Properties of Hemoglobin Solutions in Red Cells

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ABSTRACT The present studies are concerned with a detailed examination of the apparent anomalous osmotic behavior of human red cells. Red cell water has been shown to behave simultaneously as solvent water for nonelectrolytes and nonsolvent water, in part, for electrolytes. The nonsolvent properties are based upon assumptions inherent in the conventional van't Hoff equation. However, calculations according to the van't Hoff equation give osmotic volumes considerably in excess of total cell water when the pH is lowered beyond the isoelectric point for hemoglobin; hence the van't Hoff equation is inapplicable for the measurement of the solvent properties of the red cell. Furthermore, in vitro measurements of osmotic and other properties of 3.7 millimolal solutions of hemoglobin have failed to reveal the presence of any salt exclusion. A new hypothesis has been developed from thermodynamic principles alone, which predicts that, at constant pH, the net charge on the hemoglobin molecule decreases with increased hemoglobin concentration. The existence of such cooperative interaction may be inferred from the effect of pH on the changes in hemoglobin net charge as the spacing between the molecules decreases. The resultant movement of counterions across the cell membrane causes the apparent anomalous osmotic behavior. Quantitative agreement has been found between the anion shift predicted by the equation and that observed in response to osmotic gradients. The proposed mechanism appears to be operative in a variety of tissues and could provide an electrical transducer for osmotic signals.

Red cells adjust their volume according to the osmolality of the medium in which they are suspended. It has long been known that the volume change observed is less than that expected according to the van't Hoff law. Ponder (1) has developed an equation to express the deviation from the expected van't Hoff behavior in terms of the ratio, R, of the observed volume change to the expected one. Many studies have been made which indicate this ratio to be less than unity, though there has been disagreement over the exact fraction of the cell water involved (2–4). Recently Savitz, Sidel, and Solomon (5) measured cell volume by isotope dilution and found that 20% of the cell water was apparently unable to participate in osmotic phenomena.

This 20% fraction may be expressed in terms of the hemoglobin content as 0.4 g/g hemoglobin. In studies of horse methemoglobin crystals Perutz (6) had found that the water within the crystal did not contain a uniform concentration of salt and that 0.3 g H_2O/g hemoglobin contained no salt at all. Perutz called this "bound" water and calculated that it was equivalent to a layer one molecule thick surrounding the protein crystal. Drabkin (7) made a similar measurement on human hemoglobin crystals and found the "bound" water to be 0.34 g/g hemoglobin. He suggested that this bound water might be present in red blood cells. The numerical agreement is persuasive and Savitz, Sidel, and Solomon also account for the phenomenon in these terms.

However, Miller (8) has shown that all the red cell water serves as solvent for glucose, and Gary-Bobo (9) has obtained a similar result for various nonelectrolytes above 20°C. Bernal (10) has pointed out that the properties of water adjacent to a protein molecule should be different from those in bulk solution. Nonetheless it is difficult to understand how a layer of bound water may totally exclude electrolytes and yet exercise no discrimination against nonelectrolytes. In consequence the present study was undertaken to examine the problem more closely. The volume response of the cells was measured in the same system in which solute distribution was studied in order to obtain both experimental results under identical conditions.

In order to test the bound water hypothesis we looked for electrolyte exclusion in less concentrated hemoglobin solutions in vitro, but were unable to find any behavior consonant with bound water. Consequently a new mechanism has been proposed based upon the concentration-dependence of the net charge on the hemoglobin molecule. Our experiments indicate that the net hemoglobin charge varies with hemoglobin concentration and that the resultant movement of counterions is responsible for the apparent aberrations in the osmotic behavior of the red cell.

MATERIALS AND METHODS

Experiments on Red Cells

The osmotically active cell water content was measured using the ^{131}I–albumin dilution method, according to the technique of Savitz et al. (5). Red blood cells, obtained from fresh heparinized blood, were washed four times in 10 volumes of buffer isosmolal with human plasma (containing in mM: $MgCl_2$ 0.5; $CaCl_2$ 1.2; Na_2HPO_4 1.7; NaH_2PO_4 4.2; KCl 4.4; Na_2CO_3 13.5; and NaCl 117.8; a mixture of 5% CO_2 and 95% air was passed through this solution to bring it to pH 7.4). The washed red cells were resuspended in buffers of varying osmolality, obtained by varying the NaCl concentration alone. The buffer also contained ^{131}I–human serum albumin and 0.5 g/liter nonlabeled albumin which was added to prevent selective binding of labeled albumin to the cells or to glass surfaces. ^{131}I–albumin was counted in a well

counter (Nuclear Chicago, Des Plaines, Ill., Model 4222). Osmolality was measured by freezing point depression in an osmometer (Model G-62, Fiske Associates, Inc., Bethel, Conn.) calibrated by comparison with NaCl solutions of known osmolality. All experiments were carried out at room temperature (25°–26°C).

Volume Shifts with Medium Osmolality

The experiments to measure the osmotic behavior of the red cells were carried out in the following way. The red cells of each sample of blood were washed four times in isosmolal buffer, divided into three parts, which were then washed four more times in three media of different osmolality (one of these being always of the same osmolality as human plasma, which we will call isosmolal). These three osmolalities were denoted as *initial* osmolalities. For each initial osmolality, experiments were carried out as previously described by Savitz et al. (5).

To measure the effect of pH on the osmotically active cell water, the red cells of each sample of blood were again divided into three parts and washed 10 times in 10 volumes of isosmolal buffer of the desired pH (the composition of which varied only by replacing the 13.5 mM Na_2CO_3 and CO_2 with NaH_2PO_4–Na_2HPO_4). The experiments at each pH were carried out in the same way as the other determinations of the osmotically active water content.

Solvent Water Content

The solvent water content of the red cells was determined by measuring the equilibrium distribution, between the cell water and the medium, of three diffusible, hydrophilic ^{14}C-labeled solutes: ethanol, 2-propanol, and D-glucose. As Gary-Bobo has previously shown (9) the equilibrium distribution of ethanol and 2-propanol is independent of concentration in the suspension so experiments with these molecules were carried out at 0.01 M. The glucose concentration on the other hand was set at 0.1 M in order to minimize both the effect of glucose metabolism and the possible influence of the facilitated transport mechanism for this molecule. Preliminary experiments indicate that glucose distribution is independent of its concentration over the range from 0.05 to 0.25 M; identical results were obtained after 1.5 and 6 hr of exposure to labeled glucose.

The radioactivity of both supernatant and cells was measured following the method previously described by Gary-Bobo (9). A correction was made for the trapped medium in the red cell fraction based on the ^{131}I–albumin studies. The total water content of the red cells was measured gravimetrically as described by Savitz et al. (5).

Ionic Shifts with Medium Osmolality

For these experiments the bicarbonate content of the cell was thoroughly removed by washing the cell 10 times in 10 volumes of isotonic phosphate buffer of the desired pH. In the chloride shift experiments, the cells were equilibrated with a mixture of $Na^{36}Cl$ and ^{131}I–albumin in a fixed ratio. In order to be sure that the cell chloride was completely equilibrated the isotope mixture was added to the buffer of the five last washes. By the tenth wash, further washes did not change the $^{36}Cl/^{131}I$ ratio. The

osmotic experiment was carried out as usual in anisotonic phosphate buffers containing the same isotopic mixture, so that the specific activity of ^{36}Cl was kept constant through the entire osmotic range. The sodium shift experiments were carried out in the same way but cell equilibration with the $^{24}NaCl/^{131}I$ mixture was omitted. The ionic shifts were computed from changes in the isotope ratio and expressed in milliequivalents/liter initial cell H_2O. This experimental technique has the advantage of eliminating any protein precipitation. Furthermore the computation of ionic shifts is independent of water movement.

The buffer in the external medium was titrated to measure hydrogen ion movement. This movement was computed from the amount of base or acid necessary to bring the buffer pH to its value before contact with the cells. The medium contained 50 mM phosphate buffer, and the osmolality was altered by changing NaCl concentration alone. The hematocrits were 35–40%. The hydrogen movement was measured in the same set of experiments in which the sodium and chloride movements were measured, and the results were expressed in the same units.

Experiments on Hemoglobin Solutions

Hemoglobin solutions were prepared from human red cells following the procedure described by Gary-Bobo (9). The osmotic measurements on these solutions were carried out by the zero flow method. The two compartments in the chamber were separated by a cellulose acetate membrane[1] (Loeb membrane [11]). Capillary pipettes (0.1 ml) were sealed in each compartment for volumetric readings. The displacements in the two capillaries, opposite in direction, were checked and found to be equal. Thus any aberrant volume changes not due to osmotic gradients across the membrane could be discarded. The hydraulic conductivity of the membrane was found to be constant over the range of hydrostatic pressure (up to 0.5 atm) as well as over the salt concentration range (up to 0.5 M).

Distribution ratios of NaCl and KCl between hemoglobin solutions and water were measured by dialyzing hemoglobin solutions in small cellophane bags against NaCl or KCl solutions of various concentrations, which contained mixtures of either $^{24}NaCl$ and $Na^{36}Cl$ or ^{42}KCl and $K^{36}Cl$. The equilibrium concentrations were measured in both phases. ^{24}Na and ^{42}K were counted in the automatic well counter; after complete decay ^{36}Cl was counted in the same samples by a liquid scintillation counter (Nuclear Chicago Corporation, Des Plaines, Ill., Model 6801).

