibrium will equal $16 - q$, the number of ionized imidazole groups at the isoionic point, minus B, the number of added hydroxyl ions, plus the number of hydroxyl ions used up in removing hydrogen ions fron originally un-ionized carboxyl groups. This latter quantity equals $(6 + q - HC)$. Therefore

$$(HIm^+) = (16 - q) - B + (6 + q - HC)$$

which is identical with equation (2).

(26) It is assumed that no significant quantities of hydroxyl ion reacted with zinc ions under the conditions of the experiments. To a solution of zinc nitrate (pH 6.25) was added enough sodium hydroxide so that the final concentrations were equivalent to 1×10^{-3} M in zinc and 1×10^{-4} M in hydroxyl ions, respectively. The pH of the resulting solution at 0° was 7.48. At pH values of 6.6 and less (Fig. 2), therefore, the concentration of hydroxyl ion removed from the system by hydrolysis of zinc ion or by other means was much less than 1×10^{-4}. Since the quantity of alkali taken for this experiment represents not more than 2% of the quantities employed in the equilibration experiments, the above assumption appears secure, unless the hydrolysis of zinc ion were greatly enhanced by association with the imidazole group.

(27) Considering the value of the hydrogen ion association constant for imidazole group at 0°, it has been assumed that about one imidazole group would be dissociated at pH 5.48, the observed isoionic point in 0.15 M sodium nitrate, and hence that 99 positive charges would remain and would require 99 carboxyl groups to be ionized in the isoionic protein.

(28) At 0°, using the constants of Scatchard's laboratory, $w/2.303 = 0.0502 - 0.488\sqrt{\mu}/(1 + 10.53\sqrt{\mu})$, where μ is the ionic strength.

(29) Scatchard, Scheinberg and Armstrong have presented experimental evidence that sodium ions are not bound to human serum albumin.[22]

(30) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 540 (1950).

imidazole group, and suggests that a slight amount of double coördination may occur.

The value of $n + \bar{\nu}$ for the individual experiments ranged between 13.8 and 17.0 with an average of 15.0, and a mean deviation of 0.6. In keeping with this, an equally good straight line with intercepts $n = 15$, $\bar{\nu} = 15$ may be drawn through the points in Fig. 3 (broken line). This suggests the possibility that there may be a slight error in the value used for the total number of carboxyl groups.[31] In this case the slope of 45° indicates a precise one-to-one relationship of a zinc ion with an imidazole group.

Calculation of the Intrinsic Binding Constant

If this conclusion is correct, it should be possible to calculate an intrinsic binding constant from the competition of zinc and hydrogens ions for the imidazole group in the albumin molecule. Combining the simple mass law equations for the binding of the two ion species yields, when corrected for the different charges borne by the two ions[20]

$$k^{\circ}_{ZnIm} = \bar{\nu}\,(H^+)\,k^{\circ}_{HIm}\,e^{2w z_p}/(Zn^{++})(H_{Im}^+) \qquad (5)$$

where k°_{ZnIm} and k°_{HIm} are the intrinsic association constants for the imidazole groups of the protein with zinc and hydrogens ions, respectively, (Zn^{++}) represents the equilibrium concentration of unbound zinc ions, and the other symbols are as previously defined.

The value of k°_{HIm} was obtained from Tanford's value at 25° corrected to 0° by the van't Hoff equation, using 7,000 cal./mole for ΔH.[6] The value used was $\log k^{\circ}_{HIm} = 6.56$.

The protein charge, Z_p, was determined from the relation

$$Z_p = \Delta Z_p - 12$$

where ΔZ_p is defined by equation (5) and 12 represents the number of nitrate ions bound to the isoionic albumin at 0° in 0.15 N sodium nitrate. This latter figure was obtained from the data of Scatchard and Black[17] on the pH changes occurring in albumin solutions in the presence of sodium nitrate. Their data and treatment indicate slightly less than 15 nitrate ions to be bound under these conditions, but since these authors found their method to give values which are too high in this concentration range we have used 12 as a more reasonable figure. An error in the chosen value will not affect the constancy of k°_{ZnIm} but will appear as a constant factor in all cases.

