

Hemoglobin Charge Dependence on Hemoglobin Concentration in Vitro

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ABSTRACT Studies have been made of the dependence of the charge of the hemoglobin molecule on hemoglobin concentration in the concentration range between 3 and 11 mmolal. The charge has been determined by measuring the distribution of ^{42}K between a hemoglobin solution in a cellophane bag and an external solution. The pH was 6.6, the K concentration was 10 mM, and the temperature was 4°C. The charge decreased along a sigmoid curve from a value of +3 in the most dilute solutions to a value of +0.5 in the most concentrated solutions. The results were in excellent agreement with earlier studies of Gary-Bobo and Solomon in which Cl distribution was measured between human red cells and external solutions and thus give added support to the conclusion that the apparent anomalous osmotic behavior of human red cells may be attributed to concentration-dependent changes in the hemoglobin net charge. The present findings also support the view that the water in the red cell is solvent water for K and Cl and differs in no quantitatively important respect from bulk water in free solution.

The apparent anomalous osmotic behavior of red blood cells has its origin in concentration-dependent effects on hemoglobin (Hb) net charge according to a hypothesis developed by Gary-Bobo and Solomon (1). These authors presented experimental evidence from studies of the human red cell showing that the net charge on the Hb molecule at constant pH decreases with increases in Hb concentration. As a result changes in red cell volume at constant pH affect the Hb charge so that red cell counterion concentration, primarily Cl and HCO_3^- , should depend on red cell volume. The quantitative agreement Gary-Bobo and Solomon obtained between experimental and predicted Cl shifts with cell volume changes, in HCO_3^- -free human red cells, offered powerful support to the hypothesis of a concentration-dependent cooperative interaction among Hb molecules. These previous observations were all made in whole human red cells in which the interior milieu could

not be quantitatively controlled. In order to test the hypothesis further, a set of *in vitro* experiments has now been designed in which Hb interaction can be investigated without the intervention of a cellular membrane. We have measured the Hb net charge as a function of Hb concentration by observing the Donnan equilibrium of K across a cellophane membrane separating Hb and salt solutions.

EXPERIMENTAL METHOD

Preparation of Hb Solutions

20% (weight to volume, w/v) salt-free Hb solutions were prepared from human blood, as described previously (1). In order to concentrate these solutions further, a technique of ultrafiltration at moderate pressure was devised. The 20% Hb solutions were transferred to bags made of 0.98 cm wide cellophane tubing and a nitrogen tank was used to apply pressures of 0.5–1.5 atmospheres. The pressurized bags were then immersed in distilled water and kept at 4°C. In 6–10 days the Hb concentration reached 25–40% (w/v), depending upon pressure and time.

K Distribution Measurements

About 2 ml of each Hb solution was transferred to a smaller cellophane bag and the same pressure was again applied. The bags (10 at a time) were immersed in 1 liter of 10 mM KCl solution containing 1 mCi of ⁴²K (Cambridge Nuclear, Inc., Cambridge, Mass.). The pH of the solution was checked regularly and adjusted if necessary by the addition of KOH or HCl since small variations (<0.1 pH unit) were observed during the first 15 hr. 36 hr at 4°C were required to attain complete K equilibrium within the 1% accuracy of the experiment. ⁴²K was determined in an automatic well scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill., Model 4222), on weighed samples of both the Hb solutions, and the external K concentration was measured to an accuracy of better than 1% by using a flame photometer (Instrumentation Laboratory, Inc., Boston, Mass., Model 143).

The K molality in the external medium, m_+ , and in the Hb solutions, m'_+ , was computed and the average net number of positive charges per Hb molecule, z , was calculated according to the equation

$$m'_+(m'_+ + z m_{\text{Hb}}) = m_+^2 \quad (1)$$

RESULTS AND DISCUSSION

Typical results of all the determinations at pH 6.60 are given in Fig. 1, for 21 experiments in which the Hb concentration ranged from 3 to 11 mM. Similar results were obtained in eight experiments at pH 6.71. The accuracy in the latter experiments was less than in those at pH 6.60, since z approaches zero at pH 6.95. Attempts were made to carry out experiments at higher Hb concentrations but great difficulties were found with the more concentrated Hb solutions since Hb tends to aggregate and these very concentrated

