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Ionic Binding, Net Charge, and Donnan Effect of Human Serum Albumin as a Function of pH

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The ionic activities and total molalities of sodium, potassium, calcium, lithium, and chloride in a solution of human serum albumin were measured at different values of pH between 4 and 9. The same quantities were measured simultaneously in a protein-free electrolyte solution in membrane equilibrium with the albumin solution. Taking the residual liquid-junction potential and bias from unselectivity of the electrodes into account, we determined the own, bound, and net charges of albumin. Chloride was amply bound at low pH, and calcium at high pH. The varying charge of ions bound to albumin opposed the effect of acid or base on the net charge. All ions were distributed across the membrane according to the same electric potential difference, which equalled the Donnan potential. The high concordance between observation and theory favors the Donnan theory and furthermore implies that the electrodes are as accurate in a solution with albumin as in a protein-free solution.

Additional Keyphrases: *artificial kidney · dialysis · colloid osmotic pressure · ion-selective electrodes · calcium · chloride · lithium · potassium · sodium*

The colloid osmotic pressure of albumin in plasma balances the intravascular hydrostatic pressure, thereby maintaining a normal plasma volume. About half of the colloid osmotic pressure is due to the Donnan effect: The permeable cations and anions are unevenly distributed intra- and extravascularly, according to the Donnan theory (*1*), with the larger total molality (mmol/kg of water) on the vascular side. The contribution of the Donnan effect to the colloid osmotic pressure ($\pi_{colloid}$) is proportional to the squared molality of impermeable charge and inversely proportional to the molality of salt:

$$\pi_{colloid} = R \cdot T \cdot \phi[m_{albumin} + Z_{net}^2/(4m_{salt})] \cdot \rho^*_{H_2O} \quad (1)$$

where R is the gas constant, T is the temperature, ϕ is the osmotic coefficient, m is molality, Z_{net} is the molality of impermeable albumin net charge, and $\rho^*_{H_2O}$ is the density of water.

The net charge of albumin is defined as its own charge plus the charge of all bound ions, all of which depend on pH (*2*). pH and electrolyte disturbances are common in patients with abnormal plasma volume and albumin, for example, because of neonatal asphyxia, hemorrhage, shock, nephrotic syndrome, or lung edema, so a study of the influence of pH on the net charge, Donnan effect, and ionic binding of albumin is clinically relevant. Increasing the pH in a patient might be as effective for the colloid osmotic pressure as an albumin infusion.

Although plasma is the system used normally in clinical chemistry, it is the interstitial fluid that constitutes the actual environment of the cells and is regulated by homeostasis. Ion activity is not identical in plasma and interstitial fluid. The Donnan effect and the factors affecting it should be known and taken into account for the interpretation of electrolyte results, especially in patients with severe electrolyte, pH, or protein disturbances (*3*). The Donnan effect may also underlie the alkalosis of hypoproteinemia and the acidosis of hyperproteinemia.

Materials and Methods

The experimental setup is shown in Figure 1. We used an artificial kidney with a Cuprophan membrane (Gambro GF 120M Hollow Fiber Dialyzer with 1.3 m^2 effective membrane area). On the inner side of the membrane was a 200 g/L solution of human serum albumin for injection (Nordisk Gentofte, Denmark), 0.5 L, which had been dialyzed to remove its stabilizing caprylic acid. The ratio of fatty acid to albumin was 1. The high concentration of albumin was chosen to give a high precision to the measurements, but we must assume that the association constants were unaffected. We added physiological concentrations of NaCl, KCl, and $CaCl_2$ plus LiCl (1 mmol/L), imidazole (1 mmol/L), and succinic acid (1 mmol/L). All reagents were analytical grade from Merck (Darmstadt, Germany). The two buffers were necessary to stabilize pH in the protein-free solutions, and they were chosen to cover the pH interval without binding calcium. Extra $CaCl_2$ was added to compensate for the increased albumin binding of Ca^{2+} with increasing pH. Bicarbonate was not included here because P_{CO_2} could not be controlled and because bicarbonate interferes with the chloride electrode. On the outer side of the membrane was a protein-free solution with ions in equilibrium with the albumin solution.

