LETTER TO THE EDITOR

[Brief letters to the Editor that make specific reference to papers published previously in The Journal of General Physiology are invited. Receipt of such letters will be acknowledged, and those containing pertinent scientific comments and scientific criticisms will be published.]

Analysis of Hemoglobin Aggregation from Gibbs-Donnan Equilibrium Experiments

Dear Sir:

Gary-Bobo and Solomon (1968, 1971) have employed Gibbs-Donnan equilibrium studies in the assessment of the anomalous osmotic behavior of the red cell. In these studies, the interpretation was based on thermodynamic equations for ideal solutions. During the course of recent investigations, we have found that it is necessary to consider the nonideal aspects of concentrated hemoglobin solutions. In this letter, we have transformed the ideal equations of Gary-Bobo and Solomon to include nonideal contributions for the interpretation of hemoglobin-hemoglobin and hemoglobin-solution interactions.

Experimentally, Gary-Bobo and Solomon (1968, 1971) measured the Donnan equilibrium of hemoglobin-potassium chloride solutions. The Donnan ratio, \( r \), is defined in Eq. 1, where \([K^+]_{in}\) is the molal potassium ion concentration in the compartment containing hemoglobin, and \([K^+]_{out}\) is the molal potassium ion concentration in the external compartment which is free of hemoglobin.

\[
r = \frac{[K^+]_{in}}{[K^+]_{out}}
\]  

Computations for the charge, \( Z \), on the hemoglobin molecule

\[
Z = \frac{1 - r^2 [K^+]_{out}}{[Hb]}
\]  

were carried out under a variety of experimental conditions, namely changes in pH, \([KCl]\), and \([Hb]\). According to this ideal model, the charge decreased with increasing hemoglobin concentration in a sigmoid fashion. This was interpreted in terms of the removal of protons when hemoglobin molecules interacted with each other, thus decreasing their actual charge per molecule.

A nonideal expression is now derived by introduction into the equations of the mean activity coefficient of KCl in both inside and outside compartments. The conservation of charge requires that

\[
Z[Hb] + [K^+]_{in} = [Cl^-]_{in}.
\]  

Dividing both sides by \([K^+]_{out}\) gives

\[
\frac{Z[Hb]}{[K^+]_{out}} + \frac{[K^+]_{in}}{[K^+]_{out}} = \frac{[Cl^-]_{in}}{[K^+]_{out}}.
\]  

Since in the outside compartment only KCl is present, it follows that
Thus Eq. 4 can be rewritten as

\[
\frac{Z[Hb]}{[K^+]_{\text{out}}} + \frac{[K^+]_{\text{in}}}{[K^+]_{\text{out}}} = \frac{[Cl^-]_{\text{in}}}{[Cl^-]_{\text{out}}}. \tag{5}
\]

At equilibrium, the chemical potential of KCl on the outside must equal the chemical potential of KCl on the inside:

\[
\mu_{K^+_{\text{in}}} + \mu_{Cl^-_{\text{in}}} = \mu_{K^+_{\text{out}}} + \mu_{Cl^-_{\text{out}}}. \tag{6}
\]

In terms of standard chemical potentials, \(\mu^o\), and activities, \(a\), this becomes

\[
\mu^o_{K^+} + RT \ln a_{K^+_{\text{in}}} + \mu^o_{Cl^-} + RT \ln a_{Cl^-_{\text{in}}} = \mu^o_{K^+} + RT \ln a_{K^+_{\text{out}}} + \mu^o_{Cl^-} + RT \ln a_{Cl^-_{\text{out}}}
\]

and it follows that

\[
a_{K^+_{\text{in}}} a_{Cl^-_{\text{in}}} = a_{K^+_{\text{out}}} a_{Cl^-_{\text{out}}}. \tag{7}
\]

Writing the activities in terms of the product of the mean ionic activity coefficient, \(\gamma_z\), and the concentration gives

\[
\frac{[Cl^-]_{\text{in}}}{[Cl^-]_{\text{out}}} = \frac{\gamma_{KCl_{\text{in}}}[K^+]_{\text{out}}}{\gamma_{KCl_{\text{out}}}[K^+]_{\text{in}}}. \tag{8}
\]

Substituting Eqs. 10 and 1 into Eq. 6 and rearranging gives an expression for the ratio of the mean activity coefficients of KCl in and KCl out as a function of the hemoglobin concentration.