RESULTS AND DISCUSSION

Simultaneous Measurements of Solute Distribution and Volume Changes

The volume measurements were carried out following the procedure of Savitz et al. (5). According to these authors the variation of the cellular volume V with the osmolality π, can be described by:

$$V = W_{\text{eff}}(\pi_i/\pi) + b' \qquad (1)$$

[1] We are glad to express our indebtedness to Dr. R. Bloch for advice on the technique of casting the cellulose acetate membrane.

W_{eff} is the volume fraction of the cell that is apparently able to participate in osmotic phenomena, the subscript i denotes that the reference osmolality, π_i, was isosmolal with plasma, and b' is the fraction of the cell volume, including the solute and membrane volume, which apparently does not participate in the osmotic response. The solvent volume of the cell was measured in the same experiments from the distribution ratio of three readily diffusible nonelectrolytes, ethanol, 2-propanol, and D-glucose, added in tracer amounts to the external medium.

The results (Table I, last column) show that at 25°C (±0.5°C), the volume fraction of cell water that apparently participates in osmotic phenomena is 0.573. Since the average volume fraction of total cell water under isosmotic

TABLE I
MEASUREMENTS OF RED CELL WATER

Solute	Medium osmolality	Volume fraction of		
		Total cell water	Solvent water	Apparent osmotic water
	milliosmols/liter	*ml/ml cell*	*ml/ml cell*	*ml/ml cell*
Ethanol	303	0.710	0.710	0.565
	292	0.715	0.715	0.582
	291	0.710	0.715	0.575
2-Propanol	291	0.725	0.720	0.570
	290	0.720	0.720	0.577
Glucose	290	0.715	0.710	0.577
	290	0.710	0.690	0.570
Average	292	0.715	0.710	0.573
SE		0.006	0.010	0.005

conditions is 0.715 (column 3), 0.142 ml water/ml cell apparently does not participate in the osmotic response of the cells. This represents 0.198 of the isosmolal cell water content, a figure which is in good agreement with the figure of 0.202 obtained by Savitz et al.

On the other hand, the volume of distribution of the three nonelectrolytes was always found to be equal to the total cell water volume, over the entire range of osmolality as shown in Fig. 1. This result is in agreement with the findings of Miller (8). Gary-Bobo (9) also found that at this temperature, more than 98% of the water in the cell served as solvent for ethanol and 2-propanol, using chemical measurements rather than radioactive isotopes. The discrepancy between the two different types of measurement, the solvent volume (given in the fourth column of Table I) and the apparent osmotic volume (given in the fifth column), cannot be ascribed to a difference in

experimental conditions. Thus in the same experiment, we are led to the conclusion that all the cell water is solvent water by one type of measurement whereas only 80% of the cell water apparently participates in osmotic phenomena according to the other type of measurement.

Evaluation of Protein "Bound Water" Hypothesis

It might be possible to account for this discrepancy in terms of water bound to the hemoglobin molecule in such a way that electrolytes were unable to penetrate into the bound water though nonelectrolyte solutes could do so. It is difficult to understand how water might possess these apparently divergent qualities, but is is necessary to examine the evidence for water binding to hemoglobin in order to see whether this possibility may be excluded on the-

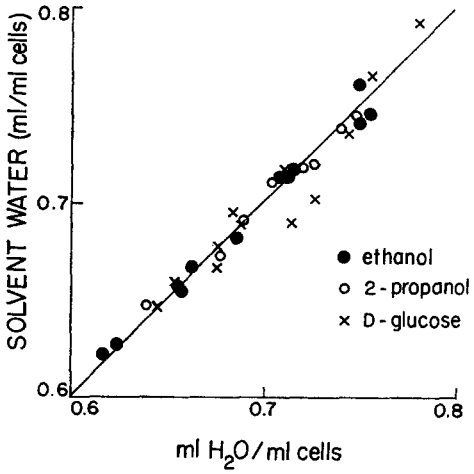

FIGURE 1. Volume fraction of solvent water for three nonionic solutes, ethanol, 2-propanol, and D-glucose in human red cells as a function of the volume fraction of total cell water. The line is the line of identity.

oretical or experimental grounds. Perutz (6) studied the swelling properties of horse methemoglobin crystals and found that the amount of bound water at the isoelectric point, pH 7, remained essentially constant over the entire concentration range from 1.5 to 4 M $(NH_4)_2SO_4$. However, when pH was varied the peak content of bound water was observed at the isoelectric point, when the number of unpaired charge groups should be minimal.

The hydrated ammonium ion has a diameter of 5.2 A, very much smaller than the 7.2 A sulfate ion, as computed according to the modified Stokes law (12) so that steric effects should be expected to play an important role in the local charge distribution in the 15 A thick liquid channel in hemoglobin crystals (6). Thus the apparent pH dependence of bound water found by Perutz may bear very little relation to the pH actually present in the water channels separating the crystalline regions. Furthermore, these con-

siderations make it quite likely that steric factors serve to exclude salt from the rigid confines of the hemoglobin crystal. Thus we should not expect Perutz's bound water to be carried into solution in its entirety.

In this respect, Tanford (13) gives an interesting discussion. At the isoionic point, ovalbumin (mol wt, 45,000) has 41 positive charges, counterbalanced by 41 negative charges. Assuming the tightly bound water for these groups to be the same as for the guanidine hydrochloride of alkyl ammonium groups, about 2 ± 1 for each pair of groups, there would be 82 ± 41 tightly bound moles of water. For ovalbumin this would correspond to a water content of 0.032 ± 0.016 g/g protein. For hemoglobin with 180 charge groups the bound water computed in this way would amount to 0.049 ± 0.025 g/g Hb which falls short of the 0.3 g/g Hb bound water content of hemoglobin crystals.

Buchanan, Haggis, Hasted, and Robinson (14) have made dielectric measurements on horse methemoglobin molecules in solution and have differentiated irrotationally bound water, amounting to about 0.1 g/g Hb, from a more loosely bound water around the charged protein groups. Similar results have been obtained by Schwan (15). The general conclusion that might be drawn from all these studies is that some water is bound to the hemoglobin molecule in free solution, but the amount would appear to be a good deal less than that characteristic of crystalline hemoglobin.

The question at issue is whether this bound water, which appears in any case to be too small to account for the anomalous osmotic behavior of the red cell, possesses the ability to dissolve nonelectrolyte molecules and exclude electrolyte molecules. This question can be answered experimentally by measuring the osmotic pressure of hemoglobin solutions in the presence and absence of electrolytes. Bound water which could exclude electrolytes should cause the osmotic pressure exerted by a salt solution containing hemoglobin to be greater than the sum of the osmotic pressure of the salt solution without hemoglobin plus the osmotic pressure of hemoglobin at the same concentration in pure water. Katchalsky, Alexandrowicz, and Kedem (16) have shown that the osmotic pressures of ternary solutions of salts and polyelectrolytes obey a simple additivity rule, and that the total osmotic pressure may be obtained by adding up the separate contributions of each component.

A two compartment chamber, separated by a cellulose acetate membrane, was used to measure the osmotic pressure of a 3.7 millimolal (20%) hemoglobin solution in water. One compartment was filled with the hemoglobin solution, and the other with water. Increasing hydrostatic pressure, P, was applied to the hemoglobin solution, to balance the volume flow, J_v. J_v was plotted against P and the straight line obtained from four experiments is shown in Fig. 2. The method of extrapolation avoids the problem of the unstirred layer, which becomes progressively less important as J_v approaches

zero. When $J_v = 0$,

$$(P)_{J_v=0} = RT\Phi_{Hb}m_{Hb} = \pi_{Hb} \quad (2)$$

in which Φ_{Hb} is the osmotic coefficient of hemoglobin, m_{Hb} its concentration, and π_{Hb}, its osmotic pressure. P was found equal to 0.115 ± 0.005 atm. The osmotic coefficient was computed from this value and Adair's molecular weight (17) of hemoglobin of 66,800. Φ_{Hb} is 1.67 in excellent agreement with the value of 1.65 taken from Adair's data at this concentration (18).

In a second set of eight experiments, KCl was added to both compartments in an amount such that the molal salt concentration was equal on both sides.

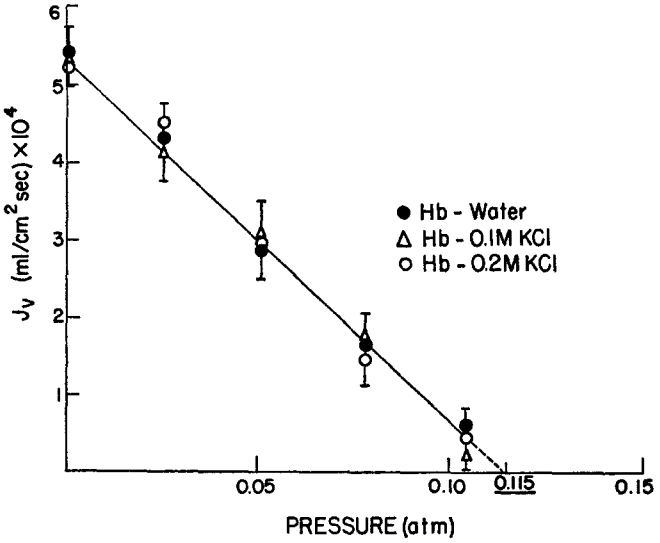

FIGURE 2. Osmotic pressures of 3.7 millimolal hemoglobin solutions in water and in the presence of potassium chloride, at 22°–24°C. The molalities of KCl solutions were equal on both sides of the membrane. The errors are standard errors.

