J. Membrane Biol. 91, 259–273 (1986)

The Journal of
Membrane Biology

Osmotic Properties of Human Red Cells

A.K. Solomon, Michael R. Toon, and James A. Dix*
Biophysical Laboratory, Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115

Summary. When an osmotic pressure gradient is applied to human red cells, the volume changes anomalously, as if there were a significant fraction of "nonosmotic water" which could not serve as solvent for the cell solutes, a finding which has been discussed widely in the literature. In 1968, Gary-Bobo and Solomon (*J. Gen. Physiol.* **52**:825) concluded that the anomalies could not be entirely explained by the colligative properties of hemoglobin (Hb) and proposed that there was an additional concentration dependence of the Hb charge (z_{Hb}). A number of investigators, particularly Freedman and Hoffman (1979, *J. Gen. Physiol.* **74**:157) have been unable to confirm Gary-Bobo and Solomon's experimental evidence for this concentration dependence of z_{Hb} and we now report that we are also unable to repeat the earlier experiments. Nonetheless, there still remains a significant anomaly which amounts to 12.5 ± 0.8% of the total isosmotic cell water ($P \ll 0.0005$, t test), even after taking account of the concentration dependence of the Hb osmotic coefficient and all the other known physical chemical constraints, ideal and nonideal. It is suggested that the anomalies at high Hb concentration in shrunken cells may arise from the ionic strength dependence of the Hb osmotic coefficient. In swollen red cells at low ionic strength, solute binding to membrane and intracellular proteins is increased and it is suggested that this factor may account, in part, for the anomalous behavior of these cells.

Key Words red cell · nonosmotic water · osmotic coefficient · hemoglobin · cell swelling

Introduction

Ponder's great monograph (1948) provided a coherent summary, probably the first, of the osmotic properties of the human red cell. Ponder pointed out that a significant fraction of the red cell water behaved anomalously, as if it were segregated and could not serve as solvent for the cell solutes, a finding that has been discussed by many others including Savitz, Sidel and Solomon (1964), Cook (1967), and Dick (1967, 1969). In 1968, Gary-Bobo and Solomon concluded that the apparent anomalous properties of human red cell water could not be explained entirely by the colligative properties of hemoglobin (Hb) and proposed that the explanation lay in an additional concentration-dependent change in the Hb charge (z_{Hb}). A number of subsequent papers have not been able to confirm the concentration dependence of z_{Hb}, particularly those of Gros et al. (1978), Freedman and Hoffman (1979), and Hladky and Rink (1978), and we now report that we have also failed to confirm it. Freedman and Hoffman have modified the Jacobs and Stewart (1947) equations for the dependence of red cell volume on osmolality to incorporate nonideal thermodynamic parameters and report that their equations fit the osmotic shrink-swell characteristics of human red cells (with an average error of 2.4 ± 0.9%, based on differences in single points) when the colligative properties of Hb are computed from the osmotic coefficients of Adair (1929).

We have made further detailed studies of the shrink-swell behavior of the red cell and find significant differences between the modified Jacobs-Stewart theory and experiment in the "apparent nonosmotic water," which is determined by the slope of the line relating cell volume to inverse osmolality. The slope differs from theory by 12.5 ± 0.8% ($P \ll 0.0005$, t test). Since the osmotic behavior of the red cell does not conform to the predictions of the nonideal thermodynamic equations, after taking exact account of established nonidealities of osmotic and activity coefficients, other physical chemical factors must play a role in the process. We have found that corrections based on the dependence of the Hb osmotic coefficient on ionic strength in concentrated solutions significantly reduce the discrepancy at high Hb concentrations. In dilute solutions, binding of ions to red cell proteins, including Hb, and to the membrane also contribute to a reduction in the discrepancy.

* *Present address:* Department of Chemistry, State University of New York, Binghamton, NY 13901

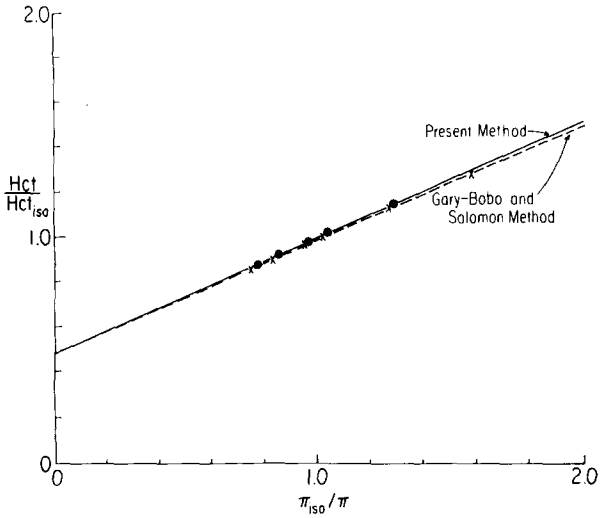

Fig. 1. Comparison of the present method (●) of determining hematocrit with that used by Gary-Bobo and Solomon (1968) (x)

Materials and Methods

Blood

Whole human blood was obtained by venipuncture from healthy adult volunteers and collected into heparinized flasks (10 units heparin/ml whole blood). The plasma and buffy coat were removed by centrifugation and aspiration. The packed cells were then washed in one of three isosmolar buffers: (i) a standard buffer containing (in mM) 117.8 NaCl, 4.4 KCl, 19.5 Na_2HPO_4 + NaH_2PO_4, 0.5 $MgCl_2$, 1.2 $CaCl_2$, and 0.5% bovine serum albumin, pH 7.4, 290 mOsm; (ii) a 50-mM phosphate buffer, similar to the one used in the Cl^- flux experiments of Gary-Bobo and Solomon, containing (in mM) 50 sodium phosphate, 96 NaCl, pH 7.4, 290 mOsm; or (iii) a HEPES buffer containing (in mM) 150 NaCl, 5 KCl, 10 HEPES, pH 7.4, 0.5% bovine serum albumin, 290 mOsm. The osmolalities were measured in a Fiske model OS osmometer (Uxbridge, MA).

Chemicals

$H^{36}Cl$, ^{14}C-methoxyinulin and Biofluor were obtained from New England Nuclear (Boston, MA). All other chemicals were reagent grade and obtained from Fisher Scientific (Pittsburg, PA) or Sigma Chemical Co. (St. Louis, MO).

Osmotic and Gravimetric Water Measurements

Cells washed in isosmolar standard buffer were resuspended to 0.8 hematocrit. 5.0-g samples of this cell suspension were added to pretared 40-ml centrifuge tubes. Each sample was washed five times in 10 volumes of buffer of the desired osmolality and pH. The osmolality was adjusted by varying the NaCl content of the isosmolar standard buffer described above; typically, six osmolalities in the range 200–400 mOsm were prepared. The pH was adjusted by adding varying amounts of NaH_2PO_4 and Na_2HPO_4 while keeping the total phosphate constant at 19.5 mM. The cells were washed by centrifugation at 3600 × *g* for 6 min. After the centrifugation, the supernatant was removed carefully so that no cells were lost. At the end of the fifth wash, the six samples were resuspended to the same final weight of cells plus buffer to give an isosmolar hematocrit of approximately 0.4. Hematocrits were determined in quadruplicate by centrifugation at 5000 × *g* for 30 min in microhematocrit tubes (Fisher, Pittsburgh, PA). Hematocrit measurements agreed to better than 0.5%. Gravimetric water was determined by the method of Savitz et al. (1964).

In each series of experiments on the effect of osmolality and pH on cell volume, the total number of cells per unit volume remained constant. Therefore, neglecting the small trapped volume correction (Savitz et al., 1964), the hematocrit is proportional to the cell volume at a given osmolality and pH. We have used the hematocrit at 290 mOsm, pH 7.4, in order to express all hematocrits as cell volumes relative to that cell volume. The hematocrit at 290 mOsm, pH 7.4, was determined from a linear, least squares fit of the hematocrit data at pH 7.4 to inverse osmolality. At each pH, the osmotic water W'_{eff}, and the remaining volume, which includes apparent nonosmotic water, solute volume and membrane volume, was calculated as

$$\frac{h}{h_{290,7.4}} = W'_{eff}\frac{290}{\pi} + b'$$

where h is the hematocrit at osmolality π, and $h_{290,7.4}$ is the hematocrit at 290 mOsm, pH 7.4. As will be shown later, the present values of W'_{eff} agree excellently with those of Savitz et al. (1964) which shows that the trapped volume correction has no effect on the determination of W'_{eff}.

In order to compare the osmotic pressure dependence of relative cell volumes with those predicted by the nonideal Jacobs-Stewart theory, as modified by Freedman and Hoffman (1979), the cell volume must be measured at low hematocrit. In our experiments the cells were washed five times in 10 volumes of the shrink/swell buffer, which fixes the suspending buffer concentrations and thus gives an effective hematocrit on the order of 10^{-5}. We have compared our hematocrit measurements with hematocrit measurements on the same blood done by the method used by Gary-Bobo and Solomon (1968). The results in Fig. 1 show close agreement between the two methods.