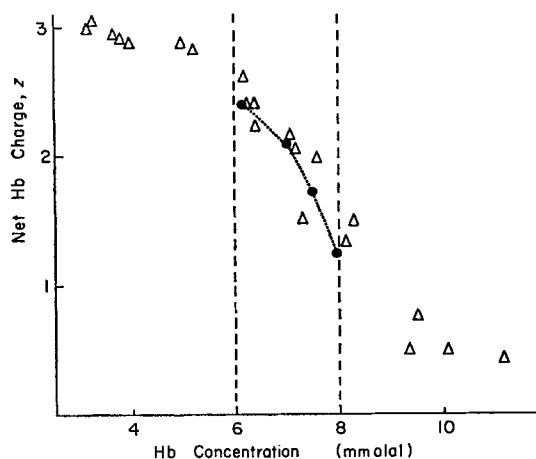


FIGURE 1. The results of the present experiments at pH 6.60 (open triangles) are compared with those previously obtained in vivo (solid circles connected by a dotted line). The previous data are averages of two experiments at each point; the vertical dashed lines indicate the attainable excursions of osmolality with intact human red cells.

solutions did not remain homogeneous in the bags. Experiments at pH's lower than 6.5 are also unpractical since they appear to favor Hb precipitation.

Fig. 1 shows that the net charge of the Hb molecule decreases significantly with increasing Hb concentration in agreement with the observations of Gary-Bobo and Solomon on human red cells. Indeed, at pH 6.60 and at a 3 mmolal Hb concentration the asymptotic value of z is equal to 3, a value in good agreement with the previous value of 3.2 at a pH of 6.6 and the same Hb concentration. The points in Fig. 1 appear to fall on a sigmoid curve. As the Hb concentration increases from 3 to 6 mmolal, z decreases only by about 0.5 unit. As the Hb concentration is increased in the range between 6 and 8 mmolal, z decreases much more rapidly. In the most concentrated region between 9 and 11 mmolal where Hb concentration on a percentage basis is $\approx 37\%$ (g Hb/g solution), z once again decreases slowly. The maximum variation of the Hb concentration achieved by Gary-Bobo and Solomon in red blood cells was from 6 to 8 mmolal. This region, indicated by the dashed lines in Fig. 1, is the region of the most rapid change of z . The mean Hb concentration in human red cells is about 7.2 mmolal when the cells are suspended in an isosmolal solution of 290 milliosmols.

It is possible to make a direct comparison of the previous results on red cells with the present in vitro data, by normalizing the previous data on human red cells to the present data. In the in vivo experiments, the data obtained were all relative. In order to express these data in absolute terms the charge on the Hb molecule at pH 7.32 was arbitrarily set (by difference) at 3.4 units as explained in the footnote¹ to Table III of reference 1. With

¹ In Table III of reference 1, there are two errors: the number of Hb charges should read 3.4 instead of 4.7. The internal pH should read 7.32 and not 7.44.

the use of this arbitrary base we then computed (see Fig. 8 of reference 1) that the net Hb charge should be 3.65 units under isosmolal conditions at pH 6.6. Our present experimental determinations give an interpolated value of 2.2 units at pH 6.6 at the same molal Hb concentration. We have therefore normalized the previous values from Fig. 8 by multiplying them by the factor 0.605 (2.2/3.65). The average normalized figures are shown by the solid circles in Fig. 1, and the agreement between the normalized figures from human red cells and the present data is found to be very good. There appears to be no significant difference between the experiments *in vivo* and *in vitro*, so that the present results confirm the conclusions reached in the previous paper.

In addition to the presence of the red cell membrane in the previous experiments there is another important difference in the milieu of the Hb molecule in the two sets of experiments. The ionic strength in the present experiments is ≈ 0.01 as compared to the value of 0.15 characteristic of the *in vivo* condition. In view of this difference in ionic strength the quantitative agreement of the data in Fig. 1 is somewhat surprising because it suggests that the Hb interaction is essentially independent of ionic strength. It therefore appears that the Hb-Hb interaction is stronger than the effect of ionic strength on the dimensions of the zeta potential shell around the Hb molecule. The Hb molecules in the red cell are packed very tightly. Under isosmolal conditions in human red cells, the Hb molecules are separated by only about 27 Å according to Bateman et al. (2) and Riley and Herbert (3) as compared to the 15 Å separation observed by Perutz (4) in crystalline horse hemoglobin. Tanford (5) computes that the Debye-Hückel electrostatic free energy of an impenetrable spherical protein of 25 Å radius would decrease from 6.7 kcal/mole to 2.5 kcal/mole when the ionic strength increases from 0.01 to 0.15 for a net charge of 10 units. Since the electrostatic free energy is a function of the square of the unit charge, the electrostatic free energy of Hb under our conditions would be less than one-tenth of these values since z does not exceed 3 in the present experiments. As discussed above the present experimental value, $z = 3$ (pH, 6.6; Hb, 3 mmolal), was obtained at an ionic strength of ≈ 0.01 and agrees very well with the previous extrapolated experimental value of $z = 3.2$ under the same conditions of pH and Hb concentration in experiments in which the ionic strength varied between 0.01 and 0.25. The relative unimportance of the ionic strength is consistent with a strong protein-protein interaction.