The solutions were maintained at 37 °C. The flow of albumin and protein-free electrolyte through the artificial kidney was maintained at 500 mL/min by two peristaltic pumps. The volume was maintained by an adjustable clamp on the tubing leading from the artificial kidney, and an increased trans-membrane pressure was necessary with increasing pH.

pH was increased from 4 to 9 in 14 steps by adding sodium hydroxide. Constant results indicated that equilibrium was reached 15 min after sodium hydroxide was added; the samples were drawn after 30 min.

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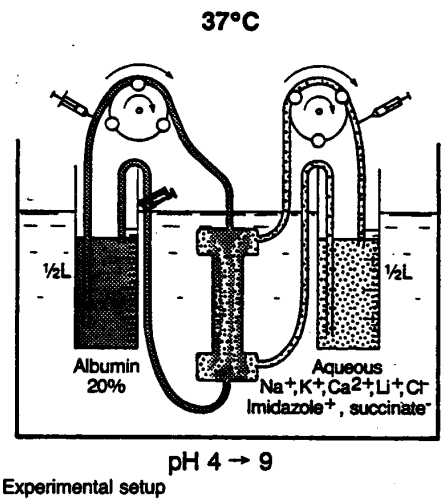

Fig. 1. Experimental setup

The hollow fiber artificial kidney (in the *middle*) and the containers for albumin and dialysis fluid were immersed in a water thermostat. Two roller pumps provided the counter-current circulation, with the albumin solution being on the inside of the hollow fibers and the dialysis fluid on the outside. The transmembrane pressure could be regulated by a clamp on the albumin line coming from the artificial kidney. Analytical samples could be taken by the syringes. The albumin solution had the same ions as the dialysis fluid, in membrane equilibrium at the time of sampling

All samples were subject to many measurements. The activity of Na^+, K^+, Ca^{2+}, Li^+, Cl^-, and pH was measured by ion-selective electrodes, with some overlap caused by an excess of electrode systems. We used one KNA1 (K^+ and Na^+), one CL1 (Cl^- and Na^+, not commercially available), two ICA1s (Ca^{2+} and pH), and one BMS2 (pH) from Radiometer (Copenhagen, Denmark), and one NOVA 11 (K^+, Na^+, and Li^+) and one STAT Profile 1 (K^+, Na^+, Ca^{2+}, and pH) from NOVA Biomedical (Waltham, MA).

Substance concentrations of sodium and potassium were measured by flame-emission photometry with an IL 343 flame photometer (Instrumentation Laboratory, Lexington, MA), calcium and lithium were measured by atomic-absorption spectrophotometry with a Perkin-Elmer 403 spectrophotometer (Norwalk, CT), and chloride was measured with a CMT10 chloride titrator from Radiometer. The mass concentration of water (ρ_{H_2O}) was determined from the weight loss of 1 mL during an overnight drying at 105 °C. The mass concentration of albumin was determined by the weight after drying, subtracting the mass concentration of salt. The molality of albumin was determined from the mass concentrations of albumin and water by using a molecular mass for albumin of 66 000 Da.

The measured substance concentrations (mmol/L) were converted to molality (mmol/kg water) by dividing by the mass concentration of water:

$$m = c/\rho_{H_2O} \quad (2)$$

We observed a variable bias due to unselectivity of the ion-selective electrodes, especially at the extremes of pH. If all ions were free in the protein-free solutions, the molality of total ions and electrode reading should have been identical, which was not always the case. We used the ratio between molality and electrode result in the protein-free solutions as a correction factor for converting the electrode results into molality of free ions in the respective albumin solutions, assuming that the activity coefficients were the same on both sides of the membrane.