Under these conditions the value of π_{Hb} would not be affected if all the water in the hemoglobin solution were solvent water for the salt. On the other hand, if any water in the hemoglobin solution was bound and could not serve as solvent water for KCl, the effective concentration of KCl would be greater in the hemoglobin compartment, and an additional osmotic pressure would develop, equal to

$$\pi_{KCl} = RT\Phi_{KCl}\sigma_{KCl}\Delta m_{KCl} \quad (3)$$

in which σ_{KCl} is the reflection coefficient of the membrane for the salt, and Δm_{KCl} the concentration difference between the two compartments.

Assuming that 0.4 g water/g Hb is bound, the effective salt concentration

in the hemoglobin solution would be 1.110 times the concentration in the protein-free compartment. For these membranes σ_{KCl} was found to be 0.187, so the additional osmotic pressure should be 0.081 atm at 0.1 M KCl and 0.160 atm at 0.2 M KCl. This would produce values for $(P)_{J_v=0}$ of 0.196 and 0.275 atm, compared to the 0.115 atm obtained in the absence of KCl. The

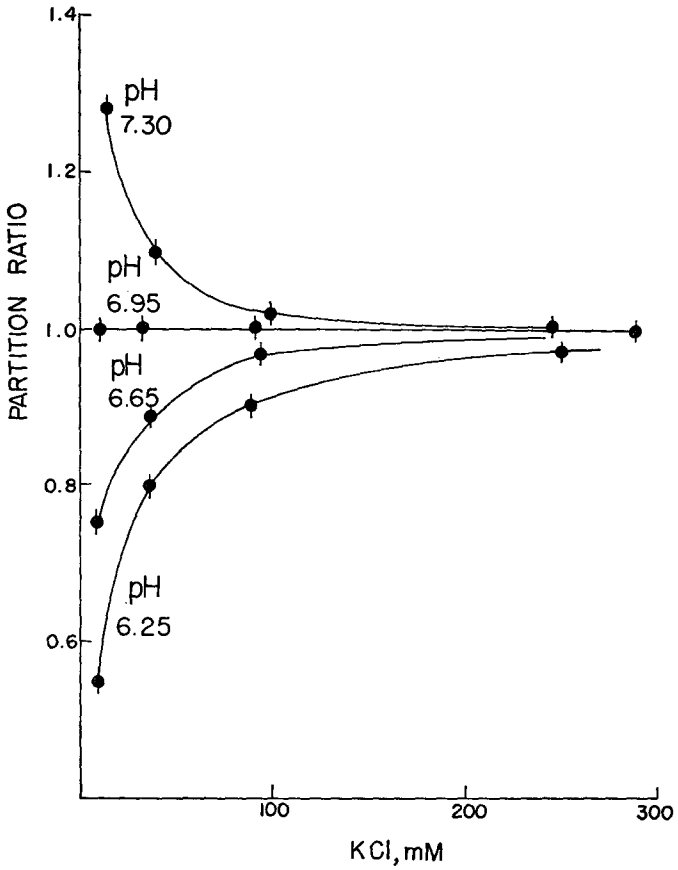

FIGURE 3. Partition ratio of potassium in 3.7 millimolal hemoglobin solutions as a function of KCl concentration.

superposition of points in Fig. 2 shows that there is no measurable difference under any of the experimental conditions. The standard error in the experiments with hemoglobin alone is 0.005 atm, so that a difference of 0.02 atm would be significant at the 1% level. Thus these results indicate that the salt–nonsolvent water is less than 0.1 g/g Hb.

Another experimental approach to the same question is provided by measuring the distribution ratios of NaCl and KCl in hemoglobin solutions by equilibrium dialysis across cellophane membranes. In these experiments,

the hemoglobin concentration was also 3.7 millimolal (20% ± 1% in different preparations of Hb) and the salt concentrations ranged between 10 and 250 mM. 12 experiments were carried out at 0°C, over a pH range from 6.0 to 7.4, using ^{42}K, ^{24}Na, and ^{36}Cl. The partition ratio, r, was computed in terms of counts per minute per kilogram of H_2O. The results obtained with potassium are given in Fig. 3; those with sodium are indistinguishable. The partition ratios agree fully with expectations based on the Gibbs-Donnan principle. Since we are concerned with salt–nonsolvent water in the protein solution, the results obtained at the isoelectric point, pH 6.95, are of major interest. At this point, r for NaCl and KCl is equal to 1.0 ± 0.005 instead of the value of 0.90, which would be expected if 0.4 g water/g Hb were nonsolvent for the salts. Also, as expected, the limiting value of r at high concentration is also unity. The results are therefore completely described by the classical equations from which the number of charges, z, of the hemoglobin molecule can be computed.

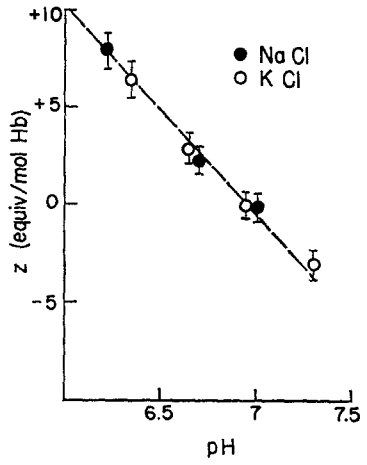

FIGURE 4. Net hemoglobin charge as a function of pH in sodium and potassium chloride solutions.

$$m''_+(m''_+ + zm_{Hb}) = m''_-(m''_- - zm_{Hb}) = m'_+m'_- \quad (4)$$

in which the protein side of the membrane is denoted by ″, m is the molality of cations (+) and anions (−), and z is the number of charges per mole of hemoglobin.

On both sides of the isoelectric pH, z increases by about 10 units per unit of pH, as shown in Fig. 4. These data are in reasonable agreement with the data obtained by Pauling et al. (19) from titration curves on human carbonmonoxy hemoglobin. These authors found a linear relation such that a shift by one pH unit in the neighborhood of the isoelectric point was associated with a net change of about 13 charges per molecule.

Effects of Red Cell Elasticity

The experiments of Rand and Burton (20) lead to the conclusion that red cell elasticity does not exert a significant force. These authors have shown the surface tension of the red cell membrane to be 0.02 dyne/cm and the pressure difference across the membrane to be only 2.3 mm H_2O both at normal osmolality and also when the red cell becomes spherical under hypo-osmolar conditions. Their experiments also indicate that a pressure of less than 2 mm H_2O is sufficient to distend the membrane, a negligible pressure in the present context.

Evaluation of Equation 1

The validity of equation 1 may be tested in experiments in which the midpoint of the range of osmotic excursion is shifted. If the water apparently unable to participate in osmotic phenomena is indeed bound water, it should either be constant in amount and independent of the midpoint osmolality in accordance with the suggestions of Perutz (6) and Drabkin (7), or it might increase with increased water activity, according to the law of mass action. The results of experiments to investigate this question are given in Table II. The volume fraction of apparent nonosmotic water is given in the fifth column and may be seen to increase as the osmolality increases. Part of this increase may be attributed to the shifts in cell volume. However, the results in the last column show that the effect persists even after expressing the results relative to the isosmolal cell volume. Thus, far from being constant, the apparent nonosmotic water increases with a decrease in water activity.

It is difficult to find a physical principle to account for such anomalous behavior. Instead we might examine equation 1 which provides the basis for interpreting the results in terms of apparent nonosmotic water. Empirically it is true that cell volume is linearly related to $1/\pi$ over limited ranges of osmolality. Furthermore, if the volume behavior followed our expectations from the van't Hoff relation, we could be justified in interpreting the slope in terms of the volume fraction of red cell water, and the intercept as the nonosmotic volume fraction. However, this justification disappears when the behavior is anomalous, and the interpretation of the slope of the line in terms of the fraction of water that apparently participates in osmotic phenomena, though widely accepted, is only an assumption. No direct experimental evidence exists which supports this assignment, and the experimental results in Table II raise serious doubts about its validity.

