Net Charge and Oxygen Affinity of Human Hemoglobin Are Independent of Hemoglobin Concentration

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ABSTRACT The dependence of net charge and oxygen affinity of human hemoglobin upon hemoglobin concentration was reinvestigated. In contrast to earlier reports from various laboratories, both functional properties of hemoglobin were found to be independent of hemoglobin concentration. Two findings indicate a concentration-independent net charge of carbonmonoxy hemoglobin at pH 6.6: (a) the pH value of a given carbonmonoxy hemoglobin solution remains constant at 6.6 when the hemoglobin concentration is raised from 10 to 40 g/dl, indicating that there is no change in protonation of titratable groups of hemoglobin; (b) the net charge of carbonmonoxy hemoglobin as estimated from the Donnan distribution of $\text{Na}^+$ shows no dependence on hemoglobin concentration in this concentration range. The oxygen affinity of human hemoglobin was determined from measurements of oxygen concentrations in equilibrated samples using a Lex-O$_2$Con apparatus (Lexington Instruments, Waltham, Mass.). $P_{50}$ averaged 11.4 mm Hg at 37°C, pH = 7.2, and ionic strength = 0.15. Neither $P_{50}$ nor Hill’s $n$ showed any variation with hemoglobin concentrations increasing from 10 to 40 g/dl.

INTRODUCTION

Gary-Bobo and Solomon (1) have proposed that the anomalous osmotic behavior of the erythrocyte is due to a decrease of the net charge of hemoglobin which occurs when the hemoglobin concentration inside the erythrocyte is raised. Employing Donnan equilibrium studies, they subsequently (2) produced evidence to show that a hemoglobin charge dependence on hemoglobin concentration also exists in vitro. This phenomenon, which has been interpreted by Gary-Bobo and Solomon (1, 2) in terms of aggregation of hemoglobin molecules, is often quoted as an example of altered functional properties of hemoglobin at high concentrations (3, 4), and has been the subject of two Letters to the Editor in this Journal (5, 6). During recent studies on the diffusivity of hemoglobin, whose dependence on hemoglobin concentration increases sharply above a concentration of 30 g/dl (7), we became interested in the behavior of other functional properties of hemoglobin when the hemoglobin concentration rises up to and above the values found inside erythrocytes. In this paper, we report...
the results of a reinvestigation of the concentration dependence of hemoglobin net charge, along with that of oxygen affinity, which also has been reported by some authors (8, 9) to vary with concentration.

METHODS

Preparation of Isoionic Hemoglobin Solutions

Solutions of human hemoglobin were prepared from ACD blood, usually 2 wk old. Cells were washed three times in 0.15 M NaCl and lysed by the addition of distilled water. After restoring an ionic strength of 0.15, ghosts were removed by centrifugation at 35,000 g. Hemoglobin solutions were dialyzed against distilled water for 2 days and then passed through a mixed bed ion exchange column (Bio-Rad Laboratories, Richmond, Calif., AG 501-X8D). Hemoglobin solutions leaving the column had a conductivity identical within 1 ~S to that of double-distilled water, and total phosphate was <0.04 mol/mol of hemoglobin tetramer. Concentrated hemoglobin solutions were obtained by ultrafiltration (Amicon cell, membrane PM10, Amicon Corp., Lexington, Mass.) of the isoionic hemoglobin solutions under a pressure of 5 atm CO or O₂, respectively. All preparative steps were carried out at 0~17°C. Hemoglobin concentrations were determined spectrophotometrically after conversion to the cyanmet derivative (ε₄₅₀ = 44 cm⁻¹mM⁻¹ on tetramer basis; ε₅₅₀ = 6.82 cm⁻¹).

pH Measurements

pH measurements were carried out in a thermostatted titration vessel that was flushed with water vapor-saturated oxygen. Sample volume was about 10 ml. Electrodes were Radiometer glass electrode G202C and reference electrode K401; the pH meter used was a Radiometer PHM64 (Radiometer Co., Copenhagen, Denmark).

Ion Concentrations

Na⁺ and K⁺ concentrations were determined in a flame photometer (Eppendorf), Cl⁻ concentrations coulometrically (Aminco chloride titrator, Amicon Corp.). Both methods were applied to protein-free solutions only.