Values of $\log k^{\circ}_{ZnIm}$ calculated from equation (5) ranged from 2.71 to 2.91 with an average value of 2.82, and a mean deviation of 0.05. Considering that the parameters $\bar{\nu}$, (H^+), (Zn^{++}) and HIm^+ were varied over roughly 4-, 15-, 18- and 2-fold ranges, respectively, the high order of constancy of k°_{ZnIm} is strong support for the formulation that the imidazole groups in serum albumin are the sites of zinc binding, and that the binding is almost entirely a result of a coördination of each zinc ion with one imidazole group.

(31) If we assume that the total number of carboxyl groups in human serum albumin is 107 instead of 106, a possibility admitted by Tanford's data,[6] the total average of $n + \bar{\nu}$ rises from 15 to 16, in perfect agreement with the total number of imidazole residues found in the protein.

Studies on Guanidinated Albumin

As already mentioned, the interpretation of the results of the studies on normal serum albumin involved the assumption that binding of zinc ions to amino groups did not occur to a detectable extent. In order to test this assumption experimentally, the interaction of zinc ions with guanidinated albumin was studied. Since almost 100% of the amino groups in this preparation had been converted to guanidinium groups, if the binding sites were the amino groups, or if the amino groups were important to the binding in any way, the zinc binding capacity of this material would have been almost completely lost.[32]

The value calculated[16] for the logarithm of the intrinsic binding costant, $\log k^{\circ}_{ZnIm}$, for the guanidinated albumin was 2.69, which shows that the degree and manner of interaction with zinc ions was virtually the same as for the native albumin ($\log k^{\circ}_{ZnIm} = 2.82$). The value of (HIm^+), which enters into equation (5) was calculated from the pH of the albumin solution in the absence of zinc ions (6) and from equation (2). The agreement of 2.69 with the value of $\log k^{\circ}_{ZnIm}$ obtained from the previous experiments is quite satisfactory, particularly when one considers that the binding of nitrate ion may be affected by guanidination, and hence that the electrostatic correction in equation (5) is probably not fully valid.

This conclusively demonstrates that no significant binding of zinc ions to amino groups occurred under the conditions of the present study. Whether or not it can occur under conditions of higher pH cannot be answered at present. Preliminary experiments involving the addition of much larger quantities of sodium hydroxide to the normal albumin indicate that $\bar{\nu}$ seems to reach a maximum at 15 or 16. Since the formation of complexes between zinc and hydroxyl ions was clearly demonstrated in these studies, it appears that the reaction of zinc ions with hydroxyl ions supervenes before binding to amino groups can be elicited. Work is continuing on this problem, especially in relation to the solubility of the protein.

Studies on Diazo-esterified Albumin

Experiments have been performed to study the binding of zinc ions to diazo-esterified albumin. The preparation used had an average of 12 carboxyl groups replaced by the grouping $-COCH_2-CONH_2$ (see Methods and Materials). Since the cationic centers in the albumin molecule had not been altered, the zinc binding capacity of the protein should have remained unchanged. This was found to be the case. The observed values of $\bar{\nu}$ for a given (Zn^{++}) fell between the curves for 10 and 14 equivalents of sodium hydroxide added to unmodified albumin (Fig. 1).[16] Since, in terms of free imidazoles, the blocking of 12 equivalents of alkali is roughly equivalent to the addition of 12 equivalents of alkali, these results are in keeping with the conclusion that zinc binding was unimpaired.