Liquid-junction potentials (E_j) caused by diffusion between the bridge solution (solution 1) and the test solution (solution 2) were calculated with the Henderson equation:

$$E_j = R \cdot T \cdot F^{-1} \cdot (f_1 - f_2)/(g_1 - g_2) \cdot \ln(g_1/g_2) \quad (3)$$

where F is the Faraday constant, $f = \Sigma\, m_i \cdot \lambda_i \cdot z_i^{-1}$, $g = \Sigma\, m_i \cdot \lambda_i$, m_i is the molality of free ion i, and z_i is the charge number. The following limiting equivalent conductivities were used (unit $S \cdot cm^2 \cdot mol^{-1}$): $\lambda_{Na^+} = 66$, $\lambda_{K^+} = 92$, $\lambda_{Cl^-} = 96$, $0.5\,\lambda_{Ca^{2+}} = 78$, $\lambda_{HCO_2^-} = 68$, and $\lambda_{Li^+} = 49$. The electrodes from NOVA had a bridge solution of KCl, 2 mol/kg water, and those from Radiometer had a bridge solution of $NaHCO_2$ (sodium formate), 4.0 mol/kg water. All results with ion-selective electrodes in the albumin solutions were subsequently corrected for the calculated difference in liquid-junction potential between the protein-free solution and the albumin solution.

The uneven distribution of ions as measured with the ion-selective electrodes was reported as an electric equilibrium potential, of size and direction irrespective of the ionic charge number. To make the results comparable, we expressed all the observed ionic distribution ratios as equilibrium potentials (in mV). The equilibrium potential for each ion was calculated with the Nernst equation:

$$E_{ISE} = R \cdot T \cdot z_i^{-1} \cdot F^{-1} \cdot \ln(m_i^a/m_i^w) \quad (4)$$

where the superscripts a and w denote the albumin and protein-free (water) solutions, respectively.

The binding of each ionic species to albumin was calculated as the difference between the molality of total and free ion in the albumin solutions:

$$m_i(\text{bound}) = m_i(\text{total}) - m_i(\text{free}) \quad (5)$$

The molality of albumin's own charge without bound ions (Z_{own}) was calculated from the neutrality condition, because albumin's own charge balances the total charge of all other cations and anions:

$$Z_{own} = -\Sigma\, m_i(\text{total}) \cdot z_i \quad (6)$$

The charge molality of all ions bound to albumin (Z_{bound}) was calculated as

$$Z_{bound} = \Sigma\, m_i(\text{bound}) \cdot z_i \quad (7)$$

The molality of net charge of albumin (Z_{net}) equalled

$$Z_{net} = Z_{own} + Z_{bound} \quad (8)$$

which is the molality of impermeable charge determining the Donnan distribution. The number of ions and charges per albumin molecule (z_{bound}, z_{own}, and z_{net}) were obtained by dividing by the molality of albumin.

We calculated the Donnan potential (E_{Donnan}) from the molality of net charge of albumin in the albumin solutions and the molality of monovalent electrolyte (m_{salt}) on the protein-free side of the membrane:

$$E_{Donnan} = R \cdot T \cdot F^{-1} \cdot \ln\{-Z_{net}/2m_{salt} + [1 + (Z_{net}/2m_{salt})^2]^{0.5}\} \quad (9)$$

The theoretical Donnan distribution ratio, r_{Donnan}, for ion i can be calculated from E_{Donnan}:

$$r_{Donnan} = \exp(z_i \cdot F \cdot E_{Donnan}/R \cdot T) \quad (10)$$

Results

The results were constant after 15 min, indicating equilibrium, and did not depend on the preceding pH, indicating reversibility.

The binding of chloride is shown in Figure 2. At physiological pH, seven chloride ions were bound per albumin molecule. The binding increased with decreasing pH, especially below pH 5.4, which is the isoelectric pH, where $Z_{own} = 0$ and where 11 chloride ions were bound per albumin molecule. The number of chloride ions bound to one albumin molecule increased to ~22 at pH 4.2.

The calcium binding to albumin is shown in Figure 3. No calcium was bound between pH 5.0 and 4.5, but the binding curve indicated a beginning calcium binding below pH 4.5. The calcium binding increased strongly with increasing pH, especially above pH 6.5. The curve was S-shaped with maximum slope at physiological pH of 7.4, where one calcium ion was bound per albumin molecule.

