groups in proteins and the temperature coefficients of these dissociation constants are given the same values as those observed in simple compounds containing the same groups (see Edsall and Wyman[23]). Yet we have seen in the last section that a change of 5 to 10 ml. from the normal value of $\Delta V$ might well be accompanied by changes of three or more kilocalories in $\Delta F$, which would bring about a change in the dissociation constant of at least two powers of ten. There is, of course, no indication that such changes actually occur.

We are unable to offer an explanation for the anomalous behavior of these volume changes in proteins. In view of the small effect of the carboxylate ion in $\alpha$- and $\beta$-amino acids on the $\Delta V$ of the amino group, it hardly seems likely that carboxylate ions near the protein amino groups can account for the anomaly. That hydrophobic regions in the vicinity of the amino groups could in some way decrease the volume changes seems unlikely because of the small effects on $\Delta V$ resulting from the substitution of alkyl groups directly onto the amino group, as in mono-, di- and trimethylamines and ethylamine. If the amino groups were embedded to any appreciable extent in a non-aqueous environment of low dielectric constant, the electrostriction would be much reduced because $V(\mathrm{d}D/\mathrm{d}V)$ in equation 7 is much smaller for such an environment than for water. It is, however, difficult to believe that this could be the case here, because such a situation would surely be accompanied by a large increase in the acidity of the protonated amino groups, which is not observed. It is conceivable that the ionization of the basic groups of proteins is accompanied by a conformation change that would bring about the anomalous volume change that we have observed. One would then expect that other properties of proteins that are sensitive to conformation (intrinsic viscosity, optical rotation) would change in the *p*H range in which the amino groups titrate. There is no evidence that this is the case, but it is conceivable that the conformation changes may be involved here which are of a type that do not affect, say, the optical rotation. We are inclined to suspect that this is the basic reason for the anomaly, but it is difficult to see why such conformation changes are not accompanied by free energy changes that would be reflected in anomalous dissociation constants of the basic groups of proteins. The layers of frozen water postulated by Klotz[24] may also be responsible for the anomaly, but aside from the objections that have been raised to this hypothesis,[25] it is not evident to us how these layers could bring about the observed behavior. Furthermore, Klotz has used his hypothesis in order to explain the anomalous reactivities of specific groups, such as sulfhydryl groups, and the groups we are concerned with here are not believed to have an anomalous reactivity.

Studies also have been made recently of the volume changes that accompany the reaction of wool and hair with acids and bases.[26] The studies show that in these substances reaction 1 is accompanied by a volume change of 9 ml., and reaction 2 is accompanied by a volume change of only 11 ml. Thus the carboxyl groups of wool and hair appear to react normally, but the basic groups deviate to an even greater extent from their behavior of simple molecules than do the basic groups in soluble proteins.

**Acknowledgments.**—This work was supported by a grant from the National Science Foundation.

(23) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press, Inc., New York, N. Y., 1958, pp. 525–526.

(24) I. M. Klotz, *Science*, **128**, 815 (1958); *Brookhaven Symposia in Biology*, **13**, 25 (1960).

(25) W. Kauzmann, *Advances in Protein Chem.*, **14**, 1 (1959); *Brookhaven Symposia in Biology*, **13**, 43 (1960).

(26) D. D. Kasarda and W. Kauzmann, to be published.

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# A Study of the Interaction of Nickel(II) with Bovine Serum Albumin[1a]

BY M. S. NARASINGA RAO

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The binding of $Ni^{II}$ by bovine serum albumin at *p*H 6.5 (acetate buffer of ionic strength 0.20) has been measured by the pM indicator method, using murexide as the indicator, and by the equilibrium dialysis technique. There is good agreement in the values obtained by the two methods. The electrophoretic mobility of albumin has been measured as a function of the bound $Ni^{II}$. The binding data supplemented by the mobility data lead to a value of $\log k^0 = 3.17$ for the intrinsic association constant. This compares well with the first association constant, $\log k = 3.27$, for $Ni^{II}$–imidazole interaction, suggesting that the $Ni^{II}$ is bound at the imidazole groups of the albumin molecule. The values of $\log k^0$ decrease with $\bar{\nu}$. This has been attributed to the increased participation of carboxyl groups in the interaction.

## Introduction

The interaction of $Cu^{II}$, $Zn^{II}$, $Cd^{II}$ and $Co^{II}$ with bovine serum albumin has been studied and the mode of interaction appears to differ among the metals.[1b] It was of interest, therefore, to study nickel–albumin interaction and to compare it with the other systems.

Recently, Ettori and Scoggan[2] have measured the binding of calcium ions by albumin with the pM indicator, murexide. Quantitative binding data have been obtained by this and the equilibrium dialysis technique to examine the applicability of this method to nickel–albumin interaction.

