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INFLUENCE OF IONIC STRENGTH ON THE BINDING OF SODIUM AUROTHIOSULPHATE TO HUMAN SERUM ALBUMIN

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Abstract—The effect of ionic strength on the binding of aurothiosulphate to human serum albumin has been studied at 37° and neutral pH by equilibrium dialysis in unbuffered solutions. The effect of ionic strength is more pronounced on the lower association constants K_2–K_4 than on the high association constant K_1. Furthermore a reduction in the number of lower affinity binding sites is observed at low ionic strength. The main ionic strength dependence on the association constants agrees with the Debye–Hückel theory. The extrapolated values of K_1 and the sum of K_2 to K_4 at zero ionic strength are $7.6 \times 10^5\ M^{-1}$ and $1.1 \times 10^5\ M^{-1}$, respectively. It is shown that the observed changes in pH of the albumin solutions during dialysis contains valuable information of the aurothiosulphate–albumin interaction. A molecular binding mechanism is discussed.

Although gold compounds for many years have been important agents in the treatment of rheumatoid arthritis, important aspects of their pharmacology are still unknown. Some information can be obtained from binding studies, but only a few quantitative data concerning the binding of gold compounds to serum proteins are available [1–3]. These studies are mainly concerned with the effect of pH and temperature on the gold-compound–albumin interaction. It is known from other binding studies, e.g. calcium to serum proteins [4, 5] and digitoxin to human serum albumin [6] that the degree of binding varies with the ionic strength. However, in general the influence of ionic strength has received little attention in binding studies making comparisons of binding results dubious.

In the present study the effect of ionic strength on the binding of sodium aurothiosulphate to human serum albumin has been investigated for the first time. Sodium aurothiosulphate is a gold compound widely used in the treatment of rheumatoid arthritis, and a variation of the association with serum albumin with ionic strength in the pathophysiological range may thus have clinical relevance. Furthermore, by implying the Debye–Hückel equation for activities, and extrapolating to zero ionic strength the true thermodynamic association constants are determined. This procedure also determines the algebraic charge of the combining gold complex, and sheds light on the molecular binding mechanism.

MATERIALS AND METHODS

Materials. The albumin preparation was purified, lyophilized human albumin (Behringwerke AG, Marburg, West Germany). The albumin preparation fulfilled the criteria for purity specified in [7]. Crossed-immunoelectrophoresis [8] performed against rabbit antihuman serum (DAKO, Copenhagen, Denmark) showed that no peaks attributable to other proteins than albumin were detectable. Polyacrylamide gradient gel (PAA 4/30, Pharmacia, Uppsala, Sweden) electrophoresis of a 0.5% albumin solution showed only one distinct band of monomer albumin and one very faint band due to dimer albumin. The sodium aurothiosulphate ($Na_3Au(S_2O_3)_2$, Sanocrysin®), was purchased from Ferrosan, Søborg, Denmark. The Visking seamless cellophane tubing (8/32 in., Union Carbide, Chicago) was used for dialysis. All initial solutions of albumin, sodium aurothiosulphate (and blanks) were unbuffered solutions prepared in distilled, sterile water containing increasing amounts of dry NaCl so that the final solutions had ionic strengths in the range 0.03–0.20 M. Various amounts of acid (0.1 N HCl) or base (0.1 N NaOH) where added to adjust pH to 7.50 in each initial solution, in order to obtain pH ≈7.4 at equilibrium [2].

Equilibrium dialysis. The binding of sodium aurothiosulphate to human albumin in unbuffered solutions at 37°, and pH 7.33–7.46, at five different ionic strengths in the range 0.03–0.20 M, was studied in an equilibrium dialysis system previously described [2, 3]. The range of concentration of total sodium aurothiosulphate was 82–1224 μM. At each ionic strength the albumin solution on the inside and the sodium aurothiosulphate solutions on the outside of the membrane was initially identical with respect to pH and ionic strength. After equilibrium was reached the pH and the concentration of albumin was measured inside the dialysis membrane. The concentration of sodium and aurothiosulphate (gold) were measured on each side of the membrane.