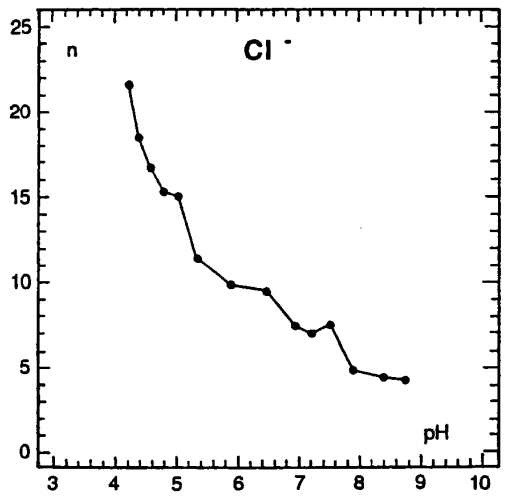

Fig. 2. Binding of chloride
Number of chloride ions bound per albumin molecule (n) as a function of pH

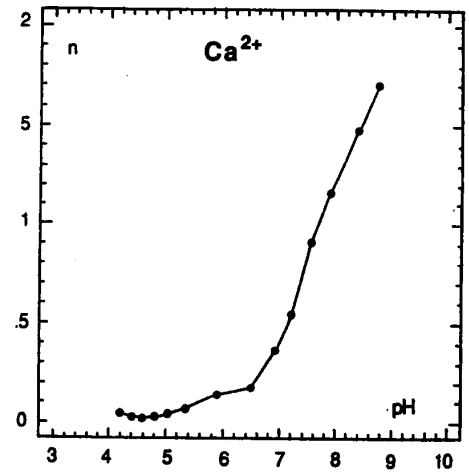

Fig. 3. Binding of calcium

Sodium, potassium, and lithium were not measurably bound to albumin at physiological pH. At pH 5 both sodium and potassium showed negative binding, the total molality being 5–10% less than the molality of free ions measured with ion-selective electrodes. The apparent binding of lithium varied in an unsystematic manner below the isoelectric pH, probably because of the lithium electrode having low selectivity towards H^+. The relative bias of the lithium electrode ranged from +25% to −30% in the pH interval 4–9, but that of the other electrodes was far less.

Figure 4 shows the charge of one albumin molecule as a function of pH. Albumin's own charge decreased to zero at the isoelectric pH (pH 5.4) and became negative with increasing pH because of the added sodium hy-

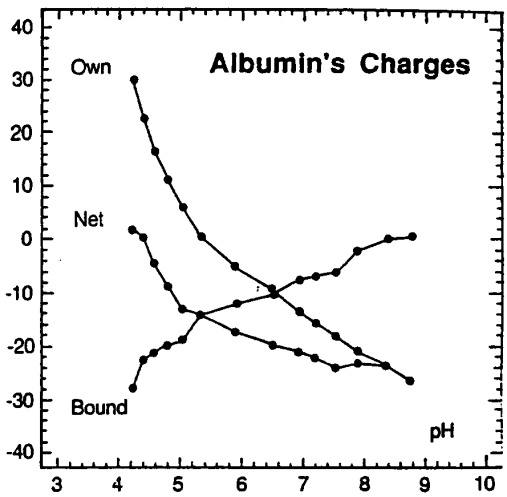

Fig. 4. The number of charges per albumin molecule as a function of pH
Albumin's own charge, defined by the neutrality condition, equalled the titration curve; bound charge was the total charge of all bound ions; and net charge was the sum of own and bound charges