Donnan Distribution of ²²Na⁺

A stainless steel dialysis chamber was used for these experiments. The lower compartment (vol ≈ 7 ml) of this chamber was filled with the CO₂-free hemoglobin solution to be investigated. The upper compartment (vol ≈ 14 ml) was filled with CO₂-free 0.010 M NaCl solution containing traces of ²²Na⁺. The two compartments were separated by a membrane cut from Visking dialysis tubing (Serva GmbH., Heidelberg), which was mechanically supported toward the upper compartment by a stainless steel grid. At a temperature of 4°C, Donnan equilibrium was reached after 20 h when both compartments were stirred. At the end of the experiment, radioactivity was determined on weighed samples from both compartments in a Gamma Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill., model 5001), and the Donnan ratio, r, was calculated from

\[
r = \frac{[²²\text{Na}^+]_i}{[²²\text{Na}^+]_o} = \frac{[\text{Na}^+]_i}{[\text{Na}^+]_o},
\]

where i refers to the lower compartment, containing the hemoglobin, and o refers to the upper compartment, which is free of hemoglobin. All concentrations are expressed as
molarities (moles Na\(^+\) per kilogram water). In addition, the following measurements were made: total concentration of sodium in the upper compartment by flame photometry, hemoglobin concentration and pH of the hemoglobin solution in the lower compartment. The pH was measured at the temperature at which the equilibrium dialysis had been performed (4\(^\circ\)C). The Donnan distributions of Na\(^+\) were used to calculate the net charge, z, of hemoglobin according to (2, 6):

\[
z = \frac{1 - r^2}{r} \left[ \frac{\text{[Na\(^+\)]}_o}{[\text{Hb}]} \right]
\]

where \(r\) is the Donnan ratio for sodium, \(\text{[Na\(^+\)]}_o\) is the sodium concentration in the upper hemoglobin-free compartment, and \([\text{Hb}]\) is the concentration of hemoglobin in the lower compartment of the dialysis chamber (in moles hemoglobin tetramer per kilogram water). It may be noted that Eq. 2 holds whether there is chloride binding or not, but requires that free Cl\(^-\) and Na\(^+\) do not exhibit differences in their activity coefficients between the hemoglobin-free and the hemoglobin-containing compartment (see, for example, the derivation of Eq. 2 given in reference 6).

**Oxygen Dissociation Curves**

O\(_2\) binding curves were obtained on isoionic hemoglobin solutions to which bis-Tris buffer and NaCl were added to give final concentrations of 0.075 moles of bis-Tris and NaCl, respectively, per kilogram of water. pH values were adjusted to 7.20 at 37\(^\circ\)C. The hemoglobin solutions were equilibrated at 37\(^\circ\)C with O\(_2\)-N\(_2\) mixtures in Laue tonometers (10) which contained siliconized glass beads (diameter 3 mm) to improve convection and shorten the equilibration time of the highly viscous solutions. Total oxygen concentration of the equilibrated solutions was measured in a Lex-O2-Con apparatus (Lexington Instruments), pH was measured in a thermostatted capillary glass electrode (Ingold, Frankfurt). For each hemoglobin concentration at least six measurements at pO\(_2\) values ranging from 7 to 17 mm Hg were made. Oxygen capacities were determined in samples equilibrated with 100% O\(_2\). Oxygen saturations were calculated from oxygen concentrations and capacities after subtracting dissolved O\(_2\). When necessary, pO\(_2\)s were corrected for pH 7.20 using a Bohr factor of ~0.48. Oxygen half-saturation pressure, \(P_{50}\), and Hill's coefficient, \(n\), were obtained from the linear regression equation of a Hill plot made from data between 20 and 80% oxygen saturation (\(r > 0.995\)).

**RESULTS AND DISCUSSION**

**Concentration Dependence of Hemoglobin Net Charge**

This question was studied using two different approaches. First, the pH of hemoglobin solutions was measured as a function of hemoglobin concentration. This is of interest because changes of hemoglobin charge are reflected by changes of pH. Second, the hemoglobin net charge at various hemoglobin concentrations was directly evaluated from the Donnan distribution of Na\(^+\).

**pH as a Function of Hemoglobin Concentration**

Gary-Bobo and Solomon (1) postulated that at pH values below the isoelectric point the (positive) charge of the hemoglobin molecules decreases as the hemoglobin concentration increases. If this decrease in charge is brought about by a dissociation of protons from hemoglobin, as postulated by Gary-Bobo and Solomon (see Eq. 13 in reference 1), one should expect the concentration of free protons to increase, i.e. the pH value to decrease, when the hemoglobin concentration rises. The
decrease of hemoglobin net charge from +3 to +0.5 reported by Gary-Bobo and Solomon (2), for example, should then be accompanied by a decrease in pH by ~0.25 U. To test this prediction, solutions of positively charged hemoglobin were concentrated, and their pH value was measured at various stages of concentration.