Ionic strength. It was found that in the concentration range of sodium aurothiosulphate used the contribution of aurothiosulphate to the ionic

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Table 1. Changes in pH of the albumin solutions during dialysis

Ionic strength 0.03 M			Ionic strength 0.20 M	
$\bar{\nu}$*	$-\Delta$pH†	$-\Delta pH_D$‡	$\bar{\nu}$	$-\Delta$pH
0.46	0.14	0.07	0.40	0.16
0.91	0.10	0.03	0.97	0.15
1.59	0.06	−0.01	1.24	0.15
2.03	0.03	−0.04	–	–

* The average number of gold atoms bound per albumin molecule.

† The observed changes in pH of the albumin solutions during dialysis.

‡ The pH changes corrected for the Donnan effect.

strength in each albumin solution could be neglected; and according to general practice the contribution of albumin to ionic strength was ignored.

pH. When salt was added to the original albumin solutions, an increase of pH was observed. This increase of pH decreased with increasing ionic strength. Therefore, various amounts of acid or base were needed in order to obtain pH = 7.5 before dialysis. Furthermore, it was found that pH decreased during dialysis as previously described [2]. This decrease diminished with decreasing ionic strength as illustrated in Table 1.

Measurements. For albumin determinations a quantitative electroimmunoassay technique was used according to the principles of Laurell [9] (Albumin standard; Standard-Human-Serum, Behringwerke AG). All pH measurements were performed at 37° with a Radiometer pH meter (BMS MK2 blood Micro System). The gold concentrations were determined with a flameless atomic absorption spectrophotometer (Beckman model 485 fitted with a Masmann Cuvette model 1268) as described by Pedersen and Graabæk [10]. The sodium concentrations were determined with a IL 343 Digital Flame Photometer.

RESULTS

Treatment and presentation of experimental data

The concentrations of sodium on each side of the dialysis membrane displayed in Table 2 show that the Donnan effect is important. For Donnan equilibria

$$\left(\frac{a_i}{a'_i}\right)^{1/Z_i} = \text{constant} \qquad (1)$$

where Z_i denotes the algebraic charge on any diffusible ion; a_i the ion activity in the solution containing the macro-ions; and a', the ion activity in the solution free from macro-ions. From the known distribution of Na^+ across the membrane, cf. Table 2, the constant in equation (1) can be calculated for the five sets of experimental data differing in ionic strength, if the activity coefficients were known. These activity coefficients can be calculated from the Debye–Hückel equation

$$-\log f = \frac{AZ^2\sqrt{I}}{1 + Ba\sqrt{I}} \qquad (2)$$

where f is the ion activity coefficient, Z is the algebraic charge of the ion in solution, a is the ion size parameter and I is the ionic strength. The parameters A and B may be estimated at respectively 0.52 and 0.33 at 37° [11]. The results so obtained differ insignificantly from the results obtained by using concentrations instead of activities. This is due to the fact that the only important contribution to the ionic strength comes from Na^+ and Cl^- and the differences of these concentrations across the membrane are small.

Table 2. The Donnan distribution ratios for sodium ions across the semipermeable membrane as a function of ionic strength

Ionic strength (M)	$\frac{Na}{Na'}$
0.03	1.17
0.07	1.06
0.11	1.03
0.15	1.01
0.20	1.00

The ionic strength was due to NaCl. The primed symbol indicates the side of the membrane free from macro-ions. The albumin concentration was 0.47–0.54 mmoles/l and pH 7.3–7.4.