Effect of Hemoglobin Oxygenation State

Cells washed in 50 mM phosphate buffer were brought to roughly 40% hematocrit with 50 mM phosphate buffer. Aliquots of the red cell suspension were then placed in an ice bath and had the oxygenation state of their hemoglobin adjusted by exposure to O_2, N_2 or CO. These gases were bubbled through the red cell suspension for 30 min using stone bubblers to provide for maximal contact between the gas and the solution. After the exposure to various gases the standard shrink/swell experiment was performed. Samples of the cell suspension were mixed with various shrink/swell buffers (50 mM phosphate buffer, pH 7.4, NaCl to provide the desired osmolality) and allowed to equilibrate at room temperature for 15 to 30 min. In this series of experiments the gaseous atmosphere was maintained during this equilibration period. Aliquots were then removed for determinations of hematocrit, as above, and the remainder of each sample was centrifuged at top speed in a clinical centrifuge for 30 min and the osmolality of the supernatant determined.

Chloride Concentration Measurements

Experiments at the Same Hematocrit in Hyposmolar Buffer

Cells were washed five times in 10 volumes of isosmolar HEPES buffer, pH 7.4. Cells were then washed an additional five times with the same buffer containing 0.1 μCi/ml $H^{36}Cl$ and 0.1 μCi/ml ^{14}C-methoxyinulin. Buffers were made hyposmolar (or hyperosmolar) by varying the [NaCl] alone. Addition of $^{36}Cl^-$ was made to each buffer so as to maintain a constant specific activity relative to the radioactive isosmolar buffer, and pH was readjusted if necessary. After the 5th isosmolar wash in labeled buffer, 2.0 ml aliquots of packed cells were transferred by volumetric pipette into pretared centrifuge tubes, and adjustment was made, if necessary, so that equal weights of cells were added. Eight ml of radioactively labeled isosmolar buffer (290 mOsm) or hyposmolar buffer (190 mOsm) was then added, the tubes were vortexed and samples taken for hematocrit (in sextuplicate) and hemoglobin determination. ^{14}C inulin was used in these experiments to correct for trapped plasma. Hemoglobin was determined by lysing 0.1 and 0.2 ml aliquots (in duplicate) in 5.0 ml of Drabkin's solution (Fisher Scientific) in volumetric flasks, and measuring at 540 nM. One-ml aliquots of each suspension were then transferred in sextuplicate to 1.5 ml microfuge tubes (Sarstedt, Princeton, NJ) to which 0.5 ml of dibutyl phthalate had been added. The samples were centrifuged for 5 min at 10,000 × *g* in an Eppendorf Microfuge Model 3200 (Brinkman Instruments, Westbury, NY). 0.2 ml samples of the supernatant were added to 2.5 ml Biofluor (New England Nuclear) and counted in a Tracor Delta 300 scintillation counter (Tracor Analytic, Elk Grove Village, IL) to 0.5%, using the external standard method of quench correction. A small correction was made for the contribution from $^{36}Cl^-$ β-rays spilling over to the ^{14}C channel. 0.2-ml samples (in quadruplicate) were also taken of the isosmolar and hyposmolar buffers before addition to the cells. After the supernatant samples were taken for counting, the balance of the supernatant and the layer of dibutyl phthalate were aspirated, the sides of the microfuge tube swabbed with a Q-tip, and 1.0 ml of a saponin solution (10 μg/ml H_2O) was added. The tubes were vortexed, and 0.1-ml samples were taken for hemoglobin determination in 5.0 ml of Drabkin's solution. 0.1 ml of ice-cold 50% TCA was then added, the tubes were vortexed and then centrifuged, as above, for 5 min at 10,000 × *g*. 0.2-ml samples (in sextuplicate) of the protein precipitated cell lysate were counted as described above. Measurement of final osmolality and pH were also made.

Experiments at High and Low Hematocrit in Hyperosmolar Buffer

The isosmolar HEPES buffer in these experiments had the bovine serum albumin content reduced to 0.1%, but was otherwise unchanged. The hyperosmolar buffer (510 mOsm) had an increase of each label concentration to 0.2 μCi/ml. As described above, the cells were washed five times with the unlabeled isosmolar buffer. At this point cells were transferred to the labeled buffers without pre-equilibration. 3.0 ml of cells were resuspended in 7.0 ml of isosmolar (290 mOsm) or hyperosmolar (510 mOsm) buffer. Samples were taken for hematocrit (in sextuplicate) and hemoglobin determination (0.05 and 0.1 ml, in duplicate). 0.2 ml of cells were also added to 9.8 ml of the hyperosmolar buffer. As described above, 1.0-ml samples were taken in sextuplicate and centrifuged through 0.5 ml of dibutyl phthalate, and 0.2-ml aliquots were taken for counting. In the case of the 30% hematocrit samples, hemolysis was sometimes observed after this centrifugation. In such cases, the complete supernatant was transferred into new microfuge tubes, 0.03 ml of 50% ice-cold TCA added, the samples were centrifuged again, and 0.2 ml aliquots were counted. The dibutyl phthalate was then aspirated from the original samples, and the tubes were swabbed as previously described. For the 30% hematocrit samples, 1.0 ml saponin solution was added, the tubes vortexed, and 0.1-ml samples were taken for hemoglobin determination. This was followed by the addition of 0.15 ml ice-cold 50% TCA, centrifugation for 5 min at 10,000 × *g*, and counting of 0.2 ml of this supernatant. For the 2% hematocrit samples, 0.6 ml of saponin solution was added and, after vortexing, 0.2-ml samples were taken for hemoglobin measurement. This was followed by the addition of 0.025 ml ice-cold 50% TCA, centrifugation as above, and 0.2 ml samples of this supernatant were counted. As in the hyposmolar buffer experiments, 0.2-ml samples of the radioactively labeled buffers were taken (in sextuplicate) before addition to cells for determination of specific activities. Final osmolalities and pH were also determined.

Results and Discussion

Dependence of Apparent Nonosmotic Water on Intracellular Osmolality and pH

The classical equation that describes the osmotic behavior of the human red cell, as given by Ponder (1948) and used by Savitz et al. (1964) and Gary-Bobo and Solomon (1968) is, in normalized form

$$V'_{cell} = W'_{eff}\,(1/\pi') + b' \qquad (1)$$

in which V'_{cell} is the total normalized volume of the red cell (relative to isosmolal (290 mOsm, pH 7.4) cell volume), including total cell water plus solute and membrane volume; and W'_{eff} is the volume of cell water that is apparently able to participate in osmotic phenomena (expressed as a fraction of the isosmolal total cell volume). The normalized osmotic pressure is $\pi' = \pi/\pi_{iso}$, in which *iso* refers to isosmolal; b' (also expressed as a fraction of the isosmolal total cell volume) is the remainder of the cell volume, including the water volume, which apparently does not participate in the osmotic response, plus total solute and membrane volume. If the solutions were ideal, in that all osmotic and activity coefficients were unity, then W' should equal the fractional cell water, and it is the difference between W' and the fractional cell water that gives the value of the anomalous "apparent nonosmotic water" in the ideal case. This classical equation makes no overt allowance for the dependence of the os-

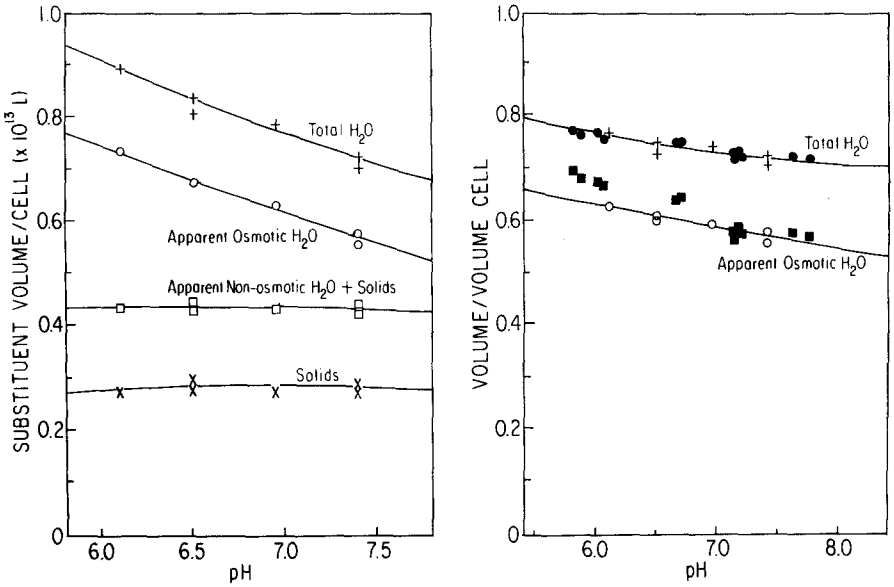

Fig. 2. Water and solids in human red cells. The left-hand panel shows the data obtained in the present experiments and the right-hand panel compares the values for total H_2O (+) and W'_{eff} (○) in the present experiments normalized to the isosmolal hematocrit at each pH, as described in the text, with those obtained by Gary-Bobo and Solomon (1968) (total H_2O, ●; W'_{eff}, ■). The data for total H_2O are those given in Fig. 5 of Gary-Bobo and Solomon (1968); the data for the apparent osmotic water come from a superseded table in an earlier draft of that manuscript. The data in the superseded table appear to have been transformed into the data published in Fig. 5 by a method we cannot now reproduce, and it was this transformation that led to the erroneous statement that apparent nonosmotic water exceeded total cell water at pH's below the Hb isoelectric point

motic coefficient of the cell contents, principally that of Hb, on cell volume, a question which has been treated extensively by Savitz et al. (1964), Dick (1967), and others. More complete sets of equations, which include additional thermodynamic relations that have to be satisfied to solve for cell volume, have been given by Jacobs and Stewart (1947), Gary-Bobo and Solomon (1968), and by Freedman and Hoffman (1979).

