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# Interaction Between Proteins and Chloride Ion

By CHARLES TANFORD

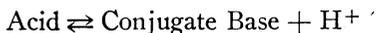
## INTRODUCTION

It has become increasingly clear in recent years that some proteins are capable of binding most common anions, and many studies with organic dye anions, detergent anions, and with some inorganic anions have been reported. On the basis of some of these studies Klotz and Urquhart<sup>4</sup> have suggested that a "binding index" exists, which can be computed from the amino acid composition of a protein, and which will predict the relative affinity of different proteins for *any* anion.

It would be desirable to test this hypothesis for chloride ion, since this is the most abundant anion in physiological systems. Unfortunately, chloride binding has been measured for only one protein, human serum albumin. The results obtained with this protein were, however, especially interesting in that they showed that an indirect method of computing chloride binding from the effect of chlorides upon the pH of an isoionic protein solution gives results in good agreement with direct determination. Since data on pH changes resulting from salt addition are available in the literature for a few proteins, it becomes possible to compute the extent of chloride binding for these proteins, and this has been done in this paper for egg albumin, beta lactoglobulin, and horse carboxyhemoglobin.

## THEORY OF THE INDIRECT METHOD

Most protein molecules contain one hundred or more groups capable of reacting with hydrogen ion according to the equation



The pH of a protein solution is then controlled by the equilibrium constants of the various ionizing groups. If all these groups are considered completely independent of one another, then each one obeys an ionization equation of the form<sup>9</sup>

$$\frac{(\text{conjugate base}) \gamma_{\text{base}} \gamma_{\text{H}^+}}{(\text{acid}) \gamma_{\text{acid}}} = K'$$

where ( ) represents concentration and the  $\gamma$ 's are activity coefficients. The ratio of activity coefficients in this equation is unlike that for the ionization of a monobasic acid because the ionizing

groups are part of a protein molecule, the charge on which can vary over wide limits, say from plus 100 to minus 100. We shall therefore incorporate the activity coefficient ratio in the equilibrium constant, and then determine the effect of protein charge upon it. We therefore write

$$\frac{(\text{conjugate base}) \alpha_H +}{(\text{acid})} = K$$

or 
$$\log \frac{(\text{conjugate base})}{(\text{acid})} - \text{pH} = -\text{pK} \quad (1)$$

where pH stands for  $-\log \alpha_H +$  and pK stand for  $-\log K$ .

The equilibrium constant is related to the free energy of ionization by the equation

$$\Delta F^\circ_{\text{ion}} = -RT \ln K = 2.303 RT \text{pK} \quad (2)$$

Now, part of this free energy is electrostatic in nature, and therefore dependent upon the protein charge. It is the free energy change accompanying a decrease in the charge of the acid by one, i.e. it is the negative of the work which must be done to increase the charge of the conjugate base molecule by one. This work is easily computed by means of the Debye-Hückel Theory. Consider that the ionizable group is on the surface of a protein molecule, which shall be taken to be a sphere, and which, we shall assume, has a charge  $Z$  evenly distributed over its surface. These assumptions are reasonably close to the true state of affairs on a dissolved protein molecule.<sup>5</sup> The electrostatic work required to increase the charge to  $(Z + 1)$  is the difference between the Debye-Hückel electrostatic free energy terms for charges  $(Z + 1)$  and  $Z$ ,

$$\begin{aligned} & \frac{(Z+1)^2 N \epsilon^2}{2D} \left( \frac{1}{b} - \frac{\kappa}{1+\kappa a} \right) - \frac{Z^2 N \epsilon^2}{2D} \left( \frac{1}{b} - \frac{\kappa}{1+\kappa a} \right) \\ &= \frac{(2Z+1) N \epsilon^2}{2D} \left( \frac{1}{b} - \frac{\kappa}{1+\kappa a} \right) = -(\Delta F^\circ_{\text{ion}})_{\text{elec}} \end{aligned} \quad (3)$$

where  $b$  is the radius of the sphere,  $a$  its radius of exclusion,  $\kappa$  has the meaning customary in the Debye-Hückel theory,  $D$  is the dielectric constant of the medium, and  $\epsilon$  is the protonic charge.