(32) It is assumed that no binding of zinc ions to guanidinium groups occurred, because of the extremely high association constant for hydrogen ions with the guanidine residues.[6]

Values of log k°_{ZnIm} of 2.85, 2.80 and 2.79 were calculated from equation (5). For this, (HIm^{+}) was obtained from the equation

$$(HIm^{+}) = 16 - (HC + 6)$$

where (HC), the number of un-ionized carboxyl groups at equilibrium was obtained in the manner already described, and 6 is the total number of cationic nitrogen atoms in the molecule (100), minus the number of carboxyl groups in the modified albumin molecule.[33] The values obtained for log k°_{ZnIm} are effectively identical with those for the unmodified albumin.

Further studies confirming the failure of zinc ions to bind to the carboxyl groups of unmodified serum albumin and showing the effects of urea denaturation on this binding will be presented in a future communication.

Comparison with the System Zinc–Imidazole

In order to define more fully the interaction of zinc ion and the imidazole groups of serum albumin, a study of the interaction of zinc ion with imidazole alone has been undertaken. Since the first association constant of zinc ion and imidazole represents an association of a zinc ion with a single imidazole nucleus, it should be of value to compare it with the measured intrinsic association constant for zinc ion with the imidazole groups in human serum albumin.

Studies conducted in this Laboratory[34] have shown that at 4.5 ± 0.5°, in an ionic strength of 0.16, the first association constant of zinc ion and imidazole has the value log $k_1 = 2.76$. The first association constant for zinc ion and imidazole is therefore nearly identical with the intrinsic association constant for zinc ion with the imidazole groups of human serum albumin (log $k^{\circ}_{ZnIm} = 2.82$).

This agreement between log k, and log k°_{ZnIm} constitutes strong evidence for the one-to-one stoichiometry of the reaction. If the zinc ions were held in chelate complexes with a second group, whether imidazole, carboxyl or amino, the value of log k°_{ZnIm} would tend to be of the order of 2 log k_1.[35]

(33) This equation is analogous to equation (2). For discussion, see footnote 25.

(34) J. T. Edsall, G. Felsenfeld, D. S. Goodman and F. R. N. Gurd, in preparation.

Discussion

The results of the study of the binding of zinc ions to human serum albumin have demonstrated the value of treating the interaction of proteins with at least certain cations as a competition between the given cation and hydrogen ions for common sites on the protein. It is obvious that for the measurement and interpretation of such competition phenomena the use of hydrogen ion buffers should be avoided.

That the binding of cations to imidazole groups in serum albumin is not restricted to zinc ions has been shown recently by Tanford, who has independently concluded that copper, zinc, cadmium and lead ions bind to bovine serum albumin through the imidazole groups in the protein molecule.[37]

The data of Klotz and Curme[38] for the interaction of copper acetate with bovine serum albumin have been reinterpreted with the same result by Scatchard.[39] It therefore seems likely that the primary sites in the serum albumin molecule at which the binding of many cations occurs are the 16 imidazole groups.

The knowledge of the sites at which zinc and other cations are bound to serum albumin will be applied to the investigations currently in progress in this Laboratory on the X-ray diffraction analysis of protein crystals containing definite numbers of heavy atoms.[40]

Acknowledgments.—The authors wish to express their gratitude to Drs. George Scatchard and I. H. Scheinberg for invaluable help with the interpretation of the results, and to Drs. W. L. Hughes, Jr., and P. E. Wilcox for generous supplies of the modified proteins.

BOSTON, MASSACHUSETTS RECEIVED JUNE 11, 1951

(35) For example, in the association of a zinc ion with one ammonia molecule log $k_1 = 2.37$,[16] whereas with one triethylenediamine molecule log $k_1 = 5.92$,[16] and with glycine log $k_1 = 4.80$.[33]

(36) J. Bjerrum, *Chem. Revs.*, **46**, 381 (1950).

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

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

(39) G. Scatchard, *American Scientist*, in press.

(40) B. W. Low and E. J. Weichel, THIS JOURNAL, **73**, 3911 (1951).