Before discussing the detailed predictions that follow from the complete nonideal thermodynamic equation, it is desirable to examine Eq. (1) in some detail because, as will be shown, it provides a useful phenomenological description of the osmotic behavior of the red cell. Gary-Bobo and Solomon (1968) had concluded that Eq. (1) did not describe the apparent osmotic properties of the human red cell, basing their conclusion on the results of two sets of experiments that we are now unable to reproduce. In the 1968 article, Gary-Bobo and Solomon reported that b' in Eq. (1) was not a constant, but varied with the intracellular osmolality; they also found, at low pH, that W'_{eff} exceeded the total water volume of the cell, determined gravimetrically. The experiments that we have now carried out do not substantiate these conclusions. The results of two experiments, typical of five, given in the left-hand section of Fig. 2 show that red cell apparent nonosmotic water is independent of pH over the range from 6.1 to 7.4. Furthermore, there is no indication that the apparent osmotic water is greater than the total water measured gravimetrically at any pH, so that Fig. 5 of the Gary-Bobo and Solomon article is clearly in error. At pH 7.4, the apparent osmotic water (expressed as fraction of total cell volume at pH 7.4, 290 mOsm) is 0.57 ± 0.01 for the two experiments in Fig. 2 and 0.55 ± 0.03 for all five experiments.

When cell volume is changed by alterations in external pH in addition to alterations in osmolality, the value used for reference volume of an isosmolal cell can be ambiguous. In the present paper, we express all volumes relative to the cell volume at pH 7.4, 290 mOsm; the cell volume at pH 7.4, 290 mOsm is taken to be 1×10^{-13} liters (*see below*). The volumes in this paper, therefore, represent volumes per cell. In the Gary-Bobo and Solomon paper (1968), volumes were taken as relative to the 290 mOsm cell volume at each pH. We have converted our data (the data shown in Fig. 2, left) into the units used by Gary-Bobo and Solomon to make the comparison shown in the right part of Fig 2. Our results in the lower pH range show no signs of a discontinuity around the isoelectric point of Hb.

We have also not been able to duplicate Gary-Bobo and Solomon's finding that apparent nonos-

motic water depends upon intracellular osmolality. Their experiments have been repeated as a part of the series of experiments used for Fig. 2. It can be seen, from the results given in Table 1, that the apparent nonosmotic water is independent of cell osmolality in the range from 200 to 400 mOsm, not only at pH 7.4, but also over the entire range of pH's studied. Table 1 also gives the Gary-Bobo and Solomon (1968) dependence of apparent nonosmotic water over roughly the same range. Our results suggest that the apparent nonosmotic water is independent of osmolality, though the error in the present measurements is large. A similar conclusion can be reached by comparing the Gary-Bobo and Solomon data over a larger range of mid-point osmolalities with those of Savitz et al. (1964) as shown in the bottom section of Table 1. In a very careful series of measurements, Savitz et al. had determined cell volume and water content in one experiment covering eight osmolalities ranging from 192 to 480 mOsm. We have divided their published data into two series of four osmolalities and have computed the ratio of apparent nonosmotic water around the two mid-points, obtaining the ratio of 0.92 ± 0.04 given in the table. This ratio is in agreement with the present data over a more limited osmolality range, but disagrees with that of Gary-Bobo and Solomon, who reported a ratio of 2.15 over a range somewhat greater than that of Savitz et al. We conclude therefore that, in this respect also, the Gary-Bobo and Solomon data are not correct. In a different approach to the same problem, Savitz et al. (1964) made a careful study of the linearity of Eq. (1) by fitting the data to a second-order polynomial and determined that the coefficient of the second term did not differ significantly from zero, thus showing Eq. (1) to be linear. The present data confirms this conclusion and leads us to accept Eq. (1), in its classical form, as a satisfactory and self-consistent phenomenological description of the osmotic behavior of human red cells.

Table 1. Effect of cell osmolality on apparent nonosmotic water

	Cell osmolality mid-point		Apparent nonosmotic water[a] higher osmolality / lower osmolality
	Lower range	Upper range	
Present study, pH 7.4	240	356	0.97 ± 0.2 (3)
Present study, all pH's	240	356	1.02 ± 0.2 (8)
Gary-Bobo & Solomon (1968)[b]	240	335	1.17
Gary-Bobo & Solomon (1968)	192	390	2.15
Savitz et al. (1964)[c]	240	405	0.92 ± 0.04

[a] Number of experiments in parentheses.

[b] The data from Gary-Bobo and Solomon were obtained from column 6 in their Table 2 by computing the ratio of apparent nonosmotic water in ml/(ml cell)$_{iso}$ at the highest mid-point osmolality to that at the lowest. Their data were divided into two groups because their experiments 3 and 4 covered a larger range of osmolalities than experiments 1 and 2. The individual values of the 240–335 group were 1.05 and 1.29; of the 192–390 group, 2.15 and 2.16.

[c] The data from Savitz et al. were obtained from the eight points in Expt 2 in their Table 1 by comparing the results at the four highest osmolalities (330 to 480 mOsm) to those at the four lowest osmolalities (192 to 282 mOsm). In all these computations the mid-point of the range was used as the reference osmolality.

Do the Colligative Properties of Hb Account for the Apparent Nonosmotic Water?

There have been many suggestions that red cell apparent nonosmotic water is a reflection of the nonideality of the osmotic coefficient of Hb. Savitz et al. (1964) examined this question carefully using a modified version of Eq. (1) which incorporated values of the molal osmotic coefficient of sheep Hb ($\emptyset_{Hb}$) computed from values of osmotic pressure that Adair (1929) determined at 0°C and pH 7.8 over the concentration range from 0.1 to 7.7 mmolal. Savitz et al. concluded that the concentration dependence of $\emptyset_{Hb}$ did not account for the apparent nonosmotic water. This view was challenged by Dick (1967, 1969) on the basis of differences between the data of Savitz et al. (1964) and those of Dick, as well as on the basis of additional values of the osmotic coefficient of ox Hb, which Adair had not himself published but made available to Hoffman (1958) and Dick (1967). Adair also showed (1928; *see also* Dick, 1967) that the osmotic coefficient of Hb is essentially independent of pH over the range of 6.8 to 8.3 but increases significantly when the ionic strength increases from 0.15 to 2. Several investigators, including Savitz et al. (1964) and Freedman and Hoffman (1979), have fitted the Adair data to polynomials. In our equations, we have used the second-order polynomial of Freedman and Hoffman, which fits all the Adair data well over an osmolality range of 0 to 12 mosmolal ($\emptyset_{Hb} = 1 + 0.0645\ [Hb] + 0.0258\ [Hb]^2$), where [Hb] is mmolal.

In order to determine quantitatively whether the colligative properties of Hb account for apparent nonosmotic water, it is necessary to develop a system of equations that includes all the thermodynamic constraints. The equation that Jacobs and Stewart (1947) developed to describe the osmotic properties of the red cell includes all possible solute movements across the cell membrane, but makes no allowance for the nonideal behavior of the osmotic

Table 2. Standard red cell

		Gary-Bobo & Solomon	Freedman & Hoffman[d]	This paper
Cell constituent				
Impermeable cations[a]	mmolal	158	150	150
Permeable anions	mmolal	108	109	111
Impermeable anions[b]	mmolal	9.3	0	0
Other solutes[c]	mmolal	20.5	12.4	29.5
Hb	mmolal	7.0	7.3	7.3
Total solutes	mOsmolal	289	277	290
Total charge	meq/liter cell H_2O	0	0	0
Other cell characteristics				
z_{Hb}	Charge/mol Hb	−4.7	−4.69	−5.39
$\emptyset_{Hb}$		2.44	2.98	2.85
r_{Cl^-}		0.694	0.803	0.737
Cell volume	femtoliter		97.2	100
Cell water	1 water/1 cell	0.717	0.689	0.717
pI_{cell}		6.95	6.80	6.80
Extracellular medium				
Cl^-	mmolal	155	150	150
pH		7.40	7.40	7.40
Osmolality	mOsmolal	289	277	290
Temperature	°C	25–26°	25°	25°

[a] Gary-Bobo and Solomon's impermeable cations comprised (meq/liter cell H_2O): ($Na^+ + K^+$), 155; Mg^{2+}, 2.5. This paper and Freedman and Hoffman (1979) neglect Mg^{2+}.

[b] Gary-Bobo and Solomon (1968) specified an intracellular phosphate concentration of 9.3 mmols/liter cell H_2O with a charge of −3. We have subsumed the charge of the phosphates in the value of the cell buffer capacity and the concentration as a component of the other solute value.