The concentrations of gold (or gold complex) in the solution containing no macro-ions C' are known from the five sets of experimental data. Using equation (1) the concentrations of unbound gold C in the solution containing the macro-ions can be calculated if the charge Z_G of gold (or gold complex) is known, which it is not. However, within experimental error the numerical value of Z_G could be determined to 3 using the procedure described below. Thus $Z_G = -3$ was used in the calculations, which means that gold is found as $Au(S_2O_3)_2^{3-}$ in the solution. The average number of gold atoms bound (in one form or another) per albumin molecule can be calculated as $\bar{\nu} = (C_{total} - C)/(\text{albumin})$ where C_{total} is the total gold concentration on the inside of the dialysis membrane. The correction for the Donnan effect, i.e. to use C instead of C' in this equation, is most important at the smallest ionic strength. The mol. wt of albumin was assumed to be 67,000 throughout.

Figure 1 shows the experimental data for the binding of sodium aurothiosulphate to human serum albumin at five different ionic strengths in the range 0.03–0.20 M; the temperature was 37°, and pH 7.33–7.46. The binding data corrected for Donnan effect are plotted as $\bar{\nu}$ vs log C. The curves in Fig. 1 are the best fit of the experimental data to equation

$$\bar{\nu} = \sum_{i=1}^{n} \frac{K_i C}{1 + K_i C} \qquad (3)$$

using a non-linear least-square curve-fitting procedure. The summation is over all n sites of the albumin molecule and K_i is the association constant for site i. Equation (3) implies that there is no interaction between the sites. In the calculations $n = 4$ was assumed for all ionic strengths in accordance

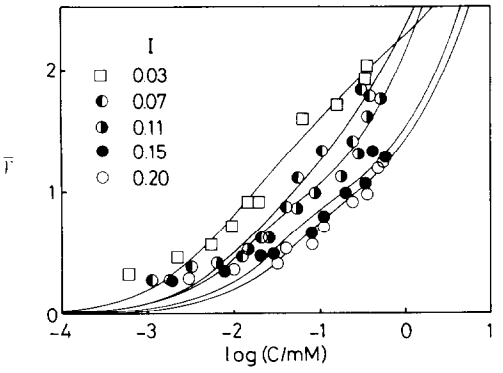

Fig. 1. The effect of ionic strength I on the binding isotherms of sodium aurothiosulphate to human albumin at 37° and pH 7.3–7.4. The curves drawn represent the best fit to equation (3) using $n = 4$. The experimental data are corrected for the Donnan effect. The unit of I is mol/l.

with previous publications [2, 3]. The association constants calculated from the experimental data with and without correction for Donnan effect are summarized in Table 3. As in previous works [2, 3] the binding data for small values of $\bar{\nu} \lesssim 0.3$ deviate from the calculated curves. The reason for this interesting discrepancy is at present unknown. It should be noted, however, that exclusion of these binding data from the fitting procedure does not lead to any significant change in the values of the association constants. The value of K_1 is affected by a decrease of the order of 10% and the other constants remain virtually unchanged.

Figure 1 and Table 3 show that the binding of gold (or gold complex) to human serum albumin is markedly affected by the ionic strength. Furthermore, it is found that when the Donnan effect is not corrected for, the association constants are underestimated increasingly with decreasing ionic strength, cf. Fig. 2. It also appears as though an increased number of binding sites become available for the gold complex binding when the ionic strength is above 0.10 M. This might indicate that a change in the conformation in albumin has occurred in the investigated range of ion strength at pH alkaline to the isoionic point. However, it should be noted that for ionic strengths above 0.10 M only a few experimental data set for $\bar{\nu} > 1$ are available. Consequently for these ionic strengths the number of binding sites cannot be determined very precisely and only the sum $K_2 + K_3 + K_4 + \ldots$ can be determined. For reasons of comparisons this sum denoted $\Sigma K_{2\text{-}4}$ are used in the following investigations. The values of $\Sigma K_{2\text{-}4}$ are displayed in Table 3.

The thermodynamic constants expressed in terms of activities of the combining species, could be obtained from the apparent association constants for instance by extrapolation to infinite dilution of all solutes, a condition under which activities become by definition equal to concentrations. This procedure will be adopted in the following, with the restriction that the results are extrapolated to zero ionic strength of supporting electrolyte only.