## Experimental

Crystallized bovine serum albumin was an Armour product lot No. CB0271. It was deionized by passing an

(1) (a) Communication No. 481 from National Chemical Laboratory, Poona-8 (India). (b) M. S. Narasinga Rao and Hira Lal, *J. Am. Chem. Soc.*, **80**, 3226 (1958).

(2) J. Ettori and S. M. Scoggan, *Arch. Biochem. Biophys.*, **91**, 27 (1960).

aqueous solution through a mixed bed of resins, Amberlite I. R. 120 and Amberlite I. R. 400.[3] Murexide was a B.D.H. product. Its purity, assayed by the gravimetric method,[4] was 92.3%. Nickel nitrate, urea and buffer salts were of reagent quality.

**Solutions.**—The solutions were prepared with deionized water. A stock solution of deionized albumin was prepared and its concentration was determined by measuring the absorption at 280 mμ. A value of 6.60 for $E^{1\%}_{280}$ was used for converting the absorption reading to concentration.[5] The nickel content of a stock nickel nitrate solution was estimated by weighing as the dimethyl-glyoxime complex. The solutions were stored in polythene bottles. All the measurements were made in acetate buffer solution of *p*H 6.5 and ionic strength 0.20.

**Optical Absorption.**—The absorption measurements were made at room temperature with a Unicam Spectrophotometer (SP 500), using cells of 1 cm. optical path.

Murexide solution of known concentration was prepared by direct weighing of the material dried over phosphorus pentoxide. The absorption spectra of an approximately $4 \times 10^{-5}M$ murexide solution alone and of solutions containing $4.11 \times 10^{-4}$, $8.22 \times 10^{-4}$, $1.64 \times 10^{-3}$ and $5.00 \times 10^{-2}M$ nickel were recorded in the range 400–600 mμ. The spectrum of the last solution corresponds to that of nickel–murexide chelate. The stability constant, $K_f$, for the formation of the chelate was calculated from the absorption readings at various wave lengths, by the method of Schwarzenbach and Gysling.[6]

For experiments with the protein, 5.0 ml. of solutions containing acetate buffer, albumin, murexide and varying amounts of nickel were prepared. The *p*H and the albumin concentration of the final solutions were 6.5 (ionic strength 0.20) and 1.4% respectively. The total nickel added varied from $1 \times 10^{-4}$ to $1 \times 10^{-2}M$. The blank for absorption measurements contained the same amount of albumin and acetate buffer. Readings were taken only at 520 and 460 mμ, they being the absorption maximum of murexide and of the chelate respectively.

**Equilibrium Dialysis.**—5.0 ml. of 1.4% albumin solution contained in a Visking Sausage tubing were equilibrated against 5.0 ml. of buffer solution containing varying amounts of nickel. Blanks in which the protein solution was substituted by the buffer solution were also run. Equilibrium at room temperature was found to be attained in 24–48 hr. on mechanical shaking. At the end of 48 hr., the solutions outside the dialysis bag of both the experimental and the blank were analysed for their nickel ion concentration. Nickel was estimated colorimetrically using quinoxaline-2,3 dithiol as the coloring agent.[7]

**Electrophoresis.**—Mobility measurements were made at 0.5° with a Tiselius Electrophoresis apparatus (Perkin–Elmer Model 38) fitted with the Longsworth scanning system. A 2-ml. cell and 1% dialysed protein solution were used.[8] Experimental procedure described previously was followed.[9] For the calculation of mobility, boundary displacements in the descending limb were used.

## Results and Calculations

For the calculation of $\bar{\nu}$, the number of metal ions bound per mole of protein, the total concentration and the equilibrium concentration of the metal ion are needed. The equilibrium concentration of nickel can be calculated from optical absorption readings at 460 mμ and 520 mμ. Following Ettori and Scoggan[2] we may write

$$[A] = \frac{1}{K_f} \times \frac{\epsilon_1^1 - \epsilon_1^2\varphi}{\epsilon_2^2\varphi - \epsilon_2^1}$$

where $[A]$ is the equilibrium concentration of $Ni^{II}$, $K_f$ the stability constant of the $Ni^{II}$–murexide chelate, $\epsilon_1^1$ the molar extinction coefficient of murexide at 460 mμ and $\epsilon_1^2$ at 520 mμ, $\epsilon_2^1$ that of the chelate at 460 mμ and $\epsilon_2^2$ at 520 mμ, and $\varphi$ the ratio of optical densities of the solution at 460 and 520 mμ. The value of $K_f$, determined at *p*H 6.5 in acetate buffer of ionic strength 0.20 and room temperature (about 25°), was log $K_f = 3.31 \pm 0.02$.[10]

This equation is valid provided there is no interaction between the protein and murexide. The absorption spectrum of murexide was not altered by the addition of albumin, indicating that there was no interaction. It was also observed that there was no binding of murexide by the protein mediated by nickel ions.