The inadequacy of equation 1 becomes even more apparent in the series of 12 experiments (on 6 different bloods) in which the effect of pH on nonosmotic water was examined. The results are given in Fig. 5, in which it will be

seen that the water volume of the cells changes smoothly in response to medium pH. On the contrary the apparent nonosmotic water shows a pronounced discontinuity around the hemoglobin isoelectric point. Indeed, at acid pH's the apparent osmotic water exceeds the total cell water. Since no physical explanation exists for such behavior, we conclude that equation 1 does not describe the system accurately, and that it is not possible to use the

TABLE II

RELATION OF APPARENT NONOSMOTIC WATER TO RED CELL VOLUME*

Experiment	Midpoint osmolality	Relative cell volume	Volume fraction of: Total cell water	Volume fraction of: Apparent non-osmotic water	Volume fraction of: Apparent non-osmotic water
	milliosmols/liter	*ml cell/(ml cells)$_{iso}$*	*ml/ml cell*	*ml/ml cell*	*ml/(ml cell)$_{iso}$*‡
1	231	1.170	0.762	0.109	0.127
	289	1.000	0.722	0.146	0.146
	331	0.962	0.707	0.138	0.133
2	247	1.083	0.752	0.116	0.126
	291	1.000	0.731	0.161	0.161
	341	0.918	0.702	0.176	0.162
3	192	1.424	0.795	0.080	0.114
	294	1.000	0.718	0.146	0.146
	390	0.881	0.680	0.280	0.246
4	194	1.333	0.790	0.070	0.093
	290	1.000	0.720	0.139	0.139
	395	0.836	0.665	0.240	0.200

* These computations have been made according to equation 1. If we consider equation 1 to be an empirical equation, it can be shown to be accurate if the experiments are referred to a single reference osmolality, π_i . Under these limited conditions, Savitz et al. (5) have shown equation 1 to be truly linear when based on an isosmotic reference, and the present results agree with their measurements in this specific situation. However, when the reference point is shifted to other osmolalities, the coefficients computed from equation 1, W_{eff} and b', do not remain constant but shift in value, as shown in the table. Under hypoosmotic conditions, the apparent nonosmotic water decreases leading to higher values for W_{eff} as discussed by Dick and Lowenstein (4) and Dick (21).

‡ (ml cell)$_{iso}$ refers to isosmolal cell volume.

slope of the line relating cell volume to π_o/π as a measure of the osmotic properties of red cell water.

Present Hypothesis

Our hypothesis had its origin in the observation that the hemoglobin molecules within the red cell are packed very closely together, virtually in a semicrystalline array. Furthermore, as Fig. 5 shows, there is a pronounced discontinuity

in osmotic behavior just at the isoelectric point of hemoglobin. Briefly our hypothesis suggests that there are cooperative phenomena between adjacent hemoglobin molecules in close packed systems which lead to a marked diminution of the effective charge on the hemoglobin molecule as the distance between adjacent molecules decreases.

The chain of reasoning follows. The experiments already described indicate that the activity of the water in the medium is equal to that in the cell. This follows from the distribution of nonelectrolytes between red cell and medium

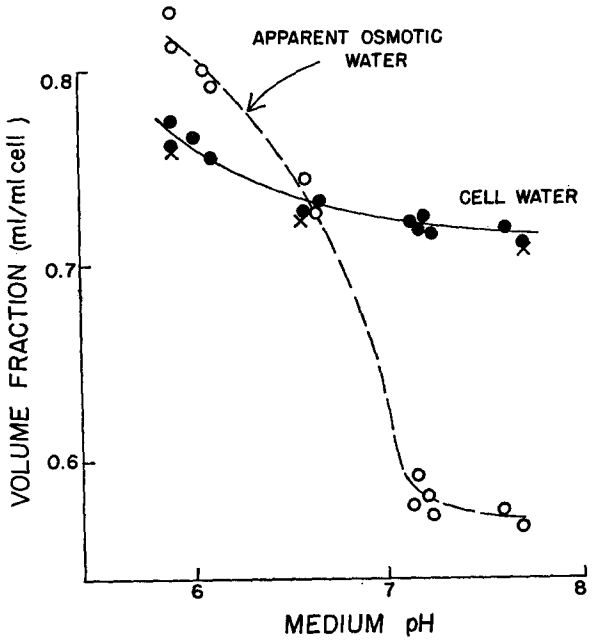

FIGURE 5. Effect of medium pH on the volume fraction of total cell water and apparent osmotic water. The filled circles are points obtained from the wet to dry weight ratio and the crosses are the distribution volumes of ethanol. The open circles are apparent osmotic water computed from equation 1.

and from the behavior of NaCl and KCl in concentrated solutions of hemoglobin in vitro. Therefore,

$$\mu_w' = \mu_w'' \tag{5}$$

in which μ_w denotes the chemical potential of water, $'$ refers to the medium, and $''$ to the cell. It follows that

$$\mu_w^{o'} + RT \ln a_w' = \mu_w^{o''} + RT \ln a_w'' \tag{6}$$

in which a is activity. Since the system is at constant temperature, and in view

of Rand and Burton's (20) demonstration that the pressure difference across the membrane is negligible, $\mu_w^{o\prime} = \mu_w^{o\prime\prime}$ and

$$\ln a_w' = \ln a_w'' \tag{7}$$

The osmotic coefficient is defined by

$$\ln a_w' \equiv -\frac{W_A}{1000}\sum_i \Phi_i m_i'$$

in which W_A is the molecular weight of water and Φ_i is the osmotic coefficient of the i*th* solute, whose molality is m_i . Hence

$$\sum_i \Phi_i m_i' = \sum_i \Phi_i m_i'' \tag{8}$$

The most important component in the medium is NaCl whose osmotic coefficient is 0.929 at 0.15 M. The osmotic coefficient for KCl is 0.92 at 0.15 M. At this concentration these osmotic coefficients are virtually independent of concentration, changing by less than 1.6% over the range from 0.1 M to 0.2 M. The osmotic coefficients for the other electrolytes, present in much smaller concentrations, do not differ markedly from these values. In consequence we have taken 0.92 for the osmotic coefficient of all electrolytes and treated this figure, which is denoted by Φ, as constant over the concentration range we have used. The nonelectrolytes such as glucose have been assumed to have an osmotic coefficient of 1. Therefore[2]

$$\sum_i \Phi_i m_i' = \pi'/RT = \Phi(m_+'' + m_{Mg}'' + m_-'' + m_p'') + \Phi_{Hb} m_{Hb} + m'' \tag{9}$$

in which m_+'' stands for the molality of the univalent cations, Na and K. The subscript, $-$, refers to all the univalent anions, and p to all the phosphate compounds including inorganic phosphate. m'' without subscript represents the molality of all the uncharged solutes, of which the principal one is glucose. Electroneutrality within the cell requires that

$$m_-'' + \nu_p m_p'' = z m_{Hb} + m_+'' + 2m_{Mg}'' \tag{10}$$

z is the net number of positive charges per mol of hemoglobin, as previously stated, and ν_p is the average charge per mol of phosphate compound. Within the cell, $m_i'' = n_i''/V_w d_w$ in which n is the number of moles and V_w is the volume of cell water of density d_w . Using equation 10 to eliminate m_-'' in equation 9

[2] No account is taken of the reflection coefficient in these equations since in human red cells σ is essentially 1 for all cations, glucose, phosphate compounds, and hemoglobin.

and solving for z, we find

$$z = (\Phi n_{Hb})^{-1}[\pi' V_w d_w/RT - \Phi(2n''_+ + 3n''_{Mg} + (1 - \nu_p)n''_p) - n'' - \Phi_{Hb} n_{Hb}] \quad (11)$$

The only variables on the right-hand side of the equation are π', V_w, and Φ_{Hb}. The constancy of the other terms may be inferred from the results of the solute leakage experiments of Savitz et al. (5), coupled with the present findings which show the cells to be impermeant to sodium over the time span of our experiments. In the experiments of Savitz et al. equal aliquots of human red cells were exposed for 40 min to electrolyte solutions of 72, 282, and 492 milliosmols whose composition was similar to the medium used in the present study. At the close of the 40 min period equal volumes of solutions of complementary osmolality (492, 282, and 72 milliosmolal) were added to the three aliquots and the cells were found to reach identical hematocrit values. If there had been any diffusion of uncharged solutes, the concentration gradient would have been greater in the shrunken cells than in the swollen ones and the cells would not have returned to the same volume. Human red cells of normal composition are also generally considered to be cation impermeable with respect to net fluxes over short time periods. This has been explicitly confirmed in the case of sodium as will be discussed below. The electroneutrality condition has been used to eliminate the cell anion concentrations so these do not enter explicitly into equation 11.

The osmotic coefficient of hemoglobin varies with concentration as has been shown by Adair (18). The most concentrated solution studied by him is 7.71 mM, slightly greater than the concentration in isosmolal human red cells. In order to extrapolate Adair's results to higher concentrations, his data were fitted to a second degree polynomial by least squares using a digital computer. The equation is

$$\Phi_{Hb} = 1.048 + 0.115\, m_{Hb} + 0.0119\, m_{Hb}^2 \quad (12)$$

The fit of the polynomial to Adair's data is shown in Fig. 6; our osmotic coefficients were obtained from this curve. V_w was measured by the difference between wet and dry weights, and the medium osmolality, π', obtained by freezing point depression as already discussed.