Let us now use the subscript zero to denote that the protein sphere has zero net charge when the ionizing group in question is in the form of its conjugate base. Then

$$- \left[ (\Delta F_{\text{ion}}^{\circ})_{\text{elec}} \right]_0 = \frac{N_0^2}{2D} \left( \frac{1}{b} - \frac{\kappa}{1 + \kappa a} \right)$$

If we assume that the ionization constant of a particular group on the protein sphere is always the same, whether neighboring groups are ionized or not, except in so far as the electrostatic part of  $\Delta F^{\circ}$  changes with the net charge, then

$$\begin{aligned} (\Delta F_{\text{ion}}^{\circ})_Z - (\Delta F_{\text{ion}}^{\circ})_0 &= 2.303 RT (pK_Z - pK_0) \\ &= \frac{N_0^2}{2D} \left( \frac{1}{b} - \frac{\kappa}{1 + \kappa a} \right) - \frac{(2Z+1)N_0^2}{2D} \left( \frac{1}{b} - \frac{\kappa}{1 + \kappa a} \right) \\ &= - \frac{2ZN_0^2}{2D} \left( \frac{1}{b} - \frac{\kappa}{1 + \kappa a} \right) \\ \text{or } pK_Z - pK_0 &= - \frac{2Z}{2.303} \frac{N_0^2}{2DRT} \left( \frac{1}{b} - \frac{\kappa}{1 + \kappa a} \right) \end{aligned} \quad (4)$$

It is customary to place

$$\frac{N_0^2}{2DRT} \left( \frac{1}{b} - \frac{\kappa}{1 + \kappa a} \right) = w \quad (5)$$

so that, at any charge  $Z$ ,

$$pK = pK_0 - \frac{2Zw}{2.303}$$

Substituting this value of  $pK$  into equation (1) we obtain

$$\log \frac{(\text{conjugate base})}{(\text{acid})} = \text{pH} - pK_0 + \frac{2Zw}{2.303} \quad (6)$$

It should be noted that the value of  $w$  depends only upon the dimensions of the protein sphere, and upon the properties of the solution in which it is placed: it is not affected by the nature of the ionizing group under consideration, i.e. it is the same whether amino, carboxyl, phenolic or any other group is being considered by an equation of the form of (6).

Now, any given protein will have a limited number of types of ionizing groups, usually about half a dozen. Let there be  $n_i$  groups of the  $i$ th type on a protein molecule, and, at any  $\text{pH}$ , let  $r_i$  of these

be in the form of the conjugate base, and  $n_i - r_i$  therefore in the acid form. For each type of group there will be a fundamental dissociation constant,  $(K_o)_i$ , and equation (6) can be written

$$\log \frac{r_i}{n_i - r_i} = \text{pH} - (\text{p}K_o)_i + \frac{2Zw}{2.303} \quad (7)$$

It is now possible to solve equation (7) for  $r_i$  for each type of ionizing group, and to evaluate the total number of groups on a protein molecule which are in the form of the conjugate base at any pH, designated by  $r = \sum r_i$ . A plot of  $r$  against pH would give the familiar "titration curve" of a protein, illustrated by Fig. 1.