[c] Gary-Bobo and Solomon's (1968) other solutes comprised (mmol/liter cell H_2O): glucose, 5.5; other, 15. Freedman and Hoffman (1979) specified: glutathione, 3.4; P_i, 9. They also point out that the sum of intracellular solutes in their model accounts for only 96% of the external osmolality, equivalent to a discrepancy of 12 mM in other solutes (their footnote 1, p. 162). In order to use our computer program it is necessary to start with a system at equilibrium as given in Eqs. (A1) through (A4) in the appendix. In particular, the internal osmolality must equal the external osmolality, which specifies 29.5 mM of other solutes, including glutathione, 3.4; glucose, 5.5; 2,3-DPG, 5.5 and P_i, 9.3, making a total of 23.7 mM. To this we have arbitrarily added 5.8 mM of other nonspecified solutes to provide osmotic equilibrium at the measured cell volume of 10^{-13} liter.

[d] The values of the cell constituents in this column are those given in the model of Freedman and Hoffman (1979) as used for their theoretical analysis (p. 162, line 8 ff). The values of "other cell characteristics" were computed by our program from these figures and the extracellular medium composition, setting the extracellular osmolality to equal the Freedman and Hoffman intracellular osmolality as discussed in footnote c.

and activity coefficients of any solute. Gary-Bobo and Solomon (1968) introduced osmotic and activity coefficients explicitly, but did not include a term for the dependence of cell volume on cell pH. Freedman and Hoffman (1979) have modified the Jacobs and Stewart equations to include the nonideality of osmotic and activity coefficients and the pH dependence and have devised a computer program to solve the resultant equations. Freedman and Hoffman tested their set of equations in experiments with dilute suspensions (5%) of nystatin-treated red cells which, in contrast to normal red cells, are cation permeable. They found that individual values of the measured cell water content agreed, within 2.4 ± 0.9%, with that predicted as the cells were shrunken at normal ionic strength in sucrose solutions ranging from 26 to 260 mM at 25°C. Freedman and Hoffman also tested their set of equations on red cells with normal cation permeability by using their equations to predict the equilibrium pH dependence of the Cl^- distribution ratio and water contents that had been observed by Gunn, Dalmark, Tosteson and Wieth (1973) in dilute suspensions of human red cells. Freedman and Hoffman did not determine the apparent osmotic water from the slope of the linear relation in Eq. (1). If the differences from predicted behavior are consistent, with the points at higher osmolalities above the theoretical line and those at lower osmolalities below, the apparent agreement of individual points can mask a significant departure of experiment from theory, as will be shown below.

We have developed a computer program to solve the Freedman-Hoffman equations using their

polynomial fit for $\emptyset_{Hb}$, their equation for the dependence of the average net red cell charge, z (expressed in equivalents/mole Hb), on temperature and pH, and their standard red cell. This program was then tested against the pH dependence of cell water content and Donnan ratio of Gunn et al. (1973), and the results agreed with those of Table V in Freedman and Hoffman. We next extended the Freedman-Hoffman equations to cover shrinking and swelling experiments at high hematocrits. This was necessary because we were anxious to examine the predictions for the Cl^- shift at high hematocrit in order to compare our theoretical results with those of Gary-Bobo and Solomon (1968). Since the shift was expected to be small, it was necessary to be able to make predictions in concentrated suspensions in which extracellular Cl^- shifts would be maximized. Our computer program required the solution of 10 simultaneous equations as described in the appendix. We tested the high hematocrit program by lowering the hematocrit in the simulation and determining that the predicted osmotic dependence of cell volume was the same as for the low hematocrit program.

In order to predict the effect of external osmolality on the volume of normal cation impermeable red cells, it is necessary to specify the composition and properties of a standard red cell, as has been done in Table 2, which compares the parameters of our standard cell with those of Gary-Bobo and Solomon and Freedman and Hoffman. Since the cell volume depends on extracellular osmolality, anion concentration, pH and the presence of extracellular albumin, we have set the standard cell volume as 1.0×10^{-13} liter, in agreement with the values given by Jay (1975) of 0.92×10^{-13} liter in the presence of albumin (1 g/liter) and 1.06×10^{-13} liter in the absence of albumin in 306 mOsm buffer at pH 7.4 and that of 1.08×10^{-13} liter given by Canham and Burton (1968) in buffer (310 mOsm, pH 7.4). The osmotic dependence of cell volume in shrink/swell experiments is not very dependent on the choice of a standard volume. Changes in isosmolal cell water volume by ±10% change the theoretical cell volume by ±2.5% over the range of 200–400 mOsm.

The osmotic response does depend on the Donnan ratio; lower values of pH increase the apparent osmotic water and higher values of pH decrease it. Gary-Bobo and Solomon used $r_{Cl} = 0.694$ (25°C, 155 mM Cl^-, 289 mOsm) as determined by Bernstein (1954). There are many determinations of r_{Cl} in the literature and they differ considerably, probably because r is conventionally measured in plasma, and r, like cell volume, depends sensitively on the composition of the suspending medium. Brahm (1977) determined r_{Cl} in buffer as a function of pH at 38°C and Gunn et al. (1973) determined it in buffer at 0°C. We have computed $r_{Cl} = 0.737$ (pH 7.4, 25°C, 150 mM extracellular Cl^-, 290 mOsm) by linear interpolation from these two sets of data. As Table 2 shows, our standard red cell preserves electroneutrality and is at osmotic equilibrium with an external solution of 290 mOsm at pH 7.4. Our standard buffer contains 150 mM Cl^-, which specifies the internal Cl^- content for a given Donnan ratio. We have set the cell buffer capacity, m, at 12.5 eq/mol per pH to give $r = 0.737$ in agreement with the experimentally determined buffer capacity of 12.6 eq/mol per pH given by Freedman and Hoffman (their Fig. 5). We have also set the other solute content at 29.5 mM in order to bring the intracellular osmolality to 290 mOsm. This standard cell has equilibrium properties similar to those used by Gary-Bobo and Solomon (except for the difference in Donnan ratio), though the actual composition of our standard cell in Table 2 has been chosen to make it as similar to the Freedman and Hoffman model cell as possible.

Our equations were next used to predict the dependence of cell volume on extracellular osmolality. This can be done phenomenologically by comparing the observed slope of the line in Eq. (1), which provides an exact determination of W'_{eff}, with the predicted value of 0.653 given by the nonideal thermodynamic equations as applied to our standard red cell. The most precise determination of W'_{eff} can be obtained from the data of Savitz et al. (1964), which is shown in Fig. 3, together with the theoretical line. The experimental value of W'_{eff} is 0.563 ± 0.006, which is significantly different ($P \ll 0.0005$, t test) from the theoretical value of 0.653. At first sight, it may seem paradoxical that points that lie as close to the theoretical line as those in Fig. 3 should be characterized by a slope that differs from theoretical with so high a level of significance. The reason is that all the points in Fig. 3 fall very exactly on a straight line and thus define the slope very exactly, as evidenced from the small standard deviation. At 290 mOsm, the difference in nonosmotic water between theory and experiment in Fig. 3 amounts to 12.5 ± 0.8% of total isosmotic cell water (P value for difference $\ll 0.0005$). Our measurements are not as precise as those of Savitz et al. but the differences from theory are almost as significant. The two values used in Fig. 2 are 0.58 ± 0.02 and 0.56 ± 0.03, both of which differ from theory with $P < 0.0025$. The average for all five measurements in that series of experiments is 0.55 ± 0.03, which differs from theory with the same value of P. Operationally, we have found that W'_{eff} virtually never exceeds the upper bound of 0.60 (and occasionally exceeds a lower bound of 0.54) in many

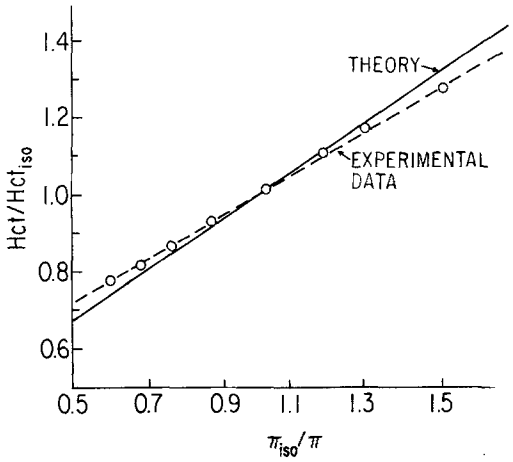

Fig. 3. Fit of experimental shrink/swell data to non-ideal thermodynamic theory. Comparison of the shrink/swell behavior of cells as determined by Savitz et al. (1964) (Table 1, exp 2) with that predicted. The data are plotted according to Eq. (1), after normalizing their data to the isosmolal Hct_{iso}. The slopes and intercepts are: exp data [slope: 0.563 ± 0.006; intercept 0.436 ± 0.007] theory [slope: 0.653, intercept: 0.348]. The difference between these figures = 12.5% of total isosmotic water [(0.653 − 0.563)/0.717]

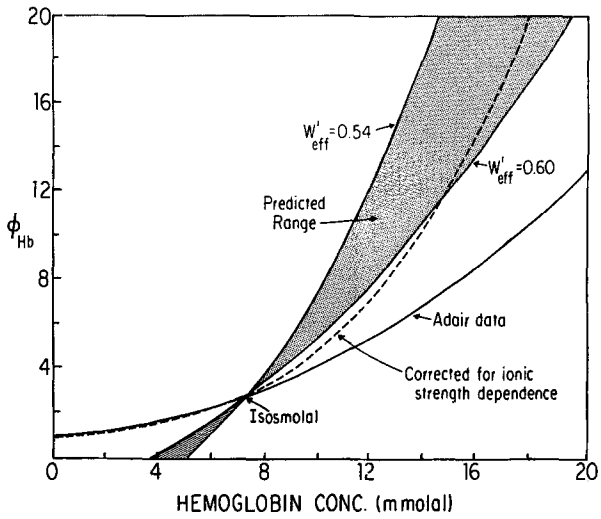

Fig. 4. $\emptyset_{Hb}$: Comparison of experimental and predicted values. The range of values of $\emptyset_{Hb}$, as computed from the observed range of W'_{eff} by the nonequilibrium thermodynamic equations (stippled area), is compared with the experimental curve of $\emptyset_{Hb}$ from data given by Adair (1929), extrapolated according to the virial equation of Freedman and Hoffman (1979) (full line). The stippled area covers the entire range of observed values of W'_{eff}, which has virtually never been observed to be greater than 0.60 and only occasionally to be less than 0.54, as described in the text. The dashed line shows Adair's data, corrected for ionic strength dependence by Eq. (3)

tens of experiments that are routinely carried out as an adjunct to stopped-flow measurements of red cell permeability. The nonideal thermodynamic equation prediction of 0.653 is outside the range of our experience.