In order to calculate z from equation 11 it is necessary to specify all the solute concentrations in the human red cell at pH 7.4 under isosmolal conditions. The data for a representative red cell are given in Table III. To compute these figures it was first necessary to make sure that electroneutrality obtained in the cell. The total cation content is given in Table III. The diffusible monovalent anion concentration was computed from the 0.694 ratio (22) of

internal to external chloride concentration and the total monovalent anion concentration in the medium. The phosphate net charge was obtained as described in the footnote to Table III, and the net hemoglobin charge was then computed by difference to be −3.4 charges/molecule. This is somewhat smaller than the value of −4.7 at a cell pH of 7.44 from Fig. 4, a finding in

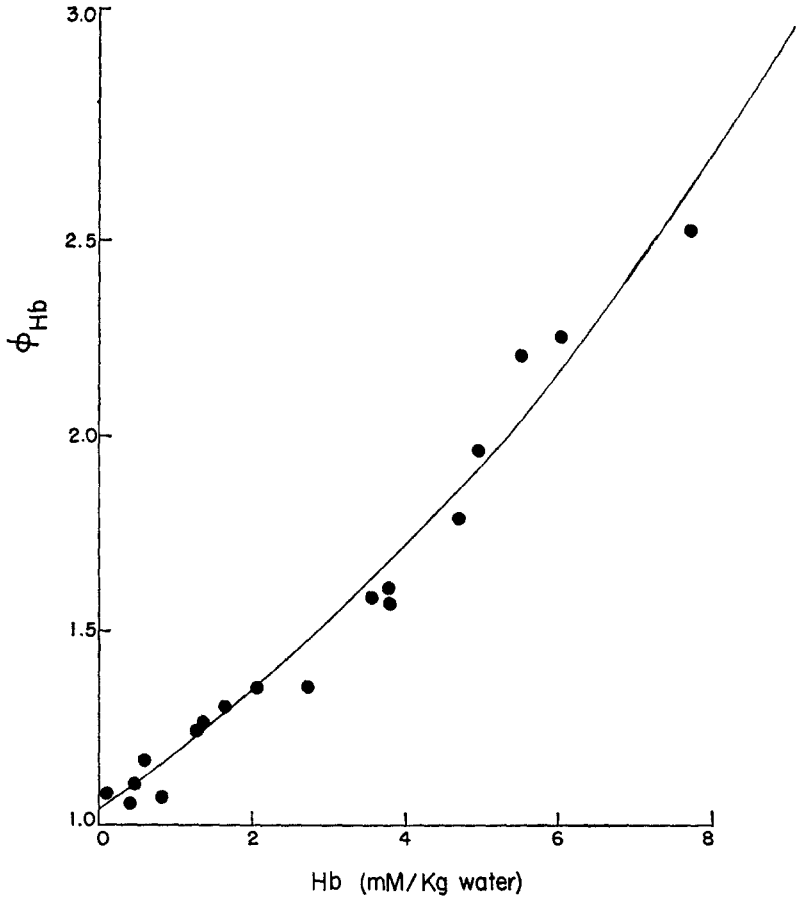

FIGURE 6. Adair's osmotic coefficient of hemoglobin fitted to a second degree polynomial by least squares.

accord with the hypothesis that increased hemoglobin concentration leads to a net charge decrease. When the osmotic contributions of all the components specified in Table III were added together, a deficit of 15 milliosmols/liter cell H_2O remained. To fill this gap we have introduced "other noncharged solutes" into the standard cell to provide the osmotic equilibrium which is characteristic of human red cells suspended in a 290 milliosmolal medium.

With these data it becomes possible to compute z quantitatively from the

measured values of cell volume and medium osmolality by equation 11. The results are given in the bottom part of Fig. 7 and it can be seen that the change of z is relatively slight in the more dilute solutions, increasing sharply as the concentration is increased above the isosmolal value. It is instructive to compare these values with the spacing between the hemoglobin molecules, as measured by x-ray diffraction. Although the actual distance between the

TABLE III
HUMAN RED CELL SOLUTES*

Solute	Mols/liter cell H_2O	Charge/mol	Osmotic coefficient	Total charge/ liter cell H_2O	Total mosmol/liter cell H_2O
Glucose	5.5	0	1.0		5.5
Other noncharged solutes	15	0	1.0		15
Na + K	155	1	0.92	+155	142
Mg	2.5	2	0.92	+5	2.3
Diffusible anions	108	−1	0.92	−108	99
Phosphate compounds	9.3	−3	0.92	−28	8.6
Hemoglobin	7	−4.7	2.44	−24	17
Total				0	289.4

* The sodium and potassium concentrations have been taken from the data of Bernstein (22) who also gives the figure of 0.694 for the ratio of cell chloride concentration to plasma chloride concentration. The cell glucose and magnesium concentrations are taken from Blood and Other Body Fluids (23). The concentrations of all the phosphate compounds in the cell have been measured by Bartlett (24). The values given in references 23 and 24 are expressed in terms of a range, and we have taken the midpoint of the range to be the average. At a medium pH of 7.4, cell pH may be computed to be 7.44, assuming that the chloride distribution ratio measured by Bernstein can be applied to hydroxyl ions. The numbers of negative charges on the phosphate groups have been taken, in general, from the reactions given by Krebs and Kornberg (25) which were specified at pH 7.5. These lead to values of 4 negative charges for ATP, 3 for ADP and 2,3-diphosphoglycerate, and 2 for each remaining phosphate group. The hemoglobin concentration is computed from the hemoglobin content of 33 g/ 100 ml cells (23), the cell water content of 0.717 given by Savitz et al. (5), and the hemoglobin molecular weight of 66,800 given by Adair (17). The osmotic coefficient for hemoglobin was taken from Fig. 6.

molecules depends on the model used in interpreting the x-ray data, the Bragg spacing provides an accurate index of changes in the spacing. This spacing has been measured by Bateman et al. (26) in suspensions of red cells and the data have been found to agree with results obtained by Riley and Herbert (27) in concentrated hemoglobin solutions. Riley and Herbert considered hemoglobin to be a cylinder, 57 A in diameter, and computed center to center distances of 79 A in 8.5 millimolal solution and 95 A in 5.2 millimolal solution. The respective channel widths are 22 A and 38 A, and the tightness of the packing may be inferred by comparison with the 15 A channel width

in crystalline horse methemoglobin (6). The data of Bateman et al. have been recomputed to cast them in the form shown in the top portion of Fig. 7. When the molecules are relatively far apart, the effect of concentration on net hemoglobin charge is relatively slight. As the solution becomes more concentrated the Bragg spacing decreases but much more slowly, presumably because of steric and electrical effects between the molecules whose distance apart is very little more than that in crystalline array. Nonetheless the x-ray

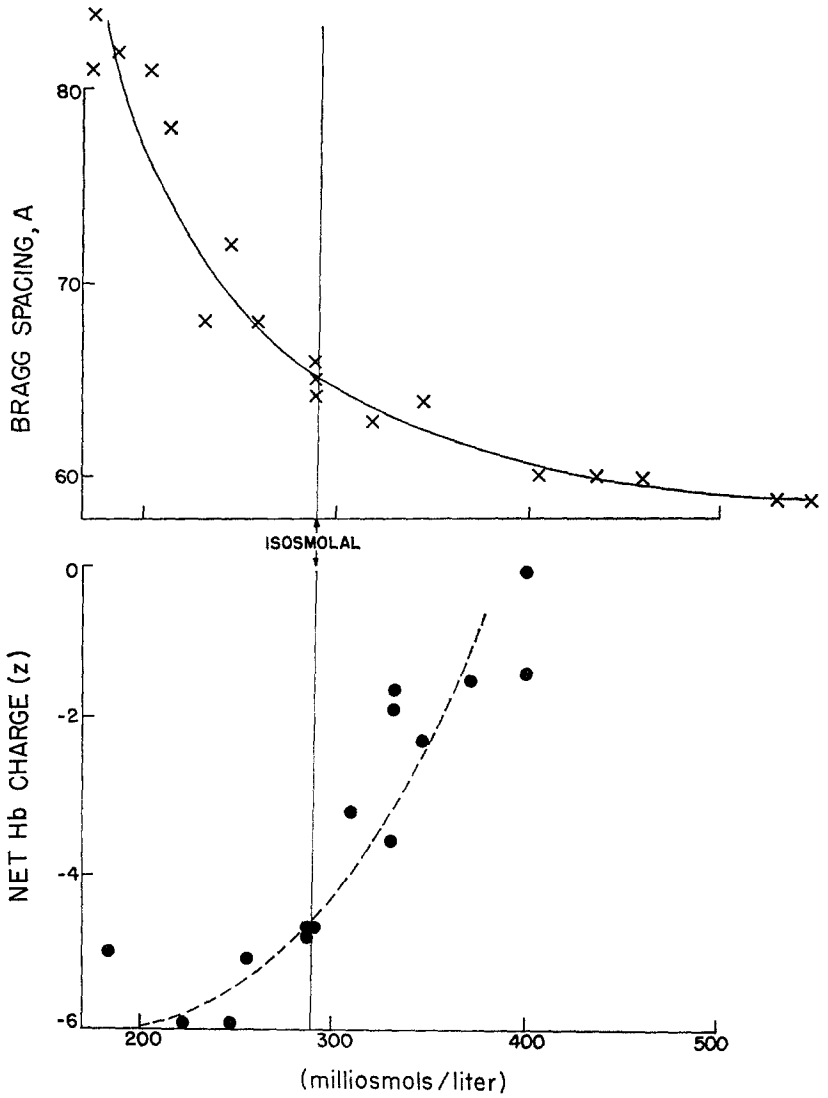

FIGURE 7. Top curve, Bragg spacing of hemoglobin molecules in red cells as a function of osmolality (computed from the data of Bateman et al. [26]). Bottom curve, hemoglobin net charge, z, computed from equation 11.

pattern is not that of a crystalline array and the molecules may still rotate freely. As close packing increases in the hyperosmolal region the effect on z is proportionately greater, presumably as a consequence of increased hemoglobin-hemoglobin interaction as the molecules get very close to one another.