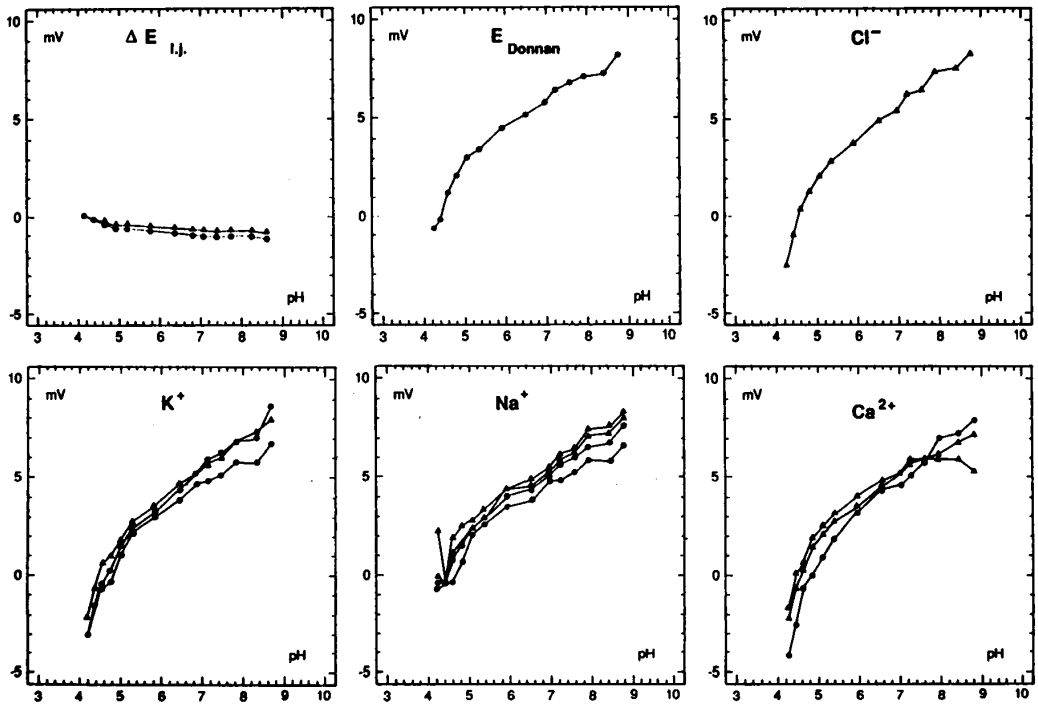

Fig. 5. Residual liquid-junction potential, Donnan potential, and membrane equilibrium potentials
●, NOVA; ▲, Radiometer

droxide. The total charge of bound ions was always negative, and it changed oppositely to albumin's own charge as a function of pH, with less chloride and more calcium being bound at increasing pH. In this way the net charge of albumin changed less than its own charge changed. At physiological pH, the approximate charges per albumin molecule were as follows: $z_{own} = -17$, $z_{bound} = -6$, and $z_{net} = -23$. The changes per albumin molecule and pH unit at physiological pH were $dz_{own}/dpH = -7.6$, $dz_{bound}/dpH = 5.2$, and $dz_{net}/dpH = -2.4$. These differential quotients are valid only at pH 7.4. z_{net} was −14 at the isoelectric pH (pH = 5.4) and became zero at one pH unit below the isoelectric pH (pH 4.4).

The residual liquid-junction potentials, the calculated Donnan potential, and the equilibrium potential determined by ion-selective electrode for each ion as a function of pH are shown in Figure 5. The calculated residual liquid-junction potentials changed from ~0 to ~−1 mV, in direction opposite to the calculated E_{Donnan}, which changed from ~−1 mV to 8 mV. The E_{ISE}s for each ion were ~0.8 mV lower than the calculated E_{Donnan}, and the curves were parallel. Table 1 gives regression data (E_{ISE} vs E_{Donnan}) for each ion, except lithium, which had a larger variation. Mean values were used for the regression when there was more than one electrode. All correlation coefficients were >0.99, the slopes were ~1 (but higher for chloride than for sodium), and the SDs about the regression lines were <0.4 mV, corresponding to a relative indeterminacy of <1.5% for a monovalent ion.

Table 1. Regression of E_{ISE} vs E_{Donnan}

	Slope	SD (slope)	r	SEE	Mean diff.
Ca^{2+}	1.06	0.03	0.995	0.34 mV	−1.0 mV
Na^+	0.90	0.04	0.991	0.38 mV	−0.5 mV
K^+	1.12	0.03	0.997	0.29 mV	−1.2 mV
Cl^-	1.16	0.03	0.997	0.29 mV	−0.5 mV

Discussion

We have compared the ion activities over a semipermeable membrane with those predicted by Donnan in 1911 (*1*). A high degree of ion binding to albumin was observed, especially of chloride and calcium, so we used the net charge of albumin including bound ions for the calculations. Theory and observation agreed well over a wide pH range, which we take as a confirmation of the Donnan theory.