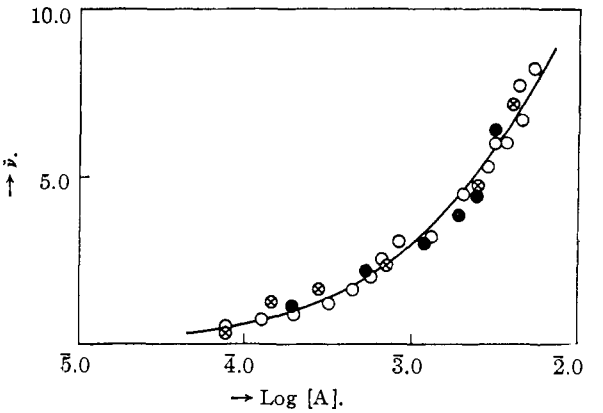


Fig. 1.—The binding of $Ni^{II}$ by bovine serum albumin (acetate buffer, *p*H 6.50; ionic strength, 0.20); -⊙-, pM indicator method; -⊗-, equilibrium dialysis method; -●-, in presence of 5 *M* urea (pM indicator method).

In Fig. 1 values of $\bar{\nu}$ are plotted as a function of log $[A]$. A molecular weight of 65,000 has been assumed for albumin for the calculation of $\bar{\nu}$. Data from the pM indicator method agree well with those from the equilibrium dialysis method; in fact, the internal consistency in values is much better in the former. However, in the higher binding region ($\bar{\nu}>8$) estimation becomes uncertain because the dye gets almost saturated with respect to the metal and absorption readings attain a constant value. Increase in the dye concentration makes the solutions too dense to permit making optical measurements. The quickness of this method and absence of membrane equilibrium uncertainties, as pointed out by Ettori

(3) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, *J. Am. Chem. Soc.*, **79**, 782 (1957).

(4) J. H. Moser and M. B. Williams, *Anal. Chem.*, **26**, 1167 (1954).

(5) C. Tanford, *J. Am. Chem. Soc.*, **74**, 211 (1952).

(6) G. Schwarzenbach and H. Gysling, *Helv. Chim. Acta*, **32**, 1313 (1949).

(7) D. A. Skoog, M. G. Lai and A. Furst, *Anal. Chem.*, **30**, 365 (1958).

(8) Addition of $Ni^{II}$ to the albumin solution at *p*H 6.5 was found to lower the *p*H. For each experiment, 5.0 ml. of 1% albumin solution containing nickel, equivalent to the bound and free metal, were equilibrated for a period of 24 hr. against 500 ml. of buffer containing nickel equivalent to the free metal concentration. The required values were taken from the binding curve.

(9) Hira Lal and M. S. Narasinga Rao, *J. Am. Chem. Soc.*, **79**, 3050 (1957).

(10) The value of log $K_f = 3.31$ is much different from that one would obtain by interpolation of the data of Reference 11. The interpolated value of log $k_f = 4.6$ refers to a medium of ionic strength 0.10. Measurements in acetate buffer of *p*H 6.5 and ionic strength 0.1 gave a value of log $K_f = 3.51$ and in an unbuffered potassium nitrate solution of *p*H 6.0 and 0.1 *M* concentration a value of log $K_f = 3.26$. The reason for the difference between the measured and literature values is not obvious.

(11) G. Schwarzenbach, "Complexometric Titrations," (Translated by H. Irving), Interscience Publishers, Inc., New York, N. Y., 1957, p. 37.

(12) N. C. Li, T. L. Chu, C. T. Fujii and J. M. White, *J. Am. Chem. Soc.*, **77**, 859 (1955).

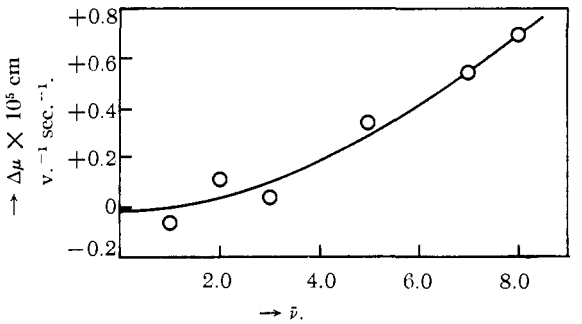


Fig. 2.—The effect of the binding of $Ni^{II}$ on the electrophoretic mobility of bovine serum albumin (0°, acetate buffer, *p*H 6.5, ionic strength 0.20).

and Scoggan, offer advantages over the equilibrium dialysis method. In Fig. 2, the mobility of albumin is given as a function of $\bar{\nu}$. It is seen that the first few bound ions do not cause any measurable change in the mobility. However, there is a pronounced decrease in the (negative) mobility at $\bar{\nu} > 4$.