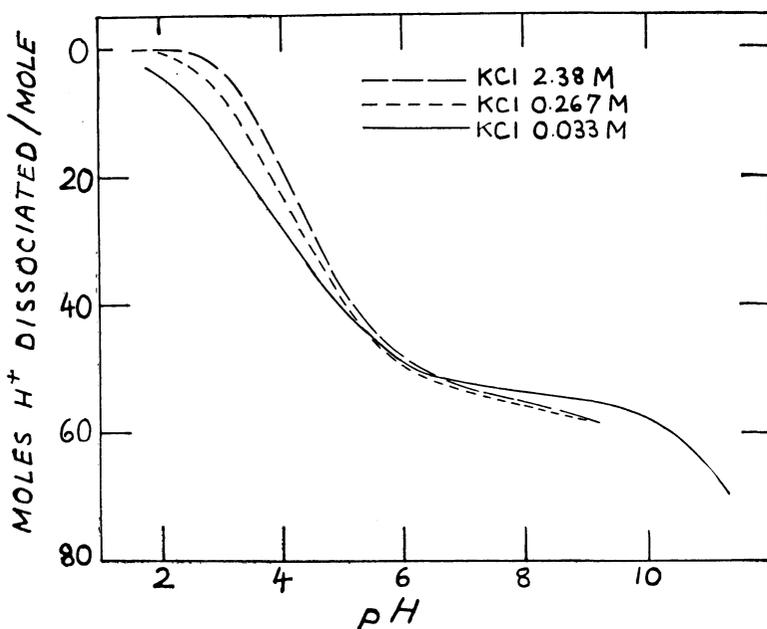


Figure 1

The value of  $r$  at any pH is, of course, equal to the number of hydrogen ions dissociated from the protein in its most acid form, when all the groups are in their acid form, and  $r$  is zero.

However, we are not interested in the construction of the titration curve at this time. We are interested, rather, in the fact that when such titration curves are obtained experimentally at different concentrations of some neutral salt, they do not coincide. This is illustrated by Fig. 1, which shows the titration curves obtained by Cannan, Kibrick and Palmer for egg albumin at various concen-

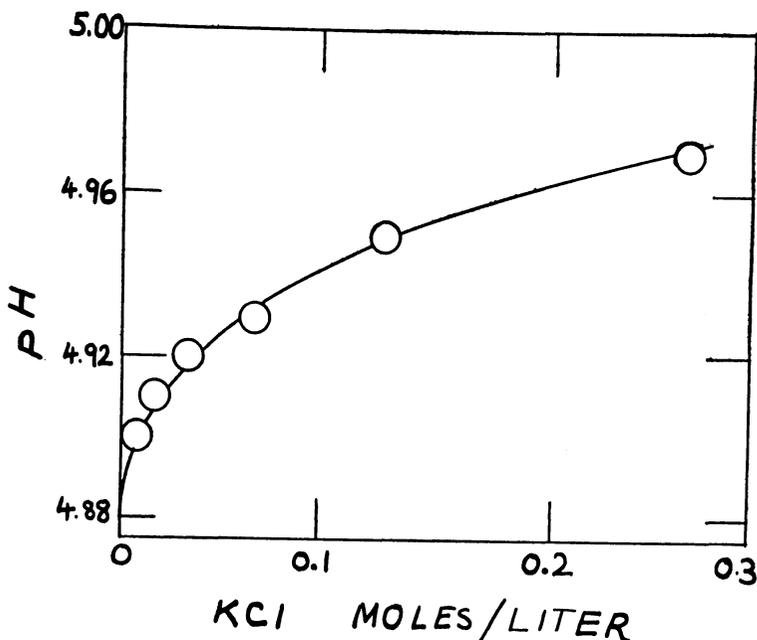


Figure 2

trations of potassium chloride.<sup>1</sup> It is possible to take a series of such curves and to plot the pH at which  $r$  has some definite value against the concentration of some neutral salt added. The result is a curve of the form of Fig. 2, which has been constructed from Cannan's data for  $r = 40$ . This value of  $r$  has been chosen deliberately, for it represents the isoionic point of the protein, i.e. that point at which the net charge on the protein molecule in the absence of added salt is, on the average, equal to zero.