Adair (18) measured the distribution of ions at pH 7.8 across a collodion sac separating two solutions of 0.165 M phosphate, one of which contained hemoglobin. Since the hemoglobin concentration was varied from 0.1 to 7.7 millimolal in these studies, we should expect to find evidence in Adair's results of the concentration effect on net hemoglobin charge. Adair computed the predicted ion accumulation in the hemoglobin solution from an equation based on a constant value for z; this computed accumulation was 40% greater than the one actually observed as determined from the measured potential difference and the equation for the Gibbs-Donnan ratio. It is very difficult to make a quantitative comparison of Adair's results with ours, because the systems are different and the assumptions also differ. It is clear, however, that the discrepancy Adair found between measured and observed ion distribution is in qualitative agreement with the present view that the net hemoglobin charge decreases as the hemoglobin concentration increases.

Effect of Medium pH on Hemoglobin Net Charge

The effect of medium pH on osmotically induced changes in hemoglobin net charge reveals several particularly interesting aspects of the process. Fig. 8 shows the values of z, computed by equation 11, as functions of osmolality and pH. Decreases in π_o/π cause a decrease in net *positive* charge below pH 6.6; above pH 7.2, decreases in π_o/π cause a decrease in net *negative* charge. Though there is a decrease in charge in both instances, the derivative, $dz/d(\pi_o/\pi)$, changes sign about the isoelectric point. This is clearly related to the discontinuity in Fig. 5.

In the pH range of 7.20 to 7.70, there appear to be instances in which the calculated net hemoglobin charge crosses zero and changes in sign. The zero on the scale is set by the hemoglobin net charge, as computed in Table III under isosmolal conditions. We are not of the opinion that this is a real phenomenon but rather that it is another reflection of the cooperative processes among tightly packed hemoglobin molecules, which have the effect of shifting the isoelectric point to a lower pH. This is equivalent to an apparent shift by a few units in a positive direction on the charge scale in Fig. 8.

Further evidence bearing on this point is provided in Fig. 9, which shows the relation between the medium pH and the degree of ionization, here expressed in units of net hemoglobin charge. The points have been obtained from the curves in Fig. 8 at values of π_o/π of 1.3, 1.0, and 0.75. The top portion of the figure compares the data at the lower osmolalities with the dashed line taken from the in vitro results presented in Fig. 4. The points fall very close

to the line, indicating that there is relatively little shift in the isoelectric point, and that the pH within the cell is very little different from that in the medium. This is to be expected since the diffusion potential, which is expressed by the $(Cl)_{in}/(Cl)_{out}$ ratio of 0.695, is equivalent to a pH shift of only 0.04 unit.

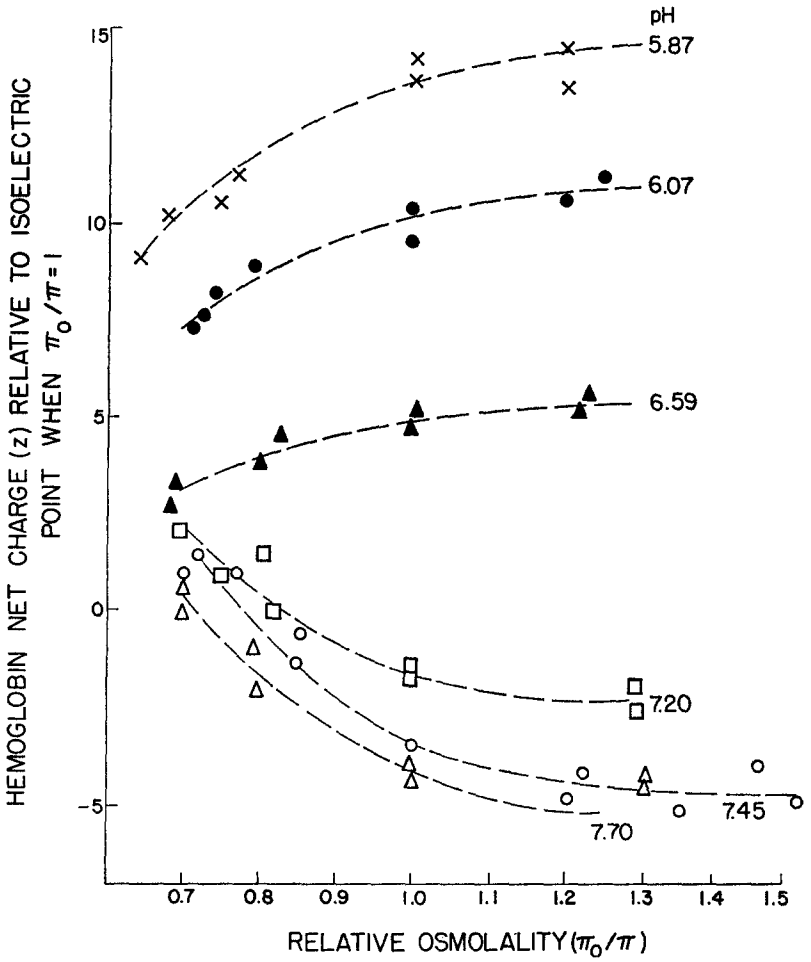

FIGURE 8. Effect of medium osmolality on the net hemoglobin charge at different pH's. The zero net charge is set by the hemoglobin net charge given in Table III under isosmolal conditions.

However, as the hemoglobin concentration is increased, the curve increases in steepness and shifts to the right, corresponding to a positive shift in the isoelectric point, as already deduced from Fig. 8.

A similar phenomenon has been observed in the titration of polyelectrolytes by Katchalsky, Mazur, and Spitnik (28). In this instance, the polymer titration curve grows steep as the isoelectric point is approached, much steeper

than is the case for the monomer, and the buffer capacity is reduced, similar to our observations in red cells. Katchalsky et al. interpret their findings as evidence of associative forces between the units in the polyelectrolyte chain which cause the release of protons to the solution. In their studies, increases

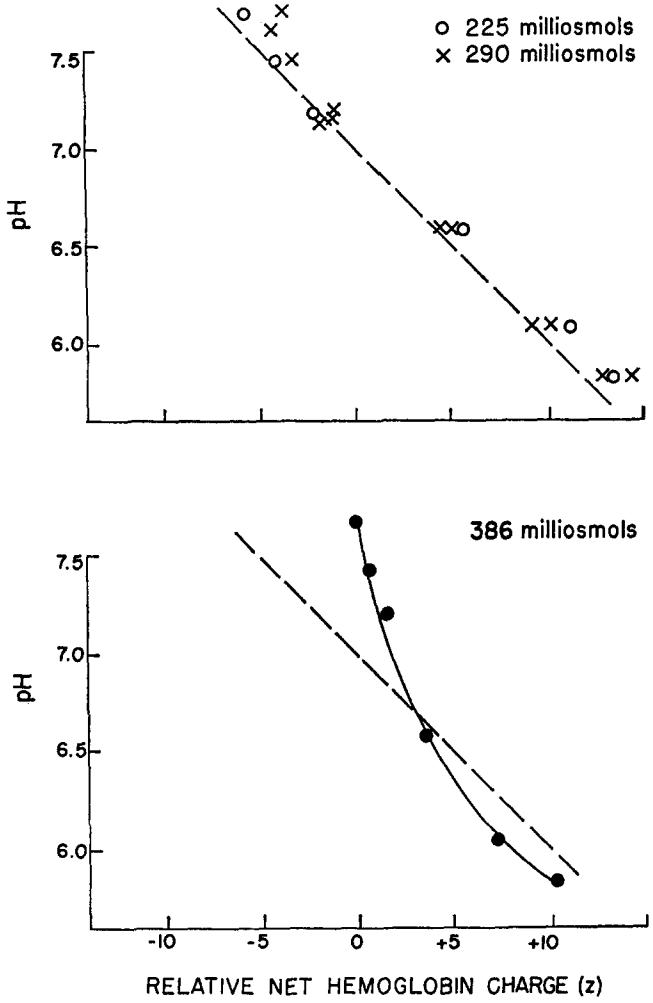

FIGURE 9. Relationship between medium pH and the degree of ionization of the hemoglobin molecule as expressed in terms of net hemoglobin charge.

in polymer concentration did not cause an increased slope of the curve, which was interpreted as evidence for cooperative interaction between separate monomers along the chain. In our studies, the effect appears to be entirely concentration dependent, which we attribute to cooperative interactions between the hemoglobin molecules.