To an isoionic solution of 10 g/dl carbonmonoxy hemoglobin in 0.1 M KCl, 0.1 N HCl was added to give a pH of 6.6 at 25°C, well below the isoelectric point. This solution, whose Cl⁻ and K⁺ concentrations then amounted to 0.10 and 0.09 molal, respectively, was concentrated at 0°C under CO. In intervals, samples were withdrawn from the ultrafiltration cell, and the pH of these samples was measured at 25°C as described above. In this way, the pH of the given hemoglobin solution was obtained at several hemoglobin concentrations. The concentrations of Cl⁻ and K⁺ in the ultrafiltrate were constant at 0.10 M during the entire process of ultrafiltration (pH of the ultrafiltrate was 6.6). This implies that no Donnan equilibrium existed between the hemoglobin solution and the ultrafiltrate. The Cl⁻ molality of the hemoglobin solution, therefore, remained constant at 0.10 M during the ultrafiltration, whereas the K⁺ molality decreased with increasing hemoglobin concentration. The ultrafiltration process was stopped after 4 days when the hemoglobin concentration had reached 46 g/dl. The results of this experiment are shown by the closed symbols (●) in Fig. 1 a.

In a second set of experiments, a 10 g/dl isoionic carbonmonoxy hemoglobin solution was adjusted to a pH of ~6.6 at 25°C using 0.1 N HCl. Chloride concentration was ~0.01 M, small cations were not present. This solution was concentrated at 0°C under CO. Again, samples were withdrawn at intervals. To these samples KCl was added to give the same final Cl⁻ and K⁺ molalities as in the first set of experiments; i.e., the total molality of Cl⁻ was again held constant at 0.10 M. The pH value of the samples was measured at 25°C as described. The results of these pH measurements are shown by the open symbols (◊) in Fig. 1 a.

Fig. 1 a shows that in both sets of experiments the pH of the hemoglobin solution does not change when the hemoglobin concentration is raised from 2 to 11 mmolal (tetramer). This indicates that no protons are liberated when the hemoglobin concentration increases. We conclude that the net charge of hemoglobin, as far as it reflects the degree of protonation of the protein molecules, is independent of hemoglobin concentration in a wide range encompassing the concentrations found inside erythrocytes (~7 mmolal).

HEMOGLOBIN CHARGE AS A FUNCTION OF HEMOGLOBIN CONCENTRATION

It may be asked whether the finding of a concentration-independent pH value is necessarily inconsistent with the finding of Gary-Bobo and Solomon (2) that the charge of hemoglobin, when estimated from Donnan distribution measurements, decreases with increasing concentration. The charge of hemoglobin is not solely determined by the state of protonation, i.e., by the pH value. There is a contribution due to the binding of other small ions. Although Na⁺ and K⁺ are not bound significantly (11), Cl⁻ is bound (12, 13) and thus should have an effect on hemoglobin charge. Were the binding of chloride by hemoglobin to change with hemoglobin concentration, a concentration dependence of hemoglobin charge could result. To be sure, not only the binding of H⁺ but also that
of Cl⁻ is associated with a change in pHH (14). But the effect on pHH may be much smaller in the case of Cl⁻ and thus might not be detected in the experiment described above. The resulting change in hemoglobin charge, however, would be observed in the type of experiment performed by Gary-Bobo and Solomon (2), which allows one to estimate the actual charge of the protein molecule. We have therefore determined the charge of hemoglobin at various hemoglobin concentrations and constant pHH from measurements of the Donnan distribution of ²²Na⁺ in a fashion similar to that described by these authors.