## Discussion

From the plot of $\bar{\nu}/A$ *vs.* $\bar{\nu}$, the values of the first association constant, $K_1$ (= $kn$, where $k$ is the intrinsic association constant) and $n$, the maximum number of binding sites are obtained. These are $K_1 = (7.5)10^3$ and $n \sim 16$. This value of $n$ suggests that the binding sites are, perhaps, the imidazole groups of the albumin molecule. Based on concepts presented in an earlier paper,[9] the intrinsic constant, corrected for the charge on the protein, has been calculated from the first association constant. The charge on the protein molecule is obtained from the electrophoretic mobility data and has a value of −16 (electron units). The intrinsic association constant thus obtained is log $k^0 = 3.17$. This compares well with the first association constant for nickel-imidazole interaction, log $k = 3.27$. The agreement between these values suggests that the imidazole groups are the sites at which nickel ions are bound by the albumin molecule.

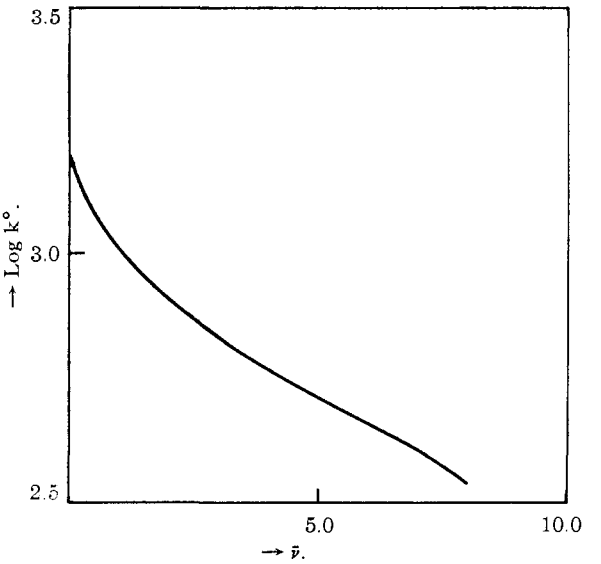


Fig. 3.—Intrinsic constants for 1:1 interaction with imidazole sites.

The binding data along with the mobility values can be utilized to calculate log $k^0$ as a function of $\bar{\nu}$.[1,9] If the binding sites are of the same class and are all equivalent, then log $k^0$ should be independent of $\bar{\nu}$. However, from Fig. 3 it can be seen that there is a gradual decrease from the initial value of 3.17 and at $\bar{\nu} = 8.0$, log $k^0 = 2.53$. This may be interpreted either as that all the sixteen imidazole groups are not equivalent or that there is an increasing participation of some group with a lower binding affinity than imidazole.

Binding measurements in the presence of 5 *M* urea, which is known to disrupt the helical structure of the protein, gave $\bar{\nu}$ values which fall on the same curve (Fig. 1). It is, therefore, likely that carboxyl groups which have a much lower affinity also compete with the imidazole groups for binding $Ni^{II}$.

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# The Hydrolysis of Chloramine in Alkaline Solution

BY M. ANBAR AND G. YAGIL

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The hydrolysis of chloramine was investigated in alkaline solutions from *p*H = 12 to 12 molar NaOH. Hydroxylamine is produced in the primary step, nitrous oxide and nitrogen are the main final products of hydrolysis. Kinetic results show that the reaction is first order in chloramine with a linear dependence on the $H_-$ acidity function. The kinetics of hydrolysis of dimethylchloramine have been investigated as well as the hydrogen exchange of this compound with water. These as well as other results suggest the nucleophilic substitution of chloride by a hydroxyl ion as the most probable mechanism of hydrolysis.

## Introduction

Chloramine has been shown to be the first product of the hypochlorite-ammonia reaction which ultimately yields hydrazine.[1] This compound undergoes a decomposition in aqueous solutions, which was found both acid and base catalysed.[2,3] Raschig examined the final products of decomposition of chloramine in alkaline solution (when $(NH_2Cl) = 0.1$ *M*) and found nitrogen, ammonia

(1) F. Raschig, "Schwefel und Stickstoffstudien," Verlag Chemie G.m.b.H., Berlin, 1924, p. 50.

(2) *Ibid.*, p. 60.

(3) J. Kleinberg, M. Tecotzky and L. F. Audrieth, *Anal. Chem.*, **26**, 1388 (1954).