The apparent association constant K obtained by using concentrations instead of activities, is related to the thermodynamic association constant K_o by

$$\log K = \log K_o + \log f_G \qquad (4)$$

where f_G is the activity coefficient of the gold complex. The reasonable assumption that the activity coefficients of the albumin–gold complex and the albumin molecule are identical has been used. Inserting the Debye–Hückel equation (2) into equation (4) leads to

$$\log K = \log K_o - \frac{AZ_G^2\sqrt{I}}{1 + Ba_G\sqrt{I}} \qquad (5)$$

Table 3. The effect of ionic strength on the association constants for binding of sodium autothiosulphate to human serum albumin at $T = 37°$, and pH 7.33–7.46*

Ionic† strength (M)	Albumin (mM^{-1})‡	K_1 ($\times 10^{-3}$ M^{-1})	K_2 ($\times 10^{-3}$ M^{-1})	K_3 ($\times 10^{-3}$ M^{-1})	K_4 ($\times 10^{-3}$ M^{-1})	$\Sigma K_{2\text{-}4}$ ($\times 10^{-3}$ M^{-1})	r.m.s.
0.028	0.47	203 (168)	14.3 (3.9)	0.50 (0.00)	0.00 (0.00)	14.8 (3.9)	0.10 (0.13)
0.068	0.51	82 (68)	3.4 (3.1)	0.40 (0.13)	0.40 (0.13)	4.2 (3.3)	0.12 (0.12)
0.107	0.52	71 (66)	0.61 (0.51)	0.61 (0.51)	0.61 (0.51)	1.8 (1.5)	0.08 (0.09)
0.154	0.54	33 (33)	0.22 (0.19)	0.22 (0.19)	0.22 (0.19)	0.66 (0.57)	0.11 (0.11)
0.200	0.49	21 760‖ (550)	0.17	0.17	0.17	0.51 112‖ (21)	0.11

* For each value of ionic strength, assuming the number of binding sites, $n = 4$, the association constants were obtained by analysis of a complete binding isotherm consisting of 10 experimental points. The values in parenthesis denote that the experimental data are uncorrected for the Donnan effect.

†‡ The indicated value is the mean value, one S.D. is typically 0.02.

§ The S.D. of $\bar{\nu}$ on C from the best least-square fit to equation (2).

‖ The thermodynamic association constants, i.e. the extrapolated value to zero ionic strength, using the curves of Fig. 2.

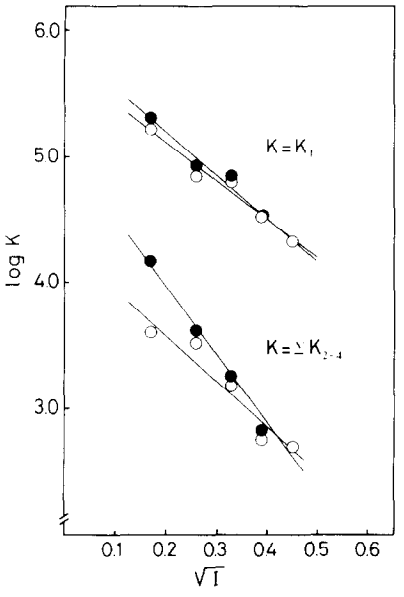

Fig. 2. The ionic strength dependence of the association constants K_1 and ΣK_{2-4}; with (●) and without (○) correction for the Donnan effect. The lines are the linear regression lines according to equation (5), cf. text. The ordinate intercept is $\log K_o$ and the slope equals AZ_G^2.