![Figure 1](image-url)

**Figure 1.** (a) pH of a carboxy hemoglobin solution at various stages of concentration. Temperature 25°C. Hemoglobin tetramer molalities, [Hb], were calculated from hemoglobin concentrations in grams per deciliter, c, according to \([\text{Hb}] = c/(6450 - 49 \cdot c)\). (●) Ultrafiltration in the presence of 0.1 M KCl; (○) ultrafiltration in the absence of KCl. In both cases the final concentration of Cl⁻ in the measured samples was 0.10 M. (b) Net charge, z, of carboxy hemoglobin, determined from the Donnan distribution of ²²Na⁺, as a function of hemoglobin concentration. Temperature 4°C. Average pH of the hemoglobin solution pHH = 6.90. In the cases in which pHH exhibited a small deviation \(\Delta\)pHH from this average, a correction \(\Delta z\) was calculated from \(\Delta z = \beta \cdot \Delta\)pHH, where \(\beta\), the hemoglobin buffer capacity, was taken to be 10. The absolute value of \(\Delta z\) never exceeded 0.5. The NaCl molality in the hemoglobin-free compartment was approximately 0.01.

Isoionic hemoglobin solutions with different concentrations were all adjusted to a pH of 6.6 at 25°C. The hemoglobin solutions were filled into the lower compartment of the dialysis chamber described above, and 0.010 M NaCl containing traces of ²²Na⁺ was filled into the upper compartment. When the Donnan equilibrium was established, the pH of the hemoglobin solutions was measured at the temperature of the experiment, 4°C. It was found to average 6.90 with very little variation (SE ± 0.01) and no significant correlation with hemoglobin concentration (\(P > 0.05\)). This is to be expected because all hemoglobin solutions had been adjusted to the same pH and, the buffer capacity of the hemoglobin compartment being at least 4 orders of magnitude larger than that of the upper compartment, no significant change in pHH can result from proton transfer across the dialysis membrane. The hemoglobin net
charge, \( z \), was calculated from the Donnan ratio of Na\(^+\), the hemoglobin concentration, and the sodium concentration in the upper compartment using Eq. 2. The values of \( z \) thus obtained are plotted in Fig. 1b as a function of hemoglobin concentration. It can be seen that for concentrations ranging from 2 to 10 mmolal, \( z \) is practically constant and in no way correlated with hemoglobin concentration. The average net charge is 5.24 (SE ± 0.05), at 4°C and pH \(_i\) = 6.90. Using the empirical relation \( p_I = pH_i + z/\beta \), and a value of 10 for the buffer capacity of hemoglobin, \( \beta \), we compute a value of 7.4 for the isoelectric point of hemoglobin, \( p_I \), at 4°C and 0.01 M NaCl. This figure falls well into the range of values reported by Lin and Forster (15) for this temperature.

Summarizing the results of Fig. 1a and b, we conclude that measurements of the pH value, as well as determinations of the Donnan distribution of Na\(^+\), indicate that the net charge of hemoglobin is independent of hemoglobin concentration. It appears that neither changes of protonation nor changes in the number of hemoglobin-bound chloride ions occur that lead to a significant decrease of the charge of carbonmonoxy hemoglobin as the protein concentration increases. The constancy of protonation follows directly from the constancy of pH shown in Fig. 1a. That no significant change in chloride binding by hemoglobin occurs, follows from this latter result together with the finding of a constant total net charge of hemoglobin as demonstrated in Fig. 1b.

These results are in conflict with two earlier reports. First, Lin and Forster (15) found that the pH of an isoionic hemoglobin solution is not constant but decreases with increasing hemoglobin concentration. The reason for the discrepancy with the results of Fig. 1a is not clear. However, Lin and Forster (15) performed the pH measurements in the complete absence of small ions, whereas in the present study pH was measured in the presence of 0.1 M KCl because it was not found possible to obtain stable pH readings in the absence of salt. Second, the present results are at variance with those of Gary-Bobo and Solomon (2), who find, under conditions very similar to ours, a decrease of net charge from 3 to 0.5 when the hemoglobin concentration is raised from 2 to 10 mmolal. How can this remarkable discrepancy be understood? It appears from the experimental protocol given by these authors that they did not control the pH of the hemoglobin solution, pH\(_i\), but instead held the pH of the hemoglobin-free compartment, pH\(_o\), at a constant value by adding acid or base. For constant [Na\(^+\)]\(_o\), the Donnan ratio decreases with increasing hemoglobin concentration. pH\(_o\) being constant, this implies that pH\(_i\) increases and \( z \) decreases with rising hemoglobin concentration. We suggest that this provides a qualitative explanation of Gary-Bobo and Solomon's findings.

