Osmotic Properties of Human Red Cells

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ABSTRACT The hematocrit method as a technique for determining red cell volume under anisotonic conditions has been reexamined and has been shown, with appropriate corrections for trapped plasma, to provide a true measure of cell volume. Cell volume changes in response to equilibration in anisotonic media were found to be much less than those predicted for an ideal osmometer; this anomalous behavior cannot be explained by solute leakage or by the changing osmotic coefficient of hemoglobin, but is quantitatively accounted for by the hypothesis that 20 per cent of intracellular water is bound to hemoglobin and is unavailable for participation in osmotic shifts.

When the chemical potential of the water surrounding a red cell is altered, the cell responds by a shift in volume. The magnitude of this shift provides important clues concerning the state of solvent and solute within the cell. Dick and Lowenstein (1) and Hendry (2) have challenged the long standing observation (3) that a portion of the water of red cells does not appear to take part in these osmotic responses. We have therefore reexamined the hematocrit method, on which most of the earlier work had been based, as a method for measuring cell volume. Our results show that this method provides an accurate estimate of cell volume, even under anisotonic conditions, so long as suitable corrections are made for the medium trapped in the cell column. We have also made new measurements of the osmotic behavior of red cells. The change in red cell water content is less than expected on an ideal basis, an observation we have attributed to the layer of water of hydration surrounding the protein, called "bound water" by Perutz (4), rather than to changes in the osmotic coefficient of hemoglobin with protein concentration as suggested by Maizels and McConaghey (5) and others.

I. EVALUATION OF THE HEMATOCRIT METHOD

In order to evaluate the hematocrit method, it is necessary to compare the results obtained by this technique with volumes determined by an independent procedure. Isotope dilution has been chosen for this purpose, a method which depends only on volume measurements of the total suspension and the medium and therefore is not influenced by the means of separating

the medium from the cells. To provide accurate comparisons, measurements were made by both methods simultaneously on the same suspensions.

EXPERIMENTAL METHOD

Blood was obtained by venipuncture from healthy young male volunteers using heparin (Lilly, 10,000 units/ml, 0.1 ml/100 ml blood) to prevent coagulation. Approximately 30 ml of plasma was removed from each 100 ml of blood to form a suspension of high hematocrit value. Equal aliquots of this enriched suspension were transferred to flasks and precise volumes of buffer solutions of various osmolalities were added. The cell suspension was stirred vigorously during the addition of the strongly hypotonic solutions and no visible hemolysis was produced at any osmolality studied.

The isotonic solution contained (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 per cent CO_2 and 95 per cent air was passed through this solution to bring it to a pH of 7.4. Anisotonic solutions were prepared by alteration of NaCl concentration alone. The osmolality at equilibrium after mixing the anisotonic buffers with the cell suspension ranged from 0.192 osmolal at the hypotonic extreme to 0.480 at the hypertonic; the corresponding range of hematocrit values was approximately 55 to 35 ml cells/100 ml suspension. Osmolality was measured by freezing point depression (Aminco-Bowman, American Instrument Co., 5-2050).

Albumin-I^{131} was added either to the entire cell suspension prior to the pipetting of aliquots or to mixtures of suspension and buffer solution. Aliquots of the final well mixed cell suspension were taken for hematocrit determination and, in triplicate, for radioactive counting. A separate sample was centrifuged at a low gravitational force (870 *g*) to obtain supernatant for determination of freezing point and for counting. The samples of supernatant for counting were taken with the same micropipet used to obtain the samples of suspension. In order to verify that equal volumes were being pipetted, aliquots were weighed in one experiment; the results showed that the pipetted volumes were equal to better than 0.3 per cent.

Hematocrit Measurement

Hematocrit values were determined as previously described (6) using tubes of precision bore glass tubing with an internal diameter of 4.67 mm spun for 25 minutes at 6700 *g*. Packing was complete under these conditions in 20 minutes. The heights of the packed cell column and the total fluid column were measured with an accuracy of 1 part in 500 with an optical comparator. The latter, which consists of a movable stage activating a machinist's dial gauge, has been previously described (7). The hematocrit value was calculated as the ratio of the height of the red cell column, corrected for trapped extracellular fluid (ECF), to the total height of cells plus supernatant fluid. The coefficient of variation of replicate determinations was less than 0.5 per cent.