In order to determine whether this difference may be ascribed to the osmotic coefficient of Hb, we have modified our computer program to solve for $\emptyset_{Hb}$ given the experimentally determined cell volume and have determined the values of $\emptyset_{Hb}$ necessary to fit the volume behavior of our standard red cell at the two experimental boundary values of 0.54 and 0.60 for W'_{eff}. The area between these two limits has been stippled in Fig. 4 to facilitate comparison with the full line computed from the standard virial equation for Adair's data on $\emptyset_{Hb}$ as used by Freedman and Hoffman and ourselves. It can be seen that the stippled area does not approach Adair's data on $\emptyset_{Hb}$. It might be possible for $\emptyset_{Hb}$ to be higher than the virial equation predicts in the more concentrated Hb solutions, but it is *not possible* for $\emptyset_{Hb}$ to fall to zero at a Hb concentration of 4 mmolal and then to go negative as an extension of the plot in Fig. 4 would require. This application of the nonideal thermodynamic equations to our standard cell shows that the concentration dependence of $\emptyset_{Hb}$, observed by Adair, cannot account for the discrepancy between the observed cell volume and that predicted by the nonideal thermodynamic theory.

Is there a Cl^- Shift when the Cell Changes Its Volume?

Gary-Bobo and Solomon (1968) concluded that the apparent nonosmotic water was not a reflection of anomalous water behavior, but rather was to be attributed to shifts of Cl^- across the cell membrane, consequent to a concentration dependence of the net intracellular Hb charge. Subsequently, Gary-Bobo and Solomon (1971) studied concentrated Hb solutions in vitro and reported that the Hb charge was concentration dependent under in vitro conditions. These results have not been substantiated in subsequent experiments by others and there is a body of compelling evidence that the charge on the Hb molecule is independent of Hb concentration. The most direct study is that of Gros et al. (1978), who found in experiments in vitro, similar to the 1971 experiments of Gary-Bobo and Solomon, that the pH of Hb, and hence its net charge, remained constant over the range from 1.5 to 11 mmolal. Freedman and Hoffman (1979) also showed, in nystatin treated red cells, that the charge on all the cellular solutes was independent of cell volume, over a Hb concentration from 5 to 15 mmolal.

In view of these findings, it was important to

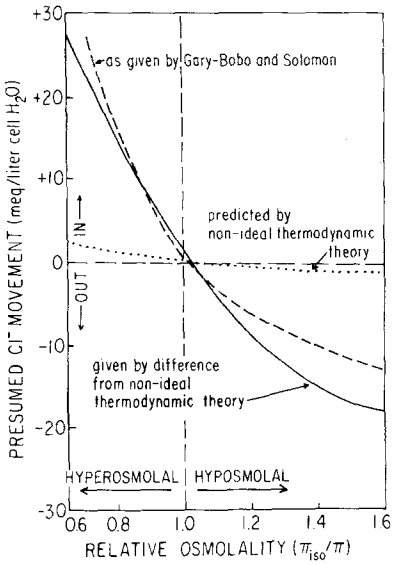

Fig. 5. Cl shift required to fit osmotic behavior. Cl^- shift required to account for "apparent nonosmotic water." The Gary-Bobo and Solomon (1968) curve (dashed line) is from their Fig. 12; the nonideal thermodynamic line (dotted) has been determined as described in the text. The full line represents the presumed Cl^- movement that would be required to account for the discrepancy between the observed cell volume and that predicted by the nonideal thermodynamic equations using our computer programs

Table 3. Calculated and observed intracellular Cl^-

Expt	Hct		Final cell $[Cl^-]$		Observed / predicted
	Initial	Final	Observed (M)	Predicted (M)	
1	0.206	0.255	0.0745	0.0744	1.00
2	0.201	0.263	0.0816	0.0814	1.00
					ave 1.00
3	0.017	0.013	0.166	0.168	0.98
	0.292	0.222	0.162	0.154	1.05
4	0.0134	0.0102	0.147	0.169	0.87
	0.271	0.225	0.141	0.156	0.90
5	0.0176	0.0135	0.160	0.169	0.95
	0.273	0.222	0.152	0.156	0.97
6	0.0197	0.0136	0.169	0.169	1.00
	0.300	0.234	0.151	0.157	0.96
					ave 0.96 ± 0.06

determine whether the discrepancy illustrated in Figs. 3 and 4 was large enough to require a Cl^- shift of the magnitude proposed by Gary-Bobo and Solomon (1968). We have modified our low hematocrit computer program in order to predict, within the framework of the nonideal Jacobs-Stewart theory, the magnitude of the Cl^- flux required to explain the discrepancy between experimental and predicted cell volumes. We have done this by treating the cell buffering capacity as a variable and solving for the value necessary to bring the predicted cell volume into agreement with the experimental cell volume. The putative Cl^- flux arising from the osmotic dependence of the cell buffering capacity is plotted in Fig. 5 as a function of external osmolality. Also plotted in Fig. 5 is the Cl^- flux predicted by Gary-Bobo and Solomon on the basis of their version of the Jacobs-Stewart equation, i.e., the Cl^- flux required to make the observed and theoretical cell volumes agree, using Adair's data for $\emptyset_{Hb}$. These data indicate that if the discrepancy between predicted and observed cell volumes is due to a Cl^- flux, the flux is appreciable and similar to that predicted by Gary-Bobo and Solomon. The only substantive difference between the theory used by Gary-Bobo and Solomon and the present one is that they did not consider the effect of cell pH on cell volume. This is a very small effect, as shown by the dotted line in Fig. 5, which shows that the nonideal thermodynamic theory predicts a negligible Cl^- shift (as a result of predicted changes in r) in response to osmotic pressure differences. Thus, when all the known thermodynamic constraints and all the known nonidealities are considered, there still remains a deficit (and surfeit) of cell solutes which is measured in tens of milliequivalents of solutes per liter of cell water.

If, as Gary-Bobo and Solomon (1968) suggested, the change is due to a Cl^- shift, it should be detectable by a double isotope experiment similar to the ones they did. Therefore we carried out a series of six experiments and obtained the results given in Table 3. In the top two swelling experiments in the table, we first washed the cells until there was no permeable anion other than Cl^- and then brought the system to constant specific $^{36}Cl^-$ activity. We then made direct measurements of $[Cl^-]$ inside and outside the cell. This enabled us to calculate r_{Cl} directly; this figure was compared with the predicted value from our high hematocrit computer program. As the table shows the observed/predicted ratio was 1.00, which means that there is no Cl^- shift. Thus, these experiments also fail to confirm the results given by Gary-Bobo and Solomon and do agree with those of Cook (1967), Gros et al. (1978) and Freedman and Hoffman (1979).

It occurred to us that there might be some difference between results obtained at high hematocrit and low hematocrit, either due to differences between the high and low hematocrit computer programs or due to solute shifts which had not been taken into account properly. Therefore, the next set of four experiments were carried out somewhat dif-

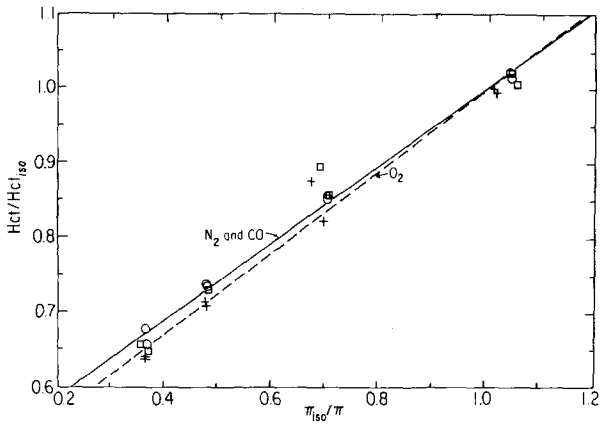

Fig. 6. Effect of state of Hb oxygenation on shrink/swell response in one experiment. The slopes and intercepts are: O_2 [slope: 0.55 ± 0.04; intercept 0.45 ± 0.02 (n = 8)]; N_2 [slope 0.52 ± 0.03; intercept 0.47 ± 0.02 (n = 8)]; CO [slope 0.52 ± 0.01; intercept 0.49 ± 0.01 (n = 8)]

ferently, as discussed in the methods section, in order to compare high and low hematocrit shrinking experiments on a single blood sample. In consequence, the measurements were somewhat less direct. But the results were the same. Although the error was somewhat larger, there was no Cl^- shift at either hematocrit. These experiments show definitively that neither the results, nor the conclusions, of Gary-Bobo and Solomon are correct. Unfortunately, this still leaves us with the same problem faced by Gary-Bobo and Solomon—that of finding an acceptable physical chemical explanation for the anomalous behavior of red cell swelling and shrinking.