where Z_G is the algebraic charge of the gold complex in solution and a_G is the ion size parameter. The ion size parameter is uncertain but a value of about 4 is probably reasonable. In fitting the determined values of K for various ionic strength to equation (5) two approaches can be taken. One can neglect the B-term in the denominator and determine AZ_G^2 by a linear regression analysis of $\log K$ vs $\sqrt{I}$. A graphical display is shown in Fig. 2. For the high affinity binding constant this gives a correlation coefficient of determination $r^2 = 0.977$, value of $\log K_o = 5.88$ and $K_o = 76 \times 10^4\ M^{-1}$, and a value of $|Z_G| = 2.6$, assuming the above value for A. For the lower affinity binding constants ΣK_{2-4} one obtains $r^2 = 0.987$, $\log K_o = 5.05$ or $\Sigma K_{2-4}^o = 112 \times 10^3\ M^{-1}$ and $|Z_G| = 3.2$. If instead one assumes that the above values of A, B, and a_G are correct, then a linear regression analysis of $\log K$ vs $A\sqrt{I}/(1 + Ba_G\sqrt{I})$ yields $r^2 = 0.965$, $\log K_o = 6.24$ or $K_o = 174 \times 10^4\ M^{-1}$, and $|Z_G| = 3.6$ for the high affinity binding constant and $r^2 = 0.991$, $\log K_o = 5.64$ or $\Sigma K_{2-4}^o = 440 \times 10^3\ M^{-1}$, and $|Z_G| = 4.52$ for the lower affinity binding constants. For the high affinity binding constant the first relationship, i.e. $\log K$ vs $\sqrt{I}$, represents the experimental data best. For the low affinity constants the two fits are equally good. For these reasons and since the value of $|Z_G|$ obtained by the first method makes more sense we adopt the values from the first method in the following, cf. Table 3. Note, however, that in any case the extrapolated values to zero ionic strength determined by the two relationships do not differ by a factor of 2 and the value of $|Z_G|$ is determined quite accurately to 3. In order to illustrate the importance of the Donnan effect the same analysis has been applied to the uncorrected data, cf. Fig. 2 and Table 3.

DISCUSSION

In the present investigation it is found that changes of ionic strength induced by NaCl have a marked effect on the binding of sodium aurothiosulphate to human serum albumin at 37° and pH 7.3–7.4. Furthermore, the results demonstrate that the Donnan effect gives rise to an underestimation of this effect increasingly with decreasing ionic strength, cf. Table 3, Fig. 1 and Fig. 2.

It is found that the effect of ionic strength shows a continuous decrease in the binding affinity with increasing ionic strength and is thereby qualitatively similar to that described previously for binding of anions and calcium to albumin [4, 12]. On the contrary, the paper of Brock [6] demonstrates a continuous increase in the binding affinity of the digitoxin–albumin interaction with increasing ionic strength at neutral pH and 37°. This indicates that different molecular mechanisms are involved in binding of digitoxin and sodium aurothiosulphate to albumin, respectively.

There is no evidence at present that sodium is bound to albumin. Chloride is bound to albumin [13] but no competition between chloride ions and aurothiosulphate ions for the same binding sites has been found [3]. The effect of ionic strength on the binding of sodium aurothiosulphate to albumin could be accounted for by the Debye–Hückel theory. It therefore implies that the effect of the added salt is an unspecific electric shielding of the gold complex and the binding sites. This is similar to the mechanism suggested for binding of calcium to albumin [4].

Figure 1 and Table 3 demonstrate an increase in the number of low affinity binding sites with increasing ionic strength, which must for reasons already mentioned be interpreted warily. Nevertheless, this result might indicate that a conformational change in the albumin molecule, presumably induced by anion binding, has occurred and that the isomeric form is favored with increasing ionic strength. This is in accordance with the following findings. Leonard, Vijai and Foster [14] have found that a transformation of the native albumin molecule to an isomeric form occurs in the pH region 7–9; that the more strongly bound anions, thiocyanate and perchlorate, shift the transition to lower pH; and that the new form is favored as the ionic strength is increased. Klotz, Burkhard and Urquhart [15] and Katz and Klotz [16] have found that an increased number of sites become available for anionic and neutral dyes in the pH range 7–9 and concluded that important changes in the conformation occur in this pH region.