Concentration Dependence of Oxygen Affinity

In Fig. 2, \( P_{50} \) and \( n \) are shown as a function of hemoglobin concentration. It is evident that neither of the two parameters of oxygen binding varies with hemoglobin concentration for hemoglobin molalities ranging from 0.7·10\(^{-2}\) M to 9·10\(^{-2}\) M. \( P_{50} \) averages 11.40 mm Hg (SE ± 0.06 mm Hg), \( n \) is 2.87 (SE ± 0.05). This \( P_{50} \) value tallies well with the value of 11.8 mm Hg given by Petschow et al. (16) for a hemoglobin molality of 0.3·10\(^{-3}\) M and identical conditions. The
finding of concentration independence of the oxygen affinity agrees with other studies covering somewhat lower ranges of hemoglobin concentration (17, 18). It seems likely that the increase in $P_{50}$ that has earlier (8, 9) been reported to occur with increasing hemoglobin concentration resulted from incomplete removal of organic phosphates. When the ratio phosphate/hemoglobin is constant, the fraction of hemoglobin that has phosphate bound, and with it $P_{50}$, rise with increasing hemoglobin concentration. It may be noted that an increase in $P_{50}$ with increasing hemoglobin concentration is also seen when the $\text{Cl}^{-}$ molarity, i.e. the number of moles of $\text{Cl}^{-}$ per liter of hemoglobin solution, instead of the $\text{Cl}^{-}$ molality is adjusted to a constant value. This implies increasing $\text{Cl}^{-}$ molalities with increasing hemoglobin concentration and leads to decreasing oxygen affinities.\(^1\)

![Figure 2](image)

**Figure 2.** Oxygen half-saturation pressure, $P_{50}$, and Hill coefficient, $n$, as function of hemoglobin concentration. Temperature 37°C, pH 7.20, salt molalities 0.075 bis-Tris and 0.075 NaCl. Methemoglobin <5%.

**Conclusions**

We have shown that two physiologically important functional properties of hemoglobin, net charge and oxygen affinity, are independent of the concentration of hemoglobin up to values well above those found inside erythrocytes. In contrast to previously held views (1, 2, 6, 9), both parameters do not show deviations from their behavior in dilute solutions up to hemoglobin molalities of $1\cdot10^{-2}$ M. It may be noted that the binding of 2,3-diphosphoglycerate to hemoglobin, previously believed to be an example of a concentration-dependent functional property of hemoglobin (4), from more recent evidence also seems to be independent of hemoglobin concentration (19).

The results reported in this paper reopen the question of the molecular mechanism behind the seemingly anomalous osmotic behavior of erythrocytes, which, upon changes in osmolarity, leads to smaller changes in erythrocyte volume than would be expected from van't Hoff's law. This anomaly could, for

\(^1\) Gros et al. Unpublished observations.
example, be due to a strong dependence of the osmotic coefficient of hemoglobin on the osmotic environment of the erythrocyte. Dick (5), quoting unpublished results of Adair, has in fact suggested that the osmotic coefficient of hemoglobin not only depends on hemoglobin concentration but also on ionic strength, and that this rather than a concentration dependence of hemoglobin charge may explain the diminished osmotic response of erythrocytes. Apparently, it has not been recognized by several workers in this field that Scatchard and Pigliacampi (20) in an extensive study indeed have shown that the osmotic coefficient of human hemoglobin, apart from its dependence on hemoglobin concentration, is strongly influenced by ionic strength as well as pH. The dependency of the osmotic coefficient on these three variables has been attributed to excluded volume effects and electrostatic interactions (20, 21). The measurements of Scatchard and Pigliacampi, though obtained at hemoglobin concentrations up to 1 millimolal only, indicate that the concentration dependence of the osmotic coefficient of human hemoglobin is much larger under the conditions of pH and ionic strength as they prevail inside erythrocytes than has been anticipated from Adair's (22) investigations of sheep hemoglobin at pH 7.8 and high ionic strength. An extension of Scatchard's and Pigliacampi's studies to higher concentrations of hemoglobin might well provide a quantitative explanation of the osmotic behavior of erythrocytes.