To interpret this effect, we return to equation (7), and examine the effect of salt concentration upon each term. Since we are interested in the effect at a constant value of  $r$ , this means that for all practical purposes the value of each individual  $r_i$  is also constant. Since the  $pK_o$  are also constant, we obtain

$$\frac{\partial \text{pH}}{\partial c} + \frac{2Z}{2.303} \frac{\partial w}{\partial c} + \frac{2w}{2.303} \frac{\partial Z}{\partial c} = 0 \quad (8)$$

If we confine ourselves to the effect of salt addition upon isoionic protein, the value of  $Z$  in the absence of salt is zero. This means that the second term of equation (8) vanishes. Actually, as salt is added, the value of  $Z$  may change, as we shall see, but the contri-

tribution of the second term will still be very small at low salt concentrations. As long as we confine ourselves to such low concentrations we can also, as a first approximation, consider  $w$  to be constant. Equation (8) therefore becomes

$$\frac{\partial \text{pH}}{\partial c} = - \frac{2w}{2.303} \frac{\partial Z}{\partial c}$$

or, for the addition of salt of concentration  $c$  to a salt-free solution,

$$\Delta \text{pH} = - \frac{2w}{2.303} \Delta Z \tag{9}$$

This equation was first derived by Scatchard and Black.<sup>7</sup>

It remains only to interpret the meaning of the term  $\Delta Z$ . Since we are investigating pH changes at constant  $r$ , this term cannot represent a change in the number of hydrogen ions bound by the protein. It must therefore be concluded that it represents the binding of some other ion by the protein, a negative ion if the pH change is positive, and a positive ion if the pH change is negative. Equation (9) can therefore be used as a measure of such binding.

#### COMPARISON WITH DIRECT MEASUREMENTS ON HUMAN SERUM ALBUMIN

Scatchard and Black<sup>7</sup> have applied equation (9) to determine the extent of chloride binding by human serum albumin, from measurement of the pH changes produced by the addition of sodium chloride to isoionic protein. In addition, Scatchard and Scheinberg<sup>8</sup> used two direct experimental methods to investigate chloride binding, (a) the distribution of ions across a membrane, and (b) electromotive force measurements with silver—silver chloride electrodes. Their results are summarized in Fig. 3, and it is seen that they agree well with one another, as well as with the results obtained by the indirect method to be used in this paper. It might also be mentioned that Scatchard and Scheinberg have shown that no sodium ions are bound by human serum albumin. If this were not true the value of  $\Delta Z$  obtained from observed pH changes might represent the difference between chloride binding and sodium binding. There is actually much other evidence to suggest that sodium and potassium cations are not usually bound by proteins.

#### RESULTS OF CALCULATIONS

For aqueous solutions of uni-univalent electrolytes at 25°C the Debye-Hückel  $\kappa$  takes on the value  $3.295 \times 10^7 \sqrt{c}$ , where  $c$  is the

electrolyte concentration in moles per liter. On substitution of this expression for  $\kappa$  and of the known values of the constants at 25°C into equation (5) we obtain for  $w$  the value

$$w = 3.59 \times 10^8 \left( \frac{1}{b} - \frac{3.295 \times 10^7 \sqrt{c}}{1 + a \times 3.295 \times 10^7 \sqrt{c}} \right) \quad (10)$$

The parameters  $a$  and  $b$ , previously defined, can be estimated with reasonable accuracy from protein dimensions. Substitution of the appropriate value of  $w$  in equation (9) will then yield a value of  $\Delta Z$  for any observed value of the pH change at a given concentration of NaCl or KCl. If we assume that the cation is not bound, then  $-\Delta Z$  will be equal to the number of chloride ions bound per protein molecule.

Calculations for isoionic egg albumin can be made from Fig. 2, and are shown in Table 1. The values of  $w$  to be used have already been computed by Cannan, Kibrick and Palmer,<sup>1</sup> and are based on the dimensions  $a = 29.5$  A,  $b = 27.5$  A. The molecular weight of egg albumin is taken to be 45,000.

Cannan, Palmer and Kibrick<sup>2</sup> have investigated the effect of the addition of potassium chloride upon isoionic betalactoglobulin. They find that there is no change in pH whatsoever, even if the solution is made 2.1 molar in KCl. Hence  $\Delta Z$  must be equal to zero, i.e. *no chloride is bound at all*.