It was possible that 2,3-DPG (2,3-diphosphoglycerate) binding to Hb (Benesch, Benesch & Yu, 1969) might play a role in the process. Since 2,3-DPG binding to Hb is markedly diminished, or abolished, when cells are treated with CO or O_2, we carried out a series of experiments to see whether the oxygenation state of the Hb affected the shrink/swell response of cells treated with O_2, N_2 or CO. As Fig. 6 shows, there is no significant difference between the slopes and intercepts of any of the lines. These data show that the anomalous osmotic behavior cannot be attributed to the binding of 2,3-DPG over the osmolality range of π_{iso}/π of 0.4 to 1.1. However, they do not exclude the participation of 2,3-DPG in shrink/swell behavior when the cells are swollen and Hb concentrations fall below about 7 mmolal.

Other Physical Chemical Properties Which May Explain Apparent Anomalous Behavior

Figure 5 shows that the discrepancy in cell volume, when expressed in terms of equivalent Cl^- concentration, ranges from a surfeit of ≈20 meq/liter cell H_2O at π_{iso}/π = 0.75 to a deficit of ≈15 meq/liter cell H_2O at π_{iso}/π = 1.4, which are significant fractions of the cell Cl^- concentration (111 meq/liter cell H_2O at 290 mOsm (Table 2)). It can easily be shown that the anomalies cannot be ascribed to elastic properties of the membrane because, as Colombe and Macey (1974) and Levin, Levin and Solomon (1980) have shown, red cell ghosts behave as essentially perfect osmometers. Therefore, the anomalous behavior must be attributed to some hitherto neglected interaction among the cell solutes or between the solutes and the membrane.

Savitz et al. (1964) proposed that the anomalous shrink/swell properties could be ascribed to water bound to the Hb molecule in agreement with previous suggestions in the literature. However, Gary-Bobo and Solomon (1968) carried out an extensive series of studies to show that this was not the case. They showed that nonelectrolytes such as ethanol and D-glucose dissolved in all the cell water, that the osmotic pressure of 3.7 mmolal Hb solutions containing up to 0.2 M KCl agreed with the classical van't Hoff theory (with Adair's $\emptyset_{Hb}$) to better than 1%, and that the limiting value of the Donnan ratio was 1.0 ± 0.005 at KCl concentrations up to 0.3 M, pH 6.95.

As pointed out above, Adair (1928) observed that the osmotic coefficient of Hb depended on ionic strength; this observation has not previously been included in the computation of the osmotic coefficient of Hb. We have fitted the data in Adair's Table VIII, which gives the ionic strength dependence of the osmotic coefficient, to the quadratic equation

$$\emptyset_\mu = 1 + a_1\mu + a_2\mu^2 \qquad (2)$$

in which $\emptyset_\mu$ is the multiplicative factor introduced by Adair to correct for the ionic strength (μ) dependence; a_1 = 1.0; a_2 = −0.53. $\emptyset_\mu$ is included in the computation of $\emptyset_{Hb}$ by the relation $\emptyset'_{Hb} = \emptyset_{Hb}\,\emptyset_\mu/\emptyset_{\mu o}$ in which $\emptyset_{\mu o}$ = the value of $\emptyset_\mu$ at μ = 0.15 M. Replacement of $\emptyset_{Hb}$ with $\emptyset'_{Hb}$ does not materially affect the discrepancy shown in Fig. 4.

Adair also pointed out that $\emptyset_\mu$ depended upon Hb concentration, though he gave only a limited amount of data. In experiments at [Hb] = 1 g/100 ml solution, the correction factor is 1.09, whereas at [Hb] = 20 g/100 ml solution, the correction factor rises to 1.63 at 2 M salt. Dick's graph (1967) showing Adair's unpublished data clearly illustrates the increase in ionic strength dependence with Hb concentration. We have therefore introduced the additional assumption that the ionic strength dependence of $\emptyset_{Hb}$ is linked to Hb concentration. We incorporated this refinement into Eq. (2) by scaling the coefficients a_1 and a_2 so that

$$\emptyset'_\mu = 1 + a_{1s}\mu + a_{2s}\mu^2 \quad (3)$$

in which

$$a_{1s} = a_{10}[\mathrm{Hb}]/[\mathrm{Hb}]_0$$

and

$$a_{2s} = a_{20}[\mathrm{Hb}]/[\mathrm{Hb}]_0.$$

a_{10}, a_{20} are values of a_1 and a_2 at μ_0 and $[\mathrm{Hb}]_0$ is the [Hb] which Adair used in Table VIII, which we have rounded[1] to 4 mmolal.

When the osmotic coefficient is corrected for $\emptyset'_\mu$, calculated as described above, the corrected osmotic coefficient for Hb is given by the dashed line in Fig. 4, and there is much better agreement between this computed value of $\emptyset_{Hb}$ and that required to fit the experimental data for concentrations of Hb above isosmolal. Even this apparent agreement may be, in part, illusory since it takes as its basis the Freedman and Hoffman second-order polynomial extrapolation to Adair's osmotic pressure data, expressed in units of molality. Ross and Minton (1977) have used excluded volume analysis to treat the same data and expressed their results as a seventh-order polynomial expressed in units of g Hb/liter of solution. Both equations fit the data equally well over Adair's concentration range, but the Freedman and Hoffman osmotic pressure rises more sharply at higher concentrations because a second-order polynomial, in units of molality, becomes a very high-order polynomial, if expressed in g Hb/liter of solution. At 12 mmolal Hb, for example, the differences are quite substantial. The value for $\emptyset_{Hb}$ of 4.7, calculated from the Freedman and Hoffman polynomial, is raised to 6.8 by using the concentration-dependent ionic strength correction. The Ross and Minton polynomial gives a value[2] of 3.9 (taking no account of the ionic strength dependence). Thus, though it is clear that the concentration-dependent ionic strength correction markedly diminishes the discrepancy, the question of quantitative agreement will not be resolved until there are reliable measurements of the osmotic pressure of red cell cytoplasm at high solute concentrations.

The ionic strength corrections are only effective at concentrations above isosmolal as Fig. 4 shows. At the lower concentrations, there is a surfeit of cellular solute, as pointed out above; at $\pi_{iso}/\pi = 1.4$, the cell contains solutes equivalent to 15 meq Cl^- more than required for osmotic balance. The experiments of Savitz et al. (1964) showed that there is no irreversible loss of solute that accompanies cell swelling in this range of osmolalities, and the present experiments show that there is no reversible loss of Cl^- on swelling. At $\pi_{iso}/\pi = 1.4$, [Hb] is 5.7 mmolal and the osmotic coefficient is about 2.2; even if the Hb osmotic coefficient fell to zero, there would still be too much cell solute to balance the cell osmotically.

At physiological and lower ionic strength, solute binding can remove solutes from solution. There are many proteins in the cell that can provide binding sites, particularly Hb and the soluble cytoplasmic enzymes of the glycolytic cycle, in addition to the membrane proteins themselves. Band 3, which comprises 25% of the cell membrane proteins contains ankyrin binding sites for attachment to spectrin (*see* Salhany, 1983) as well as sites for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and aldolase (Yu & Steck, 1975; Strapazon & Steck 1977). Furthermore, G3PDH binding to band 3 is ionic strength dependent (Kant & Steck, 1973) and about half the cell G3PDH is bound at physiological ionic strength. However, the total number of mmol of enzyme that may bind is quite small. Salhany, Cordes and Gaines (1980) and Zilber and Shaklai (1982) have shown that Hb binds to sites on the cell membrane, particularly band 3, and that Hb binding increases as ionic strength decreases. Hb binding, including nonspecific Hb binding, probably only amounts to about 0.1 mosmol, and addition of the other cellular enzymes could only multiply this figure by a small factor, say 2.

The solutes that are present in mmolar quantities are ions, particularly 2,3-DPG (5.5 mM) and anions such as Cl^-. Benesch et al. (1969) report that for salt concentrations of 0.1 M NaCl, 1 mole of 2,3-DPG binds per mole of deoxyhemoglobin; binding increases with lower salt concentrations. Benesch et al. are of the view that 2,3-DPG binds in a central cavity of deoxyhemoglobin and that other anions, such as Cl^- can also fill this cavity, though much greater concentrations are required than for 2,3-DPG. If the binding capacity of Hb is one anion/tetramer, whether it be 2,3-DPG or Cl^-, the binding

[1] Based on a molecular weight of 64,650 (Dickerson & Geis, 1983) and a density of 1.346 for sheep Hb at 0°C (Adair & Adair, 1936), 20 g Hb/100 ml solution is 3.63 mmolal Hb. For the purposes of our computation, rounding to 4 mmolal does not make a significant difference.