\[
\frac{\gamma_{KCl_{\text{in}}}}{\gamma_{KCl_{\text{out}}}} = \left(\frac{Z[Hb]}{[K^+]_{\text{out}}} + r^2\right)^{-1}. \tag{9}
\]

Note that when the activity coefficient of KCl inside and outside both approach 1, Eq. 11 reduces to the ideal Eq. 2. The results of calculations of the experimental data of Gary-Bobo and Solomon (1968, 1971) according to Eq. 11 are shown in Figs. 1 and 2. For differing concentrations of KCl, the ratio of activity coefficients is always one at the isoelectric point of hemoglobin, while pH values above and below the isoelectric point result in ratios greater than 1 and less than 1, respectively (Fig. 1). The constancy of the ratio of activity (unity) at the isoelectric pH indicates that, despite the presence of a large dipole and possibly higher order moments, the major effect comes from the monopole term. This is consistent with the fact that proteins are generally least soluble near their isoelectric point (Haurowitz, 1963).

As the ratio of activity coefficients decreases with decreasing KCl concentration (pH < pI), the free energy of the KCl in the inside compartment decreases relative to that of the KCl in the outside compartment (Castellan, 1971). Under these conditions, the charge on the hemoglobin molecule is positive, allowing for a stronger binding interaction with the negatively charged Cl\(^-\) ion. At pH values above the isoelectric point, repulsive effects between the hemoglobin ion and the chloride ion, both of which are negatively charged, lead to an increase in the free energy of the KCl inside relative to that outside. In both cases, the cation has little effect, since it does not bind significantly to hemoglobin (Carr, 1956). Increasing the concentration of KCl relative to that of hemoglobin leads to convergence of the ratio of activity coefficients toward 1. In this case, the role of hemoglobin is decreased, since the environment of each salt ion is mainly other salt ions.
LETTER TO THE EDITOR

The ratio of activity coefficients for KCl as a function of hemoglobin concentration at pH 6.60 are shown in Fig. 2. At the concentration normally found in the red cell, 6 mmolal, the ratio has a minimum value. In a study of aqueous mixtures of polyelectrolyte and simple electrolyte, Kwak (1973) observed that the mean activity coefficient of the simple electrolyte decreases with initial increases in polyelectrolyte concentration and the slope eventually becomes zero as the polyelectrolyte concentration is increased further. Similar behavior is seen in Fig. 2 at hemoglobin concentrations less than 6-7 mmolal. Increasing the hemoglobin concentration further causes an increase in the activity coefficient ratio. This may be interpreted as the initial stages of nucleation, in that the interactions between hemoglobin molecules become so pronounced that the protein molecules begin to shield one another from the KCl environment, thus increasing the activity coefficient for KCl. If the hemoglobin concentration were to increase even further, the activity coefficient ratio for KCl appears to approach unity. This limiting condition could be interpreted as a condition in which interactions between hemoglobin molecules increase to such an extent that a phase transition occurs, thus removing hemoglobin from solution and eliminating its effect on the activity of KCl.

Solomon has suggested that this latter interpretation is precluded by the observations that the osmotic coefficient for hemoglobin continues to increase as the hemoglobin concentration increases (Adair, 1928; McConaghey and Maizels, 1961) even at concentrations higher than those normally found in the red cell. Were hemoglobin to form dimers or higher order polymers in an ideal solution, one would, of course, expect a decrease in the osmotic coefficient as the fraction of monomers decreases. At the high hemoglobin concentrations found in the red cell, however, the major contribution to the osmotic coefficient would arise from nonideal components such as the large hemoglobin volume, as suggested by Williams (1973), which continues to rise with increasing concentration. It is for this reason that one must employ other physical methods, such as light scattering,
for detection of such polymerization processes as the pre-gelation aggregation of deoxygenated hemoglobin S (Wilson et al., 1974).

We have given a nonideal thermodynamic formula for treating a particular Gibbs-Donnan equilibrium for the hemoglobin system and applied it to existing experimental data. Such a treatment is of particular interest in the consideration of protein interactions in cases where polymerization does in fact occur, e.g., the gelation of deoxyhemoglobin S. The change in the mean ionic activity coefficient of the KCl solution in a compartment containing hemoglobin relative to one without hemoglobin acts as a probe in the gelation process by giving indications of the concentrations at which polymerization begins. For example, it may be that the minimum of the activity coefficient ratio as a function of hemoglobin concentration will be displaced to the left in the case of HbS deoxy relative to the normal HbA deoxy. This would indicate that the nucleating phenomenon occurs initially at a lower concentration of HbS deoxy.

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