Cohn, Green and Blanchard<sup>3</sup> have studied the effect of salt addition to horse carboxyhemoglobin. Close to the isoionic point the addition of sodium chloride to a molarity of 0.38 produces a pH

**Table 1**  
Calculations for Egg Albumin

Concentration KCl moles/liter	pH	$\Delta$ pH	$w$	$-\Delta Z$ = number of Cl ions per molecule
0	4.88 <sup>a</sup>	--	--	--
0.0085	4.90	0.02	0.0717	0.4
.017	4.91	.03	.0621	.6
.033	4.92	.04	.0523	.9
.067	4.93	.05	.0434	1.3
.133	4.95	.07	.0358	2.2
.267	4.97	.09	.0294	3.5

<sup>a</sup> By extrapolation.

change of 0.09. The dimensions of the hemoglobin molecule are close to those for serum albumin, for which  $a = 32.5 \text{ \AA}$  and  $b = 30 \text{ \AA}$ .<sup>6</sup> Using these values we obtain a value for  $w$  of 0.0237, so that the number of chloride ions bound, by equation (9), becomes 4.4. This figure must be regarded as very approximate since it is

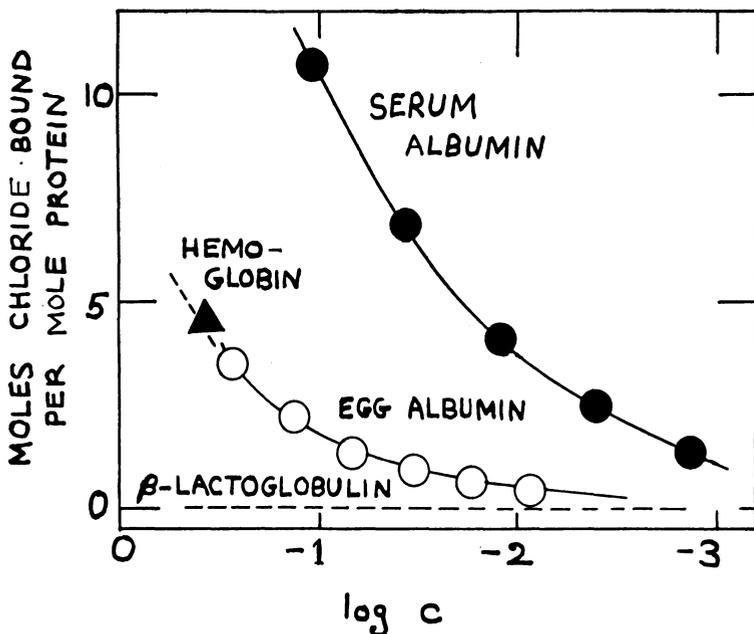


Figure 3

based upon a single experimental point, and this not precisely at the isoionic point.

All the results calculated, including those for human serum albumin obtained by Scatchard and Black, are presented graphically in Fig. 3.

#### DISCUSSION

The results obtained above are especially interesting when viewed in the light of the work of Klotz and coworkers on the binding of dye anions. These results, it has been shown by Klotz and Urquhart,<sup>4</sup> can be correlated with a "binding index," which can be calculated from the amino acid composition of a protein. Klotz and Urquhart have suggested that this index will determine the relative affinity of a protein for any anion. The "binding index" for the three proteins for which we have presented the most detailed results has been computed by Klotz and Urquhart. The values are: for serum albumin 29, for beta lactoglobulin 4.6, for egg albumin 2.5. For all other proteins for which they made calculations the index has

lower values. As predicted by the index, methyl orange is bound strongly by serum albumin, weakly by beta lactoglobulin, and virtually not at all by egg albumin.

The results reported in this paper for chloride binding, however, are not as predicted by the "binding index," for egg albumin is seen to have a much stronger affinity for chloride than does beta lactoglobulin. In fact, the latter, though it has the second highest "binding index" is seen to be incapable of binding chloride at its isoionic point at all. It therefore appears that the "binding index" does not have general validity.

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