The extrapolated value of K_1 and ΣK_{2-4} at zero ionic strength of supporting electrolyte may deviate from the true thermodynamic association constants since the albumin molecules contribute a relatively larger part of the total ionic strength as the latter becomes very small; the ionic strength induced conformational change of the albumin molecule might affect the binding, and finally the accuracy of the experimental data may not justify this refinement.

It is found that the association constant K_1 decreases from the extrapolated value $76 \times 10^4\ M^{-1}$

to $7.1 \times 10^4\,M^{-1}$ and $2.1 \times 10^4\,M^{-1}$ when the ionic strength is increased to 0.1 and 0.2 M, respectively. Similarly ΣK_{2-4} decreases from the extrapolated value $112 \times 10^3\,M^{-1}$ to $1.8 \times 10^3\,M^{-1}$ and $0.51 \times 10^3\,M^{-1}$. These observations illustrate the critical importance of constant ionic strength in binding studies and comparisons of binding results.

It has been suggested previously [3] that aurothiosulphate binds as Au^+ to the high affinity binding site by exchanging a H^+ and as $Au(S_2O_3)^{3-}$ to the lower affinity binding sites. The present results will be discussed with respect to this assumption. Returning to Fig. 2 we note, that for Donnan corrected binding data the effect of ionic strength is more marked on the lower affinity binding sites than on the high affinity binding site. This might be due to different charge of the gold (or gold complex) that binds to the high and lower affinity binding sites, respectively. However, this could conceivably also be explained by an anion induced conformational change in the albumin molecule affecting only the lower affinity binding sites.

The observed pH-changes (ΔpH) of the albumin solutions during dialysis, cf. Table 1, also support the proposed binding mechanism. As described in more detail above the two solutions separated by the dialysis membrane were initially (i.e. before dialysis) adjusted to have identical pH and ionic strength. When equilibrium was reached after dialysis a decrease of pH in all the albumin containing solutions was observed. This pH decrease increased with increasing ionic strength. It could not be explained as a Donnan effect which would produce the largest effect for the smallest ionic strength. The largest decrease of pH due to the Donnan effect can be calculated to 0.07 from the observed Donnan distribution factor for Na^+. The Donnan corrected values of ΔpH are shown in Table 1. The observed decrease in pH for $\bar{\nu} \leqslant 1$ might to some extent be explained by assuming that the gold complex binds to albumin as Au^+ by exchanging a H^+ as it diffuses into the solution. A binding to albumin of a positively charged gold complex would have the same effect, although with a smaller efficiency, since the increased positive charge of the albumin molecule would give a decreased attraction of H^+ and thus a lower binding of H^+. However, as no change in charge of the albumin molecule due to binding of the gold complex to the high affinity binding site has been observed [3], the first assumption is the most likely. Furthermore, from Fig. 1 in the study of Tanford *et al.* [17] a change in pH $\simeq$ 0.15 can be estimated assuming that one H^+ is dissociated from the albumin molecule at neutral pH, which is very close to that found in this study but cannot explain the constant value of ΔpH for $\nu < 1$. Although the tubes were capped during dialysis a decrease of pH due to concentration of the solutions cannot be excluded. The changes in pH of the albumin solutions for $\nu > 1$ can be explained by assuming that a negatively charged gold complex binds to albumin, as the change in charge of the albumin molecule due to combination with anions increases its electrostatic attraction for hydrogen ions thus increasing pH in the solution. This effect is more marked at low ionic strength, where the relative binding to sites 2–4 is strongest.

As demonstrated the binding of sodium aurothiosulphate to human albumin was markedly influenced by ionic strength. Even small variations of the ionic strength within clinical relevant range influenced the binding affinity to such a degree that variations in the unbound fraction of aurothiosulphate might give rise to unwanted effects in the individual patient, e.g. change of the ionic strength from 0.11 to 0.16 M gives rise to a twofold increase in the unbound gold concentration.

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