[2] The actual computation was made from an eighth-order polynomial using the eighth-order coefficient given by Ree and Hoover (1967) as described by Ross and Minton (1977). The coefficient, Γ_2, was taken as 3.55×10^3 and the molecular weight of Hb was taken as 70,206, which are the values used by Ross (*personal communication*) as parameters to obtain the best fit of the Ross and Minton equation to the calculated protein osmotic pressures, after Donnan corrections, given by Adair (1928). Care must be taken in computing the molal osmotic coefficient from molar data since the value of the coefficient depends upon the concentration units used.

capacity cannot exceed the [Hb] of 5.4 mmolal. However, Benesch et al. point out that at salt concentrations lower than 0.1 M, binding of more than one mol 2,3-DPG/mol of Hb occurs, so that 5.4 mmolal is not an upper limit. All of these processes are in the right direction, though their magnitude still seems too small to provide the entire explanation. Low, Waugh, Zinke and Drenckhahn (1985) have shown that denatured Hb interacts with band 3 and controls band 3 aggregation. It is possible that links between cytoplasmic proteins and the membrane could affect solute binding so that the osmotic pressure inside the cell at low ionic strength might be different from that predicted by summing the contributions computed from binding observations in free solution when only a small number of components is present.

Since white ghosts behave essentially as perfect osmometers and red cells do not, it is clear that the presence of intracellular Hb and other solutes are responsible for the deviations from ideality, which are too large to be accounted for by reasonable extrapolations of the concentration dependence of the Hb osmotic coefficient. At higher Hb concentrations, the ionic strength dependence of ϕ_{Hb} provides a plausible explanation of the discrepancy. At lower concentrations, solute binding is in the right direction to decrease the anomaly but appears quantitatively too small to account for the entire effect. What is urgently needed now, is an accurate determination of the true osmolality of a total solution of red cell solutes over the entire range from 4 to 15 mmolal Hb.

We should like to express our thanks to Mr. Matthew Frosch and Dr. Bernard Chasan for their experimental and theoretical contributions to this project and to Dr. Judith Herzfeld for discussion of the Hb osmotic coefficient. This research was supported in part by the Office of Naval Research contract N00014-83-0015 and U.S. Public Health Service grant GM15692.

References

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. London A.* **120:**573–603

Adair, G.S. 1929. The thermodynamic analysis of the observed osmotic pressures of protein salts in solutions of finite concentration. *Proc. R. Soc. London A.* **120:**16–24

Adair, G.S. Adair, M.E. 1936. The densities of protein crystals and the hydration of proteins. *Proc. R. Soc. London B.* **120:**422–446

Benesch, R.E., Benesch, R., Yu, C.I. 1969. The oxygenation of hemoglobin in the presence of 2,3-diphosphoglycerate. Effect of temperature, pH, ionic strength and hemoglobin concentration. *Biochemistry* **8:**2567–2571

Bernstein, R.E. 1954. Potassium and sodium balance in mammalian red cells. *Science* **120:**459

Brahm, J. 1977. Temperature-dependent changes of chloride transport kinetics in human red cells. *J. Gen. Physiol.* **70:**283–306

Canham, P.B., Burton, A.C. 1968. Distribution of size and shape in populations of human red cells. *Circ. Res.* **22:**405–422

Colombe, B.W., Macey, R.I. 1974. Effects of calcium on potassium and water transport in human erythrocyte ghosts. *Biochim. Biophys. Acta* **363:**226–239

Cook, J.S. 1967. Nonsolvent water in human erythrocytes. *J. Gen. Physiol.* **50:**1311–1325

Craik, J.D., Reithmeier, R.A.F. 1984. Inhibition of phosphate transport in human erythrocytes by water-soluble carbodiimides. *Biochim. Biophys. Acta* **778:**429–434

Dick, D.A.T. 1967. An approach to the molecular structure of the living cell by water studies. *In:* Physical Bases of Circulatory Transport. E.B. Reeve and A.G. Guyton, editors. pp. 217–234. W.B. Saunders, Philadelphia

Dick, D.A.T. 1969. Osmotic behavior of hemoglobin in vivo and in vitro. *J. Gen. Physiol.* **53:**836–838

Dickerson, R.E., Geis, I. 1983. Hemoglobin: Structure, Function, Evolution, and Pathology. p. 7. Benjamin/Cummings Publishing, Menlo Park

Freedman, J.C., Hoffman, J.F. 1979. Ionic and osmotic equilibria of human red blood cells treated with nystatin. *J. Gen. Physiol.* **74:**157–185

Gary-Bobo, C.M., Solomon, A.K. 1968. Properties of hemoglobin solutions in red cells. *J. Gen. Physiol.* **52:**825–853

Gary-Bobo, C.M., Solomon, A.K. 1971. Hemoglobin charge dependence on hemoglobin concentration in vitro. *J. Gen. Physiol.* **57:**283–289

Gros, G., Rollema, H.S., Jelkmann, W., Gros, H., Bauer, C., Moll, W. 1978. Net charge and oxygen affinity of human hemoglobin are independent of hemoglobin concentration. *J. Gen. Physiol.* **72:**765–773

Gunn, R.B., Dalmark, M., Tosteson, D.C., Wieth, J.O. 1973. Characteristics of chloride transport in human red blood cells. *J. Gen. Physiol.* **61:**185–206

Hladky, S.B., Rink, T.J. 1978. Osmotic behavior of human red blood cells: An interpretation in terms of negative intracellular fluid pressure. *J. Physiol. (London)* **274:**437–446

Hoffman, J.F. 1958. Physiological characteristics of human red blood cell ghosts. *J. Gen. Physiol.* **42:**9–28

Jacobs, M.H., Stewart, D.R. 1947. Osmotic properties of the erythrocyte. XII. Ionic and osmotic equilibria with a complex external solution. *J. Cell. Comp. Physiol.* **30:**79–103

Jay, A.W.L. 1975. Geometry of the human erythrocyte: I. Effect of albumin on cell geometry. *Biophys. J.* **15:**205–222

Kant, J.A., Steck, T.L. 1973. Specificity in the association of glyceraldehyde 3-phosphate dehydrogenase with isolated human erythrocyte membranes. *J. Biol. Chem.* **248:**8457–8464

Levin, S.W., Levin, R.L., Solomon, A.K. 1980. Improved stop-flow apparatus to measure permeability of human red cells and ghosts. *J. Biochem. Biophys. Methods* **3:**255–272

Low, P.S., Waugh, S.M., Zinke, K., Drenckhahn, D. 1985. The role of hemoglobin denaturation and band 3 clustering in red blood cell aging. *Science* **227:**531–533

Muller, D.E. 1956. A method for solving algebraic equations using an automatic computer. *Math. Tables Aids Comput.* **10:**205–208

Ponder, E. 1948. Hemolysis and Related Phenomena. Grune and Stratton, New York

Ree, F.H., Hoover, W.G. 1967. Seventh virial coefficients for hard spheres and hard disks. *J. Chem. Phys.* **46:**4181–4197

Ross, P.D., Minton, A.P. 1977. Analysis of nonideal behavior in concentrated hemoglobin solutions. *J. Mol. Biol.* **112:**437–452

Salhany, J.M. 1983. Binding of cytosolic proteins to the erythrocyte membrane. *J. Cell. Biochem.* **23:**211–222

Salhany, J.M., Cordes, K.A., Gaines, E.D. 1980. Light-scattering measurements of hemoglobin binding to the erythrocyte membrane. Evidence for transmembrane effects related to a disulfonic stilbene binding to band 3. *Biochemistry* **19:**1447–1454

Savitz, D., Sidel, V.W., Solomon, A.K. 1964. Osmotic properties of human red cells. *J. Gen. Physiol.* **48:**79–91

Strapazon, E., Steck, T.L. 1977. Interaction of aldolase and the membrane of human erythrocytes. *Biochemistry* **16:**2966–2971

Yu, J., Steck, T.L. 1975. Associations of band 3, the predominant polypeptide of the human erythrocyte membrane. *J. Biol. Chem.* **250:**9176–9184

Zilber, I., Shaklai, N. 1982. The interaction of hemoglobin with isolated band 3 cytoplasmic fragments. *Biochem. Int.* **4:**297–303

Received 2 January 1986; revised 6 March 1986

Appendix

The computer simulation of the Cl^- flux experiments of Gary-Bobo and Solomon (1968) was done in two stages. One stage, corresponding to the 10 washes in 10 volumes of 96 mM, NaCl, 50 mM sodium phosphate, pH 7.4, was simulated by modifying the equations given by Freedman and Hoffman (1979) to take into account Donnan equilibrium of phosphate. Although phosphate flux through the red cell is slow (Craik & Reithmeier, 1984), we assume that at the end of 10 washes phosphate has reached equilibrium. The second stage, corresponding to a shrinking of the cell at high hematocrit, was simulated by modifying the equations to take into account the high hematocrit and the buffering capacity of 50 mM phosphate. In the second stage, we assume no appreciable phosphate flux occurs and that any Cl^- flux occurs via a Cl^-/OH^- exchange.