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REFERENCES

1. Gary-BoBo, C. M., and A. K. Solomon. 1968. Properties of hemoglobin solutions in red cells. J. Gen. Physiol. 52:825-853.
2. Gary-BoBo, C. M., and A. K. Solomon. 1971. Hemoglobin charge dependence on hemoglobin concentration in vitro. J. Gen. Physiol. 57:283-289.
3. Damaschun, G., H. Damaschun, Ch. Gedcke, J. J. MÜller, H.-V. Püschel, K. Ruckpaul, and M. Zinke. 1975. Über die supramolekulare Organisation des Oxyhämoglobins im Erythrocyten: Eine Röntgen-Kleinwinkelstreuungs-Studie. Acta Biol. Med. Get. 34:391-398.
4. Garby, L., and C-H. De Verdier. 1971. Affinity of human hemoglobin A to 2,3-diphosphoglycerate: effect of hemoglobin concentration and of pH. Scand. J. Clin. Lab. Invest. 27:345-350.
5. Dick, D. A. T. 1969. Osmotic behavior of hemoglobin in vivo and in vitro. J. Gen Physiol. 33:836-838.
6. Mazziotti, A., P. N. Farnsworth, and R. H. L. Marks. 1976. Analysis of hemoglobin aggregation from Gibbs-Donnan equilibrium experiments. J. Gen. Physiol. 68:105-109.
7. Gros, G. 1978. Concentration dependence of the self-diffusion of human and Lumbricus terrestris hemoglobin. Biophys. J. 22:453-468.
8. Roughton, F. J. W., A. B. Otis, and R. L. J. Lyster. 1955. The determination of the individual equilibrium constants of the four intermediate reactions between oxygen and sheep hemoglobin. Proc. R. Soc. B. 144:29-54.
9. Radford, E. P., G. Torelli, F. Celentano, and E. D'Angelo. 1967. Concentration dependence of O2-hemoglobin equilibrium. Fed. Proc. 26:333.
10. LAUE, D. 1951. Ein neues Tonometer zur raschen Aequilibrierung von Blut mit verschiedenen Gasdrucken. Pflügers Arch. Eur. J. Physiol. 254:142-143.

11. CARR, C. W. 1956. Studies on the binding of small ions in protein solutions with the use of membrane electrodes. VI. The binding of sodium and potassium ions in solutions of various proteins. Arch. Biochem. Biophys. 62:576-584.

12. CHIANCONE, E., J. E. NORNE, S. FORSEN, E. ANTONINI, and J. WYMAN. 1972. Nuclear magnetic resonance quadrupole relaxation studies of chloride binding to human oxy- and deoxyhaemoglobin. J. Mol. Biol. 70:675-688.

13. PFISTER, R. H., and H. PAULY. 1972. Chemical potential of KCl and its ion constituents in concentrated protein salt solutions. J. Polym. Sci. C. 39:179-189.

14. ROLLEMA, H. S., S. H. DE BRUIN, L. H. M. JANSSEN, and G. A. J. VAN OS. 1975. The effect of potassium chloride on the Bohr effect of human hemoglobin. J. Biol. Chem. 250:1333-1339.

15. LIN, L., and R. E. FORSTER. 1977. Variations in pH of isoionic oxygenated hemoglobin solution with temperature, [NaCl] and [hemoglobin] including physiological concentrations. Fed. Proc. 36:272.

16. PETSCHOW, D., I. WURDINGER, R. BAUMANN, J. DUHM, G. BRAUNITZER, and C. BAUER. 1977. Causes of high blood O2 affinity of animals living at high altitude. J. Appl. Physiol. 42:139-143.

17. FORSTER, R. E. 1972. The effect of dilution in saline on the oxygen affinity of human hemoglobin. In Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status. M. Rørth and P. Astrup, editors. Munksgaard, Copenhagen. 162-164.

18. MURPHY, J. R., M. WENGERD, and R. W. KELLERMeyer. 1974. Erythrocyte O2 affinity: influence of cell density and in vitro changes in hemoglobin concentration. J. Lab. Clin. Med. 84:218-224.

19. HAMASAKI, N., and Z. B. ROSE. 1974. The binding of phosphorylated red cell metabolites to human hemoglobin A. J. Biol. Chem. 249:7896-7901.

20. SCATCHARD, G., and J. PIGLIACAMPI. 1962. Physical chemistry of protein solutions. XI. The osmotic pressures of serum albumin, carboxylhemoglobin and their mixtures in aqueous sodium chloride at 25°C. J. Am. Chem. Soc. 84:127-134.

21. ROSS, P. D., and A. P. MINTON. 1977. Analysis of non-ideal behavior in concentrated hemoglobin solutions. J. Mol. Biol. 112:437-452.

22. ADAIR, G. S. 1928. A theory of partial osmotic pressures and membrane equilibria, with special reference to the application of Dalton's law to hemoglobin solutions in the presence of salts. Proc. R. Soc. Lond. B. Biol. Sci. 120:575-603.