The symmetry of the data in Fig. 1 is very suggestive of hydrogen titration curves. The degree of ionization may be represented formally as $\beta = (z - 0.5)/2.5$ since z in Fig. 1 goes from 3.0 to 0.5; Fig. 2 shows β as a function of Hb molality. The point of inflection of the curve at 7.5 mmolal Hb could be called an "apparent pK" in formal analogy to the procedures for titration

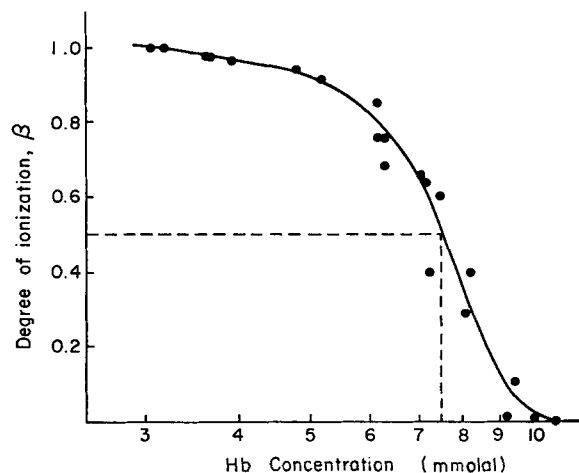


FIGURE 2. Degree of average net hemoglobin ionization as a function of hemoglobin concentration computed as described in the text from the data in Fig. 1.

curves. However, it is not possible to extend the analogy further in quantitative terms, since the appropriate symbolic equation is



Though it is theoretically possible to write the usual relationship between concentrations and the equilibrium constant, this does not lead to an advance in our knowledge, since our experiments do not provide any unequivocal method for determining n . Furthermore, though the results have been interpreted in terms of the net charge of the Hb molecule, z , this is not a unique interpretation, since our experiments determine $z m_{\text{Hb}}$ in equation 1 and there is no way to separate this product unambiguously into its separate components. We have arbitrarily assumed Hb to be a monomer in computing z , and thus provided a framework for quantitative comparison. However, any detailed theoretical treatment would require a separate experimental determination of n as a function of Hb concentration.

Dick (6) has suggested that the observation of Gary-Bobo and Solomon that the apparent osmotic water content of the red cell at low pH is greater than the total water of the cell agrees reasonably well with the observation of Dick and Loewenstein that the apparent osmotic water is 95% of the total cell water. The important point is that Gary-Bobo and Solomon's value is greater than unity, thus indicating that the apparent osmotic water as previously calculated gives imaginary results. Furthermore Gary-Bobo and Solomon showed that the apparent osmotic water was a function of pH, which played no part in Dick's calculations.

Krivacic and Rupley (7) have made direct measurements of volume

changes in solution following crystallization of horse hemoglobin. They have made computations from the observed volume change and have stated that "the solvent about the protein in the crystal is essentially the same as that in solution." This is contrary to the conclusion reached by Gary-Bobo and Solomon (1). However, Krivacic and Rupley base their reasoning upon the volume changes theoretically expected between crystalline Hb volume and the sum of the partial molal volumes of analogues of the various components of the hemoglobin, each assumed separate from one another in dilute solution. This calculation takes no account of the fact that the substituent groups in the Hb molecule are in close spatial relation to one another, so that interactions within the molecule set it clearly apart from the behavior to be expected from a sum of analogous groups in dilute solution. Hence, though the dilatometric measurements upon which the conclusions of Krivacic and Rupley are based appear to be careful and accurate, the conclusion as to the state of water in solutions of hemoglobin molecules represents an extrapolation on questionable grounds.

The present results also add a most suggestive observation, that the point of inflection, as judged by the degree of ionization, for the Hb-Hb interaction occurs at a Hb concentration of 7.5 mmolal. This concentration, which is in the center of the region of the most rapid change of ionization, is close to the 7.2 mmolal Hb concentration present in human red cells under isosmolar conditions. Thus it would be particularly appropriate for the average net Hb charge to serve as the osmotic transducer which regulates red cell volume.

The present set of in vitro experiments agrees well with expectations based on the hypothesis of Gary-Bobo and Solomon that the anomalous osmotic behavior of human red cells is attributable to hemoglobin-hemoglobin interactions. Furthermore, the results reinforce the view that the water within the human red cell is solvent water, which differs in no quantitatively important respect from bulk water in free solution.

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