The trapped ECF correction was determined with albumin-I^{131} as previously described (6). After completion of centrifugation the supernatant fluid was removed by suction without disturbing the packed cells. The surface of the packed cell column

was then washed 4 times with isotope-free buffer having the same osmolality as the cells. 33 measurements were made on blood from 3 different subjects. The trapped ECF ranged from approximately 0.8 per cent for cells swollen in 0.200 osmolal suspension to 1.6 per cent for those shrunken in 0.480 osmolal.

I^{131}-labeled human serum albumin was obtained from Abbott Laboratories (about 13 μc/mg) or from Iso/Serve, Inc. (about 54 μc/mg), and was dialyzed in the cold for at least 48 hours prior to each experiment. Since the albumin was diluted by plasma albumin in a ratio of 1:2000 to 3000, selective binding of labeled albumin to cells or to glass surfaces was minimized. I^{131} was counted in a scintillation counter to at least 10^5 counts.

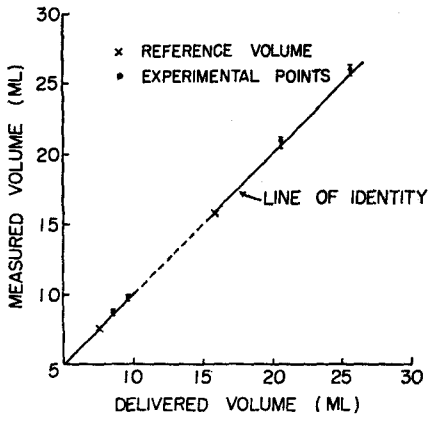

FIGURE 1. ECF volume measured by isotope dilution compared with volume delivered by pipet. The bars around the experimental points give 2 standard deviations.

Measurement of Red Cell Volume by Isotope Dilution

The fractional volume of red cells, h, in each suspension was determined from the formula:

$$h = 1 - V_{ecf}/V_s = 1 - [\text{Alb}^*]_s/[\text{Alb}^*]_{ecf} \quad (1)$$

in which V is the volume (milliliters), [Alb*] is the concentration of albumin-I^{131} (counts per minute per milliliter), and the subscripts *ecf* and *s* refer to extracellular fluid and suspension. Since precisely equal volumes of suspension and supernatant were counted, the amount of albumin-I^{131} (counts per minute) may be substituted for concentration. The standard error for each volume determination based on triplicate counts was 0.3 per cent.

The accuracy of this method for volume determination depends on the validity of two assumptions: (1) that precisely equal volumes of suspension and ECF are delivered for counting, and (2) that all of the isotope is evenly distributed in the ECF and none is either inside the erythrocyte or adsorbed on its surface. The validity of each of these assumptions was first determined independently. The gravimetric verification of assumption 1 has already been described. For assumption 2 the trapped plasma experiments indicate that no more than 0.8 per cent of the albumin can be associated with red cells. Since there must be some trapped ECF, it is clear that only a very small

fraction of the albumin is on or in the cells. This is supported by the observations of Brown (8), who showed that untreated erythrocytes adsorbed less than 1 per cent of albumin added to the ECF. Though this could be a source of error leading to an overestimate of trapped plasma in the hematocrit experiments, it is of negligible importance in the measurement of cell volume by isotope dilution.