The slopes, $dz/d(\pi_o/\pi)$, computed graphically from the curves in Fig. 8, are shown in Fig. 10 as a function of pH at the same three osmolalities used for Fig. 9. At 225 milliosmols/liter, $dz/d(\pi_o/\pi)$ is not very different from zero and virtually independent of pH. This is consonant with the observation that the

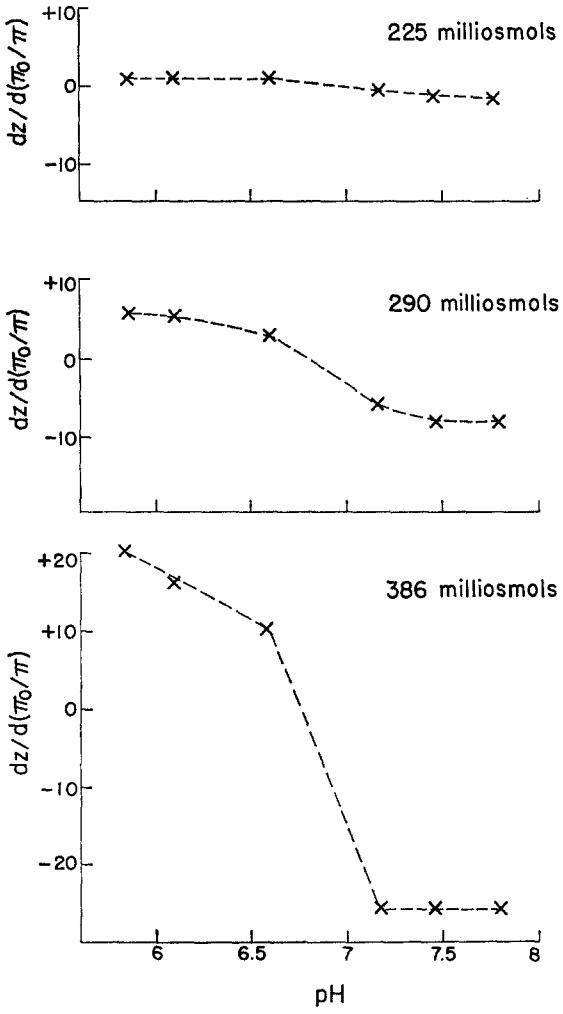

FIGURE 10. The rate of change of hemoglobin net charge with π_o/π, $[dz/d(\pi_o/\pi)]$, as a function of pH at three osmolalities. The data are obtained graphically from Fig. 8.

osmotic behavior of red cells approaches the ideal in dilute solutions, since $dz/d(\pi_o/\pi)$ is the cause of the apparent anomalous osmotic behavior. Under isosmolal conditions the slope is very much larger and there is a discontinuity around the isoelectric point for hemoglobin, tendencies which are much more apparent as the concentration is increased to 386 milliosmols/liter and the hemoglobin molecules approach one another much more closely. This con-

centration dependent increase of the discontinuity may be interpreted as further evidence of cooperative phenomena between hemoglobin molecules.

Detailed Analysis of Mechanism

The results that have been presented indicate that the charge on the hemoglobin molecule depends upon its concentration as well as the ambient pH. Increases in hemoglobin concentration lead to a decrease of the net charge at all pH's independent of the sign of the charge. At low pH when the hemoglobin net charge is positive, the reaction may be written schematically as

$$n(\mathrm{Hb}\cdot x(\mathrm{NH_3^+})) \rightleftarrows (\mathrm{Hb}\cdot x(\mathrm{NH_2}))_n + nx\ \mathrm{H^+} \tag{13}$$

assuming, for the sake of simplicity, a condition in which the net positive charge is reduced to zero. For the reaction to proceed to the right, it is necessary for the free energy of the products to be less than that of the reactants, which implies an associative force between closely apposed hemoglobin molecules. Since a high concentration of hemoglobin and a very small intermolecular spacing are necessary for the effect to become apparent, it would appear that the forces are of a relatively short range.

Two possible associative forces come to mind. The most likely would be dipole-dipole interactions between the hydrophilic side chains of the hemoglobin molecules. Another might be decreases in electrostatic free energy resulting from the entropy term as proposed by Parsegian (29) to account for the spacing between phospholipid bilayers. These forces are related to the flexibility of the polar groups and appear to be operative over spacings from 10 to 30 A in very dilute solutions.

The top portion of Fig. 9 shows that the cell pH is relatively unaffected by changes in osmolality near the isosmolal region. In consequence the hydrogen ion produced by the associative reaction should be expected to diffuse out of the cell. However, Jacobs and Parpart (30) have shown that the erythrocyte is impermeable to hydrogen ions and that apparent hydrogen diffusion is mediated by the exchange of hydroxyl ions. Consequently the hydrogen ion is neutralized by an external hydroxyl ion. To preserve electroneutrality, it is necessary for an internal anion to exchange with the hydroxyl. In the experiments to be discussed in which chloride movements were measured, chloride was the only permeant anion present in appreciable concentration, so that

$$nx\ \mathrm{Cl^-_{in}} \rightleftarrows nx\ \mathrm{Cl^-_{out}} \tag{14}$$

The sum of these several reactions at low pH is given by

$$n(\mathrm{Hb}\cdot x\,(\mathrm{NH_3^+})) + nx\ \mathrm{Cl^-_{in}} + nx\ \mathrm{OH^-_{out}} \rightleftarrows (\mathrm{Hb}\cdot x\,(\mathrm{NH_2}))_n + nx\ \mathrm{Cl^-_{out}} + nx\ \mathrm{H_2O} \tag{15}$$

which causes a net change in the osmolality of the cell. At an alkaline pH, e.g. 7.4, the reaction would be

$$n(\mathrm{Hb}\cdot y\,(\mathrm{COO^-})) + ny\ \mathrm{Cl^-_{out}} + ny\ \mathrm{H_2O} \rightleftarrows (\mathrm{Hb}\cdot y\,(\mathrm{COOH}))_n + ny\ \mathrm{Cl^-_{in}} + ny\ \mathrm{OH^-_{out}} \quad (16)$$

The overall sum of this sequence of steps is a chloride-hydroxyl exchange resulting from a shift in the net hemoglobin charge. Since water is free to move in this system, it distributes itself according to osmotic gradients and comes to equal chemical potential on both sides of the membrane. Thus the apparent anomalous osmotic behavior of the cell is the result of counterion movement as a consequence of changes in the net hemoglobin charge.

Effect of Cell Volume Changes on Sodium and Chloride Movements

The prediction of a chloride shift can be tested directly by the double labeling technique which makes it possible to avoid protein precipitation as discussed in the methods section. The results of experiments at pH 7.4 and 6.1 shown in Fig. 11 are typical of the four experiments in this series. The chloride shifts are of considerable magnitude, and the direction is as expected. When the hemoglobin is negatively charged, at pH 7.4, increases in hemoglobin concentration cause a reduction of charge, and chloride enters the cell as counterion for the cations, whose charge had previously been neutralized by the hemoglobin. When the hemoglobin is positively charged, at pH 6.1, increased concentration causes a decrease in positive charge, so that the chloride counterion is free to diffuse out of the cell.

Cook (31) had previously suggested, on an entirely different basis, that net chloride movement might be associated with red cell volume changes. The chloride fluxes that he measured were very much smaller than ours because Cook precipitated the red cell proteins, a process which irreversibly alters the state of charge of the hemoglobin molecule. Hence such experiments cannot reveal the change in z that is associated with changes of the hemoglobin concentration in the red cell.

Sodium movements and pH shifts were also measured in the same series of experiments and the results are given in Table IV. As expected, there is no net sodium movement (see column five) which verifies the assumption used in equation 11 in this respect. At pH 7.4 the computed hydroxyl movement is only about half of the measured chloride movement and the difference is even greater at pH 6.1. The discrepancy may, in part, be attributed to the buffer capacity of the red cell membrane, primarily due to the protein since the sialic acid concentration (32) is too small by three orders of magnitude to contribute effectively. The protein constituents in the membrane amount to about 20 g/kg cells. Assuming that the amino acids have an average molecular weight of 250, this would lead to an amino acid concentration of 80 mmole/kg

cells which would increase the buffer capacity of the medium by about 9 mM/liter cells. Cook also found a relative pH change of about the same size as ours. However, his system was not buffered and his conditions of measurement were different from ours. In strongly buffered systems, which we used to