The discrepancy of 0.8 mV and slopes slightly different from 1 may be due to lack of space from forbidden volumes or binding of water in the albumin solutions. These effects or their dependence on pH were not taken into account, because we had no independent way of determining them. We were confined to using the conventional molality and mass concentration of total water. Others have found ≤0.3 g of water bound per gram of protein (*4*), which would equal 6% of total water in this study. Because bound water by definition is not available for diffusible ions, water binding might explain the observed 5–10% negative binding of sodium at the lowest pH. Binding of water would imply a higher

degree of ion binding. With sodium being the most abundant free ion, the calculated net charge of albumin should then be more positive, and the calculated Donnan potential should be lower, improving the agreement between the calculated E_{Donnan} and the observed E_{ISE}. Equation 9 uses monovalent salt to calculate E_{Donnan}. Some of the ions were actually divalent Ca^{2+}. Taking this into account would further improve the agreement between E_{ISE} and E_{Donnan}.

The observed binding of chloride and calcium to human albumin agrees with earlier studies. The binding of chloride was studied by Scatchard et al. (*5*) in 1950. By dialysis, conductometry and silver/silver chloride electrodes, and Scatchard plots, they demonstrated two classes of binding sites at isoelectric pH, one of 10 binding sites with medium intrinsic affinity (K_A = 44 L/mol), and the other of 30 binding sites with low intrinsic affinity (K_A = 1.1 L/mol). A few binding experiments at pH 3.2 revealed 31 chloride ions bound per albumin molecule, in agreement with our study. Chloride binding to human albumin was rediscovered by others (*6, 7*), without reference to Scatchard. Calcium binding has been studied by many authors. We previously observed an increasing number of apparent binding sites with increasing pH, which we interpreted as exposure of calcium-binding carboxylate groups during the neutral unfolding of albumin (*8*). The emerging calcium binding at acid pH may similarly reflect an acid unfolding of albumin. This study agrees with earlier calcium-binding data. We observed no albumin binding of sodium, potassium, or lithium, but the results for lithium were less satisfactory because of interference from H^+, in accord with Okorodudu et al. (*9*). Binding of sodium was examined before (*10*), with the same result as ours.

The change in electrostatic free energy when charged particles come together can be used to convert binding data for small ions and proteins into intrinsic association constants. These are by definition independent of electric charge, and they must be distinguished from the apparent constants, describing actual binding under specific conditions. The assumptions for intrinsic constants are simple, such as the whole net charge of albumin being evenly distributed on a sphere. The total charges used for the conversion must be accurately known, which can be difficult, because several ions may be involved. The net charges of albumin depicted in Figure 3 may help to provide more accurate intrinsic constants.

The bound charge contributes about one-fourth to the net negative charge of albumin under physiological conditions, helping to maintain the plasma colloid osmotic pressure and plasma volume. Furthermore, the bound ions will have a stabilizing effect on plasma volume during disturbances of pH. Instead of albumin's own charge, which will change according to the added acid or base, the net charge of albumin will change only one-third, because of chloride and calcium. Plasma colloid osmotic pressure and plasma volume will decrease during an acidosis and increase during an alkalosis, but binding and release of chloride and calcium will counteract the changes.

Figge et al. (*11*) recently studied the role of serum proteins in acid–base equilibria by using ultrafiltration and pH. They concluded that the observed charge of serum protein of −12 mmol/L is solely attributable to serum albumin and less than hitherto assumed. We did not include bicarbonate or magnesium, but they probably bind to albumin in competition with chloride and calcium. Our data indicate that at pH 7.4 and an albumin concentration of 0.6 mmol/L, the own charge of serum protein is −10 mmol/L, and the effective net charge including bound ions is −14 mmol/L, in close agreement with Figge et al.

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