The accuracy of the method can also be tested in an experiment which checks

TABLE I

SIMULTANEOUS MEASUREMENT OF RED CELL VOLUME BY HEMATOCRIT AND ISOTOPE DILUTION METHODS

Experiment	Osmolality	Red cell volume		Ratio (1)/(2)
		Hematocrit method (1)	Isotope dilution method (2)	
	osmols	*ml cells/ml suspension*		
1	0.369	0.3876	0.3899	0.9941
	0.326	0.4137	0.4167	0.9928
	0.284	0.4474	0.4460	1.0031
	0.242	0.4932	0.4954	0.9956
	0.205	0.5494	0.5495	0.9998
2	0.480	0.3238	0.3229	1.0028
	0.422	0.3410	0.3431	0.9939
	0.376	0.3613	0.3660	0.9872
	0.330	0.3867	0.3920	0.9865
	0.282	0.4217	0.4176	1.0098
	0.245	0.4609	0.4661	0.9888
	0.224	0.4878	0.4857	1.0043
	0.192	0.5318	0.5306	1.0023
3	0.477	0.3604	0.3629	0.9931
	0.370	0.4042	0.4056	0.9965
	0.287	0.4766	0.4787	0.9956
	0.228	0.5442	0.5460	0.9967
	0.204	0.5872	0.5855	1.0029
Mean				0.997
SE				0.002

both assumptions simultaneously. To 3 identical aliquots of cell-enriched suspension containing albumin-I^{131} 3 different known volumes of isotonic buffer solution were added. Since the volume of plasma is not known precisely, the total volume of ECF is "unknown," but the increments in ECF volume between the first suspension and each of the other 2 suspensions are known precisely. Fig. 1 shows the volume of ECF as determined by albumin-I^{131} dilution, plotted against the actual volume delivered. The reference volume in each experiment is marked by an X on the line of identity; the bars about the volume determinations for the other two suspensions represent 95 per cent confidence limits. These experiments indicate that albumin-I^{131} provides a

reliable measure of the extracellular space in a red cell suspension, and thus can be used with confidence to determine that volume in the suspension from which it is excluded, the cell volume.

RESULTS

Hematocrit volumes, corrected individually for trapped plasma, and isotope dilution volumes were measured on the same suspensions over a wide range of osmolalities. The data obtained with blood from 3 different individuals are given in Table I and the results of experiment 2 are shown in Fig. 2. The mean of the ratio of the hematocrit volume to the isotope dilution volume is 0.997

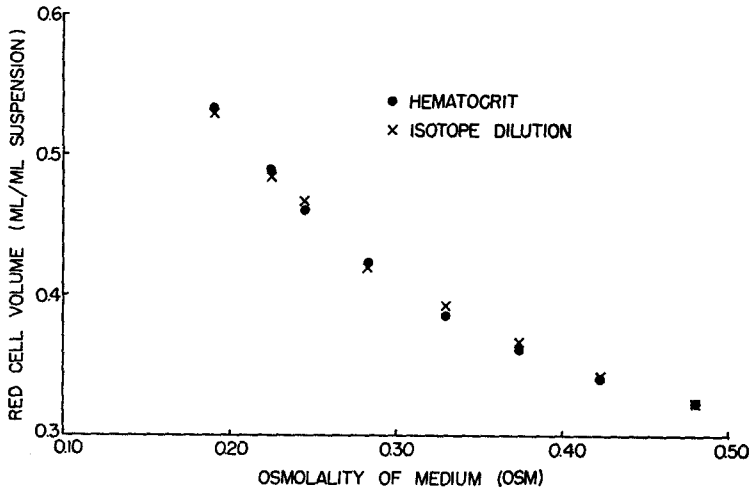

FIGURE 2. Cell volume measured simultaneously by hematocrit and isotope dilution method.

which does not differ significantly from unity ($p > 0.10$). There remains the possibility that the hematocrit volume deviates systematically from the true (isotope dilution) volume at the extremes of osmolality, for example that the hematocrit value might give readings that are too low in the hypotonic range and too high in the hypertonic range. Regression analysis of the volume ratio as a function of osmolality at 18 points gives a correlation coefficient of -0.32; this indicates the absence of significant correlation ($p > 0.10$) and demonstrates that, as expected from Fig. 2, there is no systematic deviation of hematocrit volumes from true volumes even at the extremes of osmolality.

Our results therefore indicate that the hematocrit determination, after proper correction for trapped ECF, provides a precise and accurate method for the determination of red cell volume. Hendry (2) who has questioned the validity of the hematocrit method has argued that centrifugation causes compression of cells, the effect increasing as the volume of the cell increases.

We found no evidence for compression during centrifugation at 6700 *g*, higher than the forces used for most clinical and research hematocrit determinations. The difficulty that previous workers have found with the hematocrit method may be due to lack of precise correction for trapped plasma under each new set of conditions, failure to spin to constant packing of cells, or to inaccurate measurements of the volume of cells and total volume of suspension. The first of these factors appears to be the cause of LeFevre's (9) observation that hematocrit volumes were slightly but consistently larger than cell volumes measured by isotope dilution. When these three factors are all controlled, our results indicate that the hematocrit method gives a valid measurement of the volume of normal cells over a wide range of osmotic conditions.

II. OSMOTIC EQUILIBRIUM OF ERYTHROCYTES

The second part of the study is concerned with a reexamination of the question: Is all the water in the red cell solvent water in the sense that it is free to participate in the response of the cell to osmotic gradients across the cellular membrane? For this purpose the equilibrium volumes of red cells were measured over a wide range of osmolalities.

EXPERIMENTAL METHOD

Measurement of Osmotic Pressure

Osmotic pressures of plasma, buffer solutions, and suspension media were determined by freezing point depression, expressed in units of osmolality. NaCl osmolality (molality times molal osmotic coefficient (10)) was plotted against freezing point lowering giving a linear calibration scale; experimental osmolalities were determined by interpolation of freezing points on this scale. The instrument was calibrated daily. Duplicate determinations of osmolality on the same solution agreed to within 0.003 osmolal, or approximately 1 per cent.

To determine water content gravimetrically, it is necessary to measure: the dry to wet weight ratio for the suspension, M_s, and for the plasma, M_p; the relative volume of red cells, h, in the suspension; the specific gravity of the suspension, ρ_s; and the specific gravity of the plasma, ρ_p. The fractional water content by volume, V_w/V_c (ml H_2O/ml cells; the subscripts w and c refer to water and cell) may then be calculated.

$$V_w/V_c = \rho_s(1 - M_s)/h - \rho_p(1 - h)(1 - M_p)/h \qquad (2)$$

For measurement of M_s and M_p, portions of suspension and of plasma were delivered into tared bottles and weighed. They were then evaporated to constant dry weight by heating for several days at 70°C. Duplicate determinations of each dry to wet weight ratio always agreed to within 0.35 per cent and usually to within 0.15 per cent. For measurement of ρ_s, portions of the suspensions were weighed into tared hematocrit tubes of precision bore glass tubing as described above. The volumes were

determined from the height of the column. Duplicate determinations of specific gravity usually agreed to within 0.8 per cent. ρ_p was measured directly as the weight of 1 ml aliquots of plasma.

RESULTS

Table II gives the volume ratio under isotonic conditions, V_{wo}/V_{co}, for red cells from 6 different individuals each determined in duplicate or triplicate In this case, the isotonic suspension medium was the cells' own plasma. The mean water content of 0.717 ml H_2O/ml cells agrees well with the value of 0.722 gm H_2O/ml cells found by Hald *et al.* (11). The mean value was therefore used in calculations for the first three experiments, in which V_{wo}/V_{co}

TABLE II
FRACTIONAL WATER CONTENT OF ERYTHROCYTES

Experiment	V_{wo}/V_{co}
	ml H_2O/ml cells
4	0.725
5	0.714
6	0.698
7	0.724
8	0.711
9	0.727
Mean	0.717
SE	0.005

was not directly measured. The non-water volume, b, was determined in the usual fashion as $b = V_{co} - V_{wo}$. Since, as will be demonstrated, there was no net leakage of solute into or out of the cell during the course of the experiments and b remained constant, volume changes were entirely due to water shifts, so that

$$V_w = V_c - (V_{co} - V_{wo}) \tag{3}$$

The equilibrium volume of red cells from 9 different subjects was determined by the hematocrit method over a range of osmolalities from 0.192 to 0.480. The osmotic pressure inside the red cell equals the external osmotic pressure at equilibrium as shown by freezing point measurements for the human red cell by Williams *et al.* (12). Thus any resistance to swelling or shrinking offered by the cell membrane or any internal structure is negligible compared with the osmotic forces. Therefore, using van't Hoff's law

$$\pi = RT \Sigma_i \Phi_i(n_i)/V_w \tag{4}$$

in which π is the osmotic pressure in dynes/cm^2, R is the gas constant in [ml (dynes/cm^2)/mol degree], T is the absolute temperature, Φ_i is the practical osmotic coefficient for the solute i, (n_i) is the amount of solute i in mols, and V_w is water volume in milliliters. If we make the trial assumption that all the osmotic coefficients are constant despite the change in osmolality, equation 4 may be written

$$\pi = \Phi RT(n)/V_w \tag{5}$$

in which Φ is a constant, the mean osmotic coefficient for all intracellular solutes, and (n) is the total amount of intracellular solute. This is a reasonable assumption in the case of KCl, the principal solute inside the red cell, since

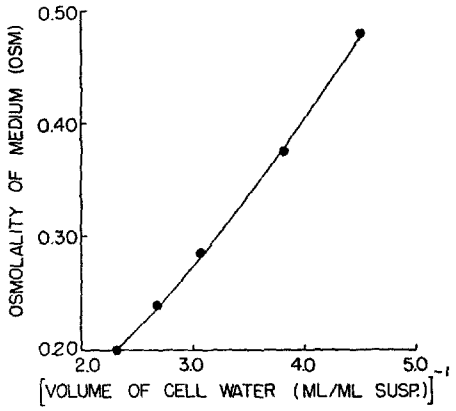

FIGURE 3. Osmolality of the medium as a function of the reciprocal of the volume of cell water, $1/V_w$, following equation 5.

Φ for KCl in water solution only changes from 0.93 at 0.2 osmolal to 0.91 at 0.5 osmolal. Equation 5 now predicts that π will be a linear function of $1/V_w$ with a zero intercept. A single experiment plotted in this fashion is shown in Fig. 3; the departure from linearity is apparent. To demonstrate this analytically, the data for each of the experiments have been examined using a 1620 IBM computer to provide a best fit by least squares to the polynomial

$$\pi = \alpha_0 + \alpha_1/V_w + \alpha_2/V_w^2 \tag{6}$$

The data from all 9 subjects give mean coefficients of $\bar{\alpha}_0 = 0.033 \pm 0.006$, $\bar{\alpha}_1 = 0.045 \pm 0.004$, and $\bar{\alpha}_2 = 0.010 \pm 0.001$. Each of these coefficients is significantly different ($p < 0.01$) from zero. The non-zero $\bar{\alpha}_0$ indicates a non-zero intercept; the non-zero $\bar{\alpha}_2$ indicates a significant departure from linearity. The data therefore confirm previous findings that the red cell does not behave as a perfect osmometer. In order to explain this deviation from expected behavior it is necessary to examine three possible causes: (1) the possibility that solute leaks into or out of the cell; (2) the concentration de-

pendence of Φ for hemoglobin; and (3) the presence of non-solvent water in the cell.

Is There a Net Change in the Amount of Intracellular Solute or in Non-Water Volume?

The first possible explanation is that the red cells leak solute in hypotonic solutions and gain solute in hypertonic solutions. The results of experiments to examine this question are given in Table III. 10 ml aliquots of a red cell suspension of hematocrit value 0.6510 were pipetted into 3 flasks. To flask 1 was added 5 ml of 0.072 osmolal buffer; to flask 2, 5 ml of 0.282 osmolal buffer (matching that of the plasma); to flask 3, 5 ml of 0.492 osmolal buffer. After 40 minutes, 5 ml of 0.492 osmolal buffer was added to flask 1, 5 ml of

TABLE III

EXAMINATION OF SOLUTE LEAKAGE HYPOTHESIS

Original suspension Hct	First addition of buffer Osmolality	First addition of buffer Hct		Second addition of buffer Osmolality	Second addition of buffer Hct
	osmols	*ml cells/ml suspension*		*osmols*	*ml cells/ml suspension*
0.6510	0.072	0.5426	←40 min.→	0.492	0.3126
	0.282	0.4213		0.282	0.3127
	0.492	0.3622		0.072	0.3129

0.282 osmolal buffer to flask 2, and 5 ml of 0.072 osmolal buffer to flask 3. The total amount of solution added to each of the 3 flasks was now equivalent to 10 ml of 0.282 osmolal buffer. Since the final hematocrit determination in all 3 flasks is equal there has been no solute leakage in either direction, allowing unequivocal rejection of the hypothesis that solute leakage accounts for the departure from expected osmotic behavior.

As Jacobs and Stewart (13) have pointed out, the possibility also exists that pH changes will cause unexpected changes in cell HCO_3 due to differences in the Donnan ratio, and hence in the distribution of Cl. Since there was no significant difference in pH over the entire range of osmolalities studied in these experiments, no significant shift in cell HCO_3 is expected to arise from this cause.

In addition, the water content of swollen and shrunken cells was calculated, using the formula $V_w = V_c - b$, from cell volumes measured by the hematocrit method and the non-water volume determined by desiccation of cells in isotonic buffer. The calculated values for V_w/V_c were 0.750 in the swollen cells and 0.601 in the shrunken cells; the V_w/V_c actually measured by desiccation were 0.749 and 0.606 respectively. It is clear that changes in water content quantitatively account for the volume shifts and that b, the non-water volume, is constant despite changes in cell volume.

Will Changes in Φ_{Hb} Account for the Deviation?

Equation 4 may be written as

$$\pi = RT[\Phi_{Hb}(Hb)/V_w + \Phi_S(S)/V_w] \tag{7}$$

in which Φ_{Hb} is the osmotic coefficient for hemoglobin, Φ_s is the mean osmotic

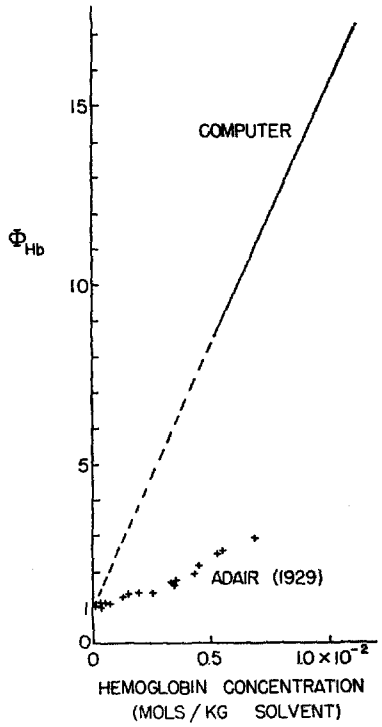

FIGURE 4. Osmotic coefficients of hemoglobin, as measured by Adair (14) for ox hemoglobin and as required by computer analysis of equation 9 to account for the disparity in the osmotic behavior of the cells.

coefficient for the remainder of the intracellular solutes, and (Hb) and (S) are the amounts of intracellular hemoglobin and of the rest of the intracellular solutes. We assume that Φ_s is constant over this range of concentrations and that Φ_{Hb} may be represented by the virial equation

$$\Phi_{Hb} = 1 + \beta_1\left[\frac{(Hb)}{V_w}\right] + \beta_2\left[\frac{(Hb)}{V_w}\right]^2 + \cdots \tag{8}$$

in which Φ_{Hb} approaches 1 as hemoglobin concentration approaches zero. Equation 7 becomes

$$\pi = RT[(Hb) + \Phi_S(S)]\left[\frac{1}{V_w}\right] + RT[\beta_1(Hb)^2]\left[\frac{1}{V_w}\right]^2 + \cdots \tag{9}$$

This polynomial may also be evaluated using the coefficients found for equation 6. According to equation 9 α_0 should be zero and α_2 should be given by $RT[\beta_1(\text{Hb})^2]$. The mean least square value[1] of $\bar{\alpha}_0 = 0.033$ is inconsistent with the prediction of equation 9 that α_0 should be zero. The value of α_2 may be used with the known concentration of Hb to calculate β_1, which is equal to 21.7 ± 1.0 ml/gm Hb. From Adair's studies (14) an estimate may be made of the osmotic coefficients of ox hemoglobin in solution in concentrations in the isotonic range. The maximum value for β_1 from his data is 3

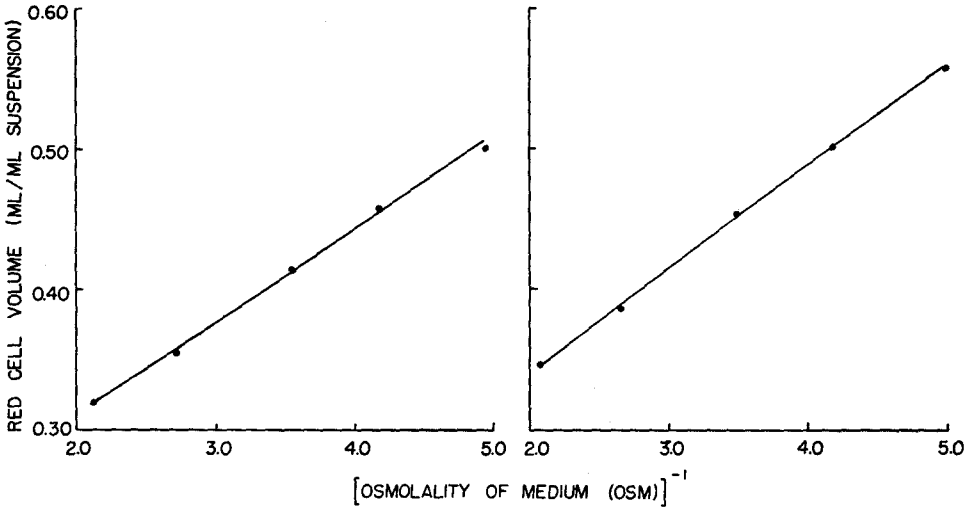

FIGURE 5. Cell volume as a function of the reciprocal of the osmolality of the medium, following equation 10. Two separate experiments are shown, the left-hand one with a slight upward convexity and the right-hand one with a slight downward convexity.

ml/gm Hb. As shown in Fig. 4, his findings differ significantly from the equation which fits our data, and the measured osmotic coefficient for hemoglobin is far too small to account for our results.

Is There Non-Solvent Water in the Cell?

In order to determine whether there is any non-solvent water in the cell, we may use equation 5 as a starting point and see whether V_w, the total water content in the cell, is equal to the solvent water in the cell, V_w'. If this is the case,

$$\pi V_w = \Phi RT(n)$$

[1] The coefficients α that we have obtained are derived from a second order polynomial and are not necessarily virial coefficients. Similarly, the values of β that we have calculated from Adair's data are derived from a second order polynomial and need not necessarily represent virial coefficients.

Since n is a constant, the right side of the equation is constant if Φ is independent of concentration. The excursion in Φ over our range of osmolalities may be computed from Adair's data expressed as a virial equation together with the tabulated values (10) for the osmotic coefficients for KCl and NaCl in water. With these assumptions the change in Φ over the entire range is only 3 per cent, so that it too may be assumed constant. Then πV_w should be equal to $\pi_0 V_{wo}$ for any conjugate values of π and V_w. Since it has been shown that $V_c = V_w + b$,

$$\pi V_w = \pi(V_c - b) = \pi_o V_{wo}$$

TABLE IV

FRACTION OF CELL H_2O THAT BEHAVES AS SOLVENT H_2O

Experiment	Fractional water content V_{wo}/V_{co}		Solvent H_2O (1)/(2)	Fractional osmotic dead space b/V_{co}		Solvent H_2O [1 − (5)]/[1 − (4)]
	Measured (1)	Derived from γ_1 (2)		Measured (4)	Derived from γ_0 (5)	
1	(0.717)*	0.589	0.822	(0.283)*	0.414	0.817
2	(0.717)	0.560	0.781	(0.283)	0.439	0.783
3	(0.717)	0.597	0.832	(0.283)	0.401	0.836
4	0.725	0.563	0.777	0.275	0.434	0.780
5	0.714	0.581	0.814	0.286	0.419	0.814
6	0.698	0.556	0.796	0.302	0.444	0.796
7	0.724	0.563	0.777	0.276	0.435	0.781
8	0.711	0.561	0.789	0.289	0.440	0.788
9	0.727	0.579	0.797	0.273	0.422	0.795
Mean			0.798			0.799
SE			0.007			0.006

* V_{wo}/V_{co} obtained from mean for experiments 4 through 9.

or

$$V_c = \pi_0 V_{wo}\left[\frac{1}{\pi}\right] + b \qquad (10)$$

Fig. 5 shows a plot of V_c against $1/\pi$ for two experiments. To obtain a quantitative measure of the agreement with equation 10, the polynomial

$$V_c = \gamma_0 + \gamma_1\left[\frac{1}{\pi}\right] + \gamma_2\left[\frac{1}{\pi}\right]^2 + \cdots \qquad (11)$$

was evaluated using the 1620 computer to obtain least squares values for the coefficients. The departure from linearity was small in every experiment; the curvature was concave upward in 5 and downward in 4 experiments, as

shown in the examples in Fig. 5. The mean coefficient of the second order term, $\bar{\gamma}_2$, was -0.001 ± 0.002 which does not differ significantly from zero. If zero were the true mean, a difference from zero of at least this magnitude would be expected in over 50 per cent of a series of 9 experiments, so that V_c is indeed linear with $(1/\pi)$.

Table IV gives values for V_{wo}/V_{co} and for b/V_{co} for each of the 9 experiments. The V_{wo}/V_{co} derived from the coefficient γ_1 is 0.80 of that actually measured by desiccation. This is exactly equal to the ratio $[(V_{co} - b_{calc})/(V_{co} - b_{des})]$ in which b_{calc} is the osmotic dead space derived from γ_0 and b_{des} is the non-water space measured by desiccation. Both parameters give a consistent result indicating that equation 10 is a good description of the system, and that only 80 per cent of the cell water participates in the osmotic behavior of the cell. Thus the value of solvent water V'_w can be expressed by $V'_w/V_w = V_{w,\,calc}/V_{w,\,des} = 0.798 \pm 0.007$. This is equivalent to setting R = 0.80 in Ponder's (3) equation, $V_c = RWV_{co}\left(\frac{\pi_0}{\pi} - 1\right) + V_{co}$. Since cell water is 0.717 ± 0.005 ml H_2O/ml cell, solvent water is 0.572 ± 0.009 ml H_2O/ml red cell, which lies between LeFevre's (9) two estimates of 0.614 and 0.562 gm H_2O/ml cells.

If this value for apparent water volume is substituted into equation 5, the polynomial of equation 6 becomes

$$\pi = \alpha'_0 + \alpha'_1\left[\frac{1}{V'_w}\right] + \alpha'_2\left[\frac{1}{V'_w}\right]^2 + \cdots \qquad (12)$$

Computer evaluation yields $\bar{\alpha}'_2 = 0.007 \pm 0.005$, $\bar{\alpha}'_1 = 0.066 \pm 0.003$, and $\bar{\alpha}'_2 = 0.0004 \pm 0.0003$. $\bar{\alpha}'_0$ and $\bar{\alpha}'_2$ are not significantly different from zero ($p > 0.10$) so the experimental data do indeed fit the equation

$$\pi = \Phi RT(n)/V_w' \qquad (13)$$

In order to demonstrate that V'_w/V_