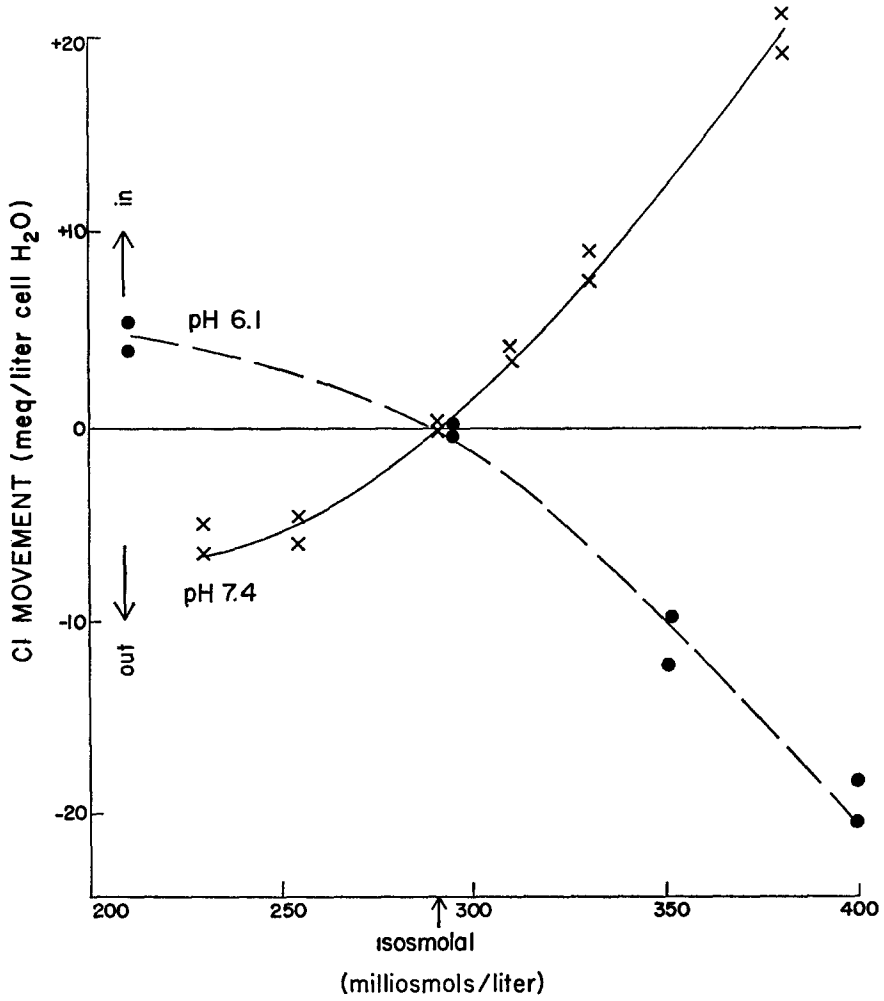

FIGURE 11. Net chloride movements in red cell as a function of osmolality and pH.

maintain the medium pH approximately constant, pH measurements are very difficult, and experiments of a different type would be required to eliminate all possible sources of error in our measurements.

Quantitative Evaluation of Theory

The data on the net chloride movement may be used for a quantitative check of the predictions of the theory as given in equation 11. Osmotically induced

changes in z have been computed at pH 7.4, relative to the value of z at π_o. When these changes are multiplied by the hemoglobin content of the cell, they give the net change in charge in milliequivalents per liter of cell H_2O which is plotted in Fig. 12. The net chloride movement at this pH has been measured in two experiments, including the one shown in Fig. 11, and these data are also plotted in Fig. 12. It can be seen that the agreement between theory and experiment is quantitative both when the cell swells and when it shrinks.

In consequence, it appears that there are no anomalous aspects in the osmotic behavior of human red cells; the apparent anomalies that have been previously observed may be entirely attributed to changes in the net hemoglobin charge. As the cells swell at pH 7.4, reaction 16 proceeds to the left, the net hemoglobin charge increases, and anions move out to preserve electroneutrality, followed by water to maintain osmotic equilibrium. As a result

TABLE IV

IONIC SHIFTS IN OSMOTIC EXPERIMENTS

pH	Medium osmolality	$^{36}Cl/^{131}I$-Albumin	Net Cl influx	$^{24}Na/^{131}I$-Albumin	Net Na influx	Net OH efflux
	millosmols/liter H_2O		*meq/liter cell*		*meq/liter cell*	*meq/liter cell*
6.04±0.01	290	1.00±0.01		1.00±0.01		
6.21±0.01	370	1.19±0.01	−18±1	1.01±0.01	−1±1	−6±2
7.45±0.01	290	1.00±0.01		1.00±0.01		
7.31±0.01	370	0.84±0.01	+20±1	1.00±0.01	0±1	+10±2

the change in red cell volume is less than would have been expected if there had been no change in net hemoglobin charge. Conversely, when the cell shrinks, the hemoglobin net charge decreases and the cell shrinks less than would have been the case had there been no change in net hemoglobin charge.

This quantitative agreement between theory and experiment provides further information about the solvent properties of water. The water that may be bound to hemoglobin is solvent water for both electrolytes and nonelectrolytes. No anomalous properties of water have been detected in our study.

Another important result of these investigations is the demonstration that no quantitative information about the osmotic properties of water may be obtained from the linear relationship of cell volume to π_o/π. It is clear that the possibility of cooperative interactions between macromolecules or other polyelectrolytes may not be neglected in studies of their behavior in solution. In consequence the van't Hoff law cannot be expected to apply to concentrated solutions, such as are commonly found in cell interiors.

A general condition for survival of all cells without walls is the preservation

of their integrity by the control of cell volume. In the present study we have demonstrated a striking effect of protein concentration upon net protein charge, which serves to translate an osmotic effect into an electric signal. Such an osmotic transducer would be well-suited to govern the regulation of cell volume and may be widely operative since the apparent osmotic behavior of the red cell is representative of a variety of tissues. For example Dydyńska and Wilkie (33) and Blinks (34) have shown that the water in striated muscle does not behave as an ideal van't Hoff osmometer. Furthermore the departure

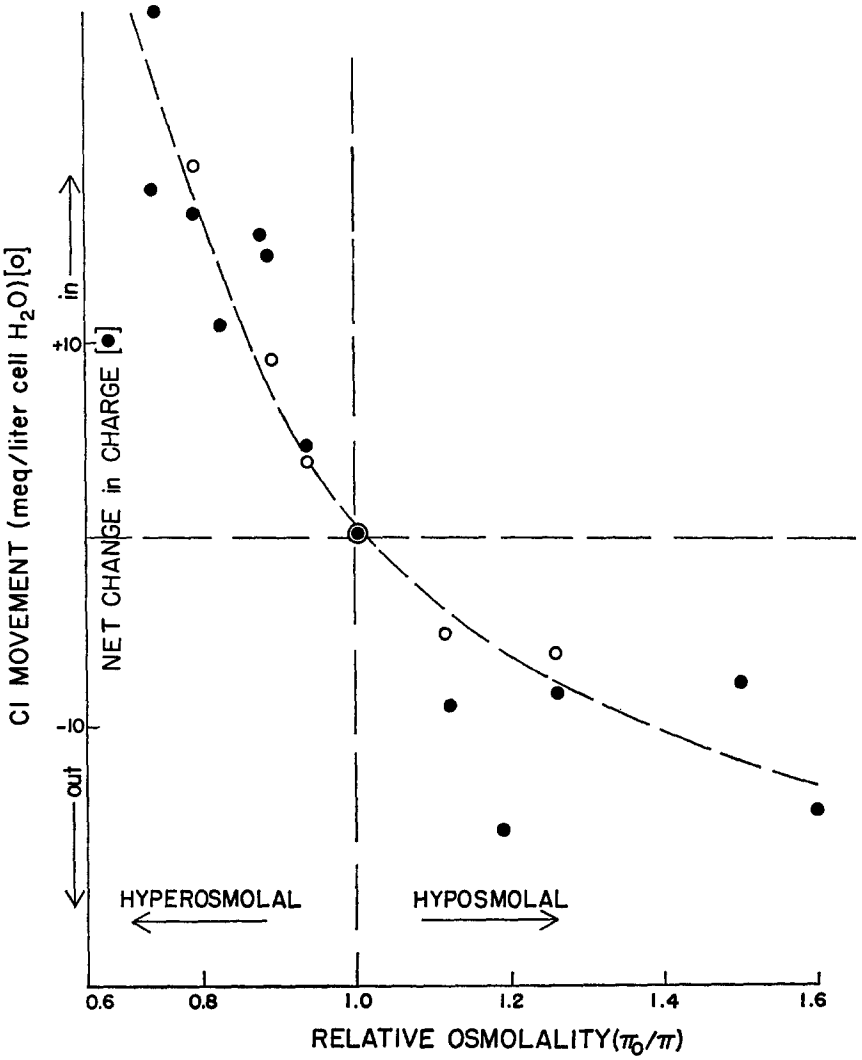

FIGURE 12. Correlation between osmotically induced change in net hemoglobin charges (filled circles) computed from equation 11, and the measured chloride movement (open circles) expressed in the same units. pH = 7.4.

from ideal behavior becomes particularly apparent at the higher osmolarities as the concentration of the muscle polyelectrolytes becomes much greater. Bentzel and Solomon (33) have observed similar osmotic behavior in mitochondrial inner compartments which contain almost all the mitochondrial enzymes. In this case the amount of apparent nonosmotic water is 0.3 g/g dry weight, similar to that in the human red cell. In spinach chloroplasts, Dilley and Rothstein (36) have also observed apparent anomalous osmotic behavior. They have not only demonstrated apparent nonosmotic water but have also shown that the chloroplast volume is related to the internal concentration of fixed charges. Though Dilley and Rothstein have attributed the change in internal charge to changes in internal pH, it is likely that the chloroplast mechanism is closely related to that in red cells. Thus osmotic responses are very similar in a wide variety of tissues, and it is probable that the present explanation of the physical basis for the process in one tissue may be generally applied to others.

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