Stage 1

The following nine simultaneous equations describe the low hematocrit wash in which phosphate comes to Donnan equilibrium:

1. Osmotic equilibrium

$$\left(\frac{\pi'}{RT}\right)_o = \frac{\emptyset_+ n''_+ + \emptyset_{Hb} n_{Hb} + \emptyset_u n''_u + \emptyset_-(n''_{Cl} + n''_{HP} + n''_{H_2P})}{V''_w}. \tag{A1}$$

2. Intracellular electroneutrality

$$n''_{Cl} + n''_{H_2P} + 2n''_{HP} = n''_+ + Z_{Hb} n_{Hb}. \tag{A2}$$

3. Donnan equilibrium of Cl^-

$$r = \frac{n''_{Cl}/V''_w}{([Cl'])_o}. \tag{A3}$$

4. Donnan equilibrium of H^+

$$0.92r = \frac{([H'])_o}{n''_H/V''_w}. \tag{A4}$$

5. Donnan equilibrium of $H_2PO_4^-$

$$r = \frac{n''_{H_2P}/V''_w}{([H_2P'])_o}. \tag{A5}$$

6. Donnan equilibrium of $HPO_4^=$

$$r = \left(\frac{n''_{HP}/V''_w}{([HP'])_o}\right)^{1/2}. \tag{A6}$$

7. pH dependence of intracellular charge

$$Z_{Hb} = -10[pH'' - (7.2 - 0.016T)]. \tag{A7}$$

8. Concentration dependence of osmotic coefficient of hemoglobin

$$\emptyset_{Hb} = 1.0 + k_1(n_{Hb}/V''_w) + k_2(n_{Hb}/V''_w)^2. \tag{A8}$$

9. Extracellular equilibrium of $HPO_4^=$ and $H_2PO_4^-$

$$K = \frac{([H])_o([HPO_4^-])_o}{([H_2PO_4^=])_o}. \tag{A9}$$

Substitution of Eqs. (3)–(9) into Eqs. (1) and (2) gives two equations in the two unknowns, r and V''_w:

$$0 = r^2\emptyset_-([HP'])_o + r[\emptyset_-([Cl'])_o + \emptyset_-([H_2P'])_o] + \frac{A}{V''_w} - \frac{\pi'}{RT} \tag{A10}$$

$$0 = 2r^2([HP'])_o + r[([Cl'])_o + ([H_2P'])_o] - \frac{n''_+}{V''_w} - \frac{Z_{Hb} n_{Hb}}{V''_w} \tag{A11}$$

where $A = \emptyset_+ n''_+ + \emptyset_u n''_u + \emptyset_{Hb} n_{Hb}$. $\emptyset_{Hb}$ implicitly contains the variable V''_w, while Z_{Hb} implicitly contains r. $([HP'])_o$ and $([H_2P'])_o$ are related to the total extracellular phosphate concentration, $[P'_t]$, and the equilibrium dissociation constant, K, by

$$([HP'])_o = \frac{K[P'_t]}{K + ([H'])_o} \tag{A12}$$

and

$$([H_2P'])_o = \frac{([H'])_0[P'_t]}{K + ([H'])_o}. \tag{A13}$$

The zero of Eq. (A11) was determined by the iterative Muller (1956) method. For each iteration a value for r was determined by solving the quadratic Eq. (A10); this value was then substi-

Table A1.

List of initial conditions (for high hematocrit shrink)	
$\left(\frac{\pi'}{RT}\right)_o$	0.290 osmolal
$([Cl'])_o$	0.096 molal
$([H'])_o$	$10^{-7.4}$ molal
$([H_2P'])_o$	0.0195 molal
$([HP'])_o$	0.0305 molal
$([P_i'])_o$	0.05 molal
$(hct)_o$	0.35–0.40
$(V_w + \gamma)_o$	1.0×10^{-13} liter
γ	0.283×10^{-13} liter
α	$(W_w + \gamma)_o/(hct)_o$ liter
β	$(V_w + \gamma)_o[0.096(1 - (hct)_o) + 0.074(hct)_o]$ moles

tuted in Eq. (A11). Convergence was assumed if the residual in Eq. (A11) was $<10^{-5}$.

Stage 2

The following 10 simultaneous equations describe the high hematocrit shrink of the red cell in 50 mM phosphate buffer assuming a Cl^-/OH^- exchange can occur:

1. Osmotic equilibrium

$$\frac{\pi'}{RT} = \frac{\emptyset_+ n''_+ + \emptyset_{Hb} n_{Hb} + \emptyset_u n''_u + \emptyset_-(n''_{Cl} + n''_{HP} + n''_{H_2P})}{V''_w}. \quad (A14)$$

2. Intracellular electroneutrality

$$n''_{Cl} + n''_{H_2P} + 2n''_{HP} = n''_+ + Z_{Hb} n_{Hb}. \quad (A15)$$

3. Donnan equilibrium of Cl^-

$$r = \frac{n''_{Cl}/V''_w}{n'_{Cl}/V'_w}. \quad (A16)$$

4. Donnan equilibrium of H^+

$$0.92r = \frac{n'_H/V'_w}{n''_H/V''_w}. \quad (A17)$$

5. pH dependence of intracellular charge

$$Z_{Hb} = -10[pH'' - (7.2 - 0.016T)]. \quad (A18)$$

6. Concentration dependence of osmotic coefficient of hemoglobin

$$\emptyset_{Hb} = 1.0 + k_1(n_{Hb}/V''_w) + k_2(n_{Hb}/V''_w)^2. \quad (A19)$$

7. Conservation of volume

$$V'_w + V''_w + \gamma = \alpha. \quad (A20)$$

8. Conservation of chloride

$$n''_{Cl} + n'_{Cl} = \beta. \quad (A21)$$

9. Dilutional effects on external medium

$$\frac{\pi'}{RT} = \left(\frac{\pi'}{RT}\right)_o - \left[\emptyset_- \left(\frac{n'}{V'_w}\right)\right]_o \frac{1 - (hct)_o}{1 - hct} + \frac{\emptyset_- [(n'_{Cl})_o - J_{Cl}]}{(V''_w + \gamma)\left(\frac{1 - hct}{hct}\right)}. \quad (A22)$$

10. Empirically determined titration of 50 mM phosphate buffer

$$pH' = pH'_o + k_3 J^*_{OH} + k_4 (J^*_{OH})^2 \quad (A23)$$

where $J^*_{OH} = \dfrac{-J_{OH}}{(V''_w + \gamma)_o \dfrac{1 - (hct)_o}{(hct)_o}}$

and $J_{OH} = -J_{Cl}$.

Equations (1), (8) and (9) can be combined to give an expression for n''_{Cl} as a function of V''_w only:

$$n''_{Cl} = \frac{(V''_w + \gamma)\beta - \dfrac{(A + B)(\alpha[V''_w + \gamma] - [V''_w + \gamma]^2)}{\emptyset_-}}{\alpha} \quad (A24)$$

where

$$A \equiv \frac{\emptyset_+(n''_+ + n''_{HP} + n''_{H_2P}) + \emptyset_{Hb} n_{Hb} + \emptyset_u n''_u}{V''_w} \quad (A25)$$

$$B \equiv \left[\left(\frac{\pi'}{RT}\right)_o - \emptyset_-(n'_{Cl}/V'_w)_o\right]\left[\frac{1 - (hct)_o}{1 - V''_w/\alpha}\right]. \quad (A26)$$

The remaining equations can be combined to give an expression containing only n''_{Cl} and V''_w:

$$0 = C - D - n''_{Cl} - 10 n_{Hb}\left[(k_3/10)\frac{n''_{Cl} - (n''_{Cl})_o}{\alpha - (V''_w)_o} + (k_4/10)\left(\frac{n''_{Cl} - (n''_{Cl})_o}{\alpha - (V''_w)_o}\right)^2 - \log\left(\frac{n''_{Cl}}{\beta - n''_{Cl}}\right) - \log\left(\frac{\alpha - V''_w}{V''_w}\right)\right] \quad (A27)$$

where

$$C \equiv n''_+ + 2n''_{HP} + n''_{H_2P}$$
$$D \equiv (pH')_o - (7.2 - 0.016T) - \log(0.92)(10 n_{Hb}). \quad (A28)$$

Substitution of Eq. (A24) for n''_{Cl} into Eq. (A27) gives an equation in V''_w only. The root of this equation was determined by Muller's method. The appropriate initial conditions, obtained from the solution of the equations described under stage 1, are listed in Table A1. The convergence criterion is the same as for stage 1.

List of Symbols

$\frac{\pi}{RT}$	osmolality
$\emptyset$	osmotic coefficient (molal units)

n	moles of solute
V_w	volume of water
Z_{Hb}	intracellular charge per mole of hemoglobin per pH
T	centigrade temperature
r	Donnan ratio
γ	volume of red cell solids per cell
α	total volume of intracellular water, extracellular water and cell solids, per cell
β	total number of moles of intracellular and extracellular chloride per cell
J	ion number flux across cell ($J > 0$ for inward flux)

List of Subscripts

Cl	chloride
OH	hydroxide
HP	$HPO_4^=$
H_2P	$H_2PO_4^-$
Hb	hemoglobin
+	positively charged solutes
−	negatively charged solutes
u	uncharged solute
$(\ )_o$	initial condition, before water and ion flow

List of Superscripts

$'$	extracellular
$''$	intracellular

List of Constants

k_1	64.5 molal^{-1}
k_2	25.8 molal^{-2}
k_3	631.6 pH/mole OH$^-$
k_4	2.91×10^5 pH/(mole OH)^2
K	6.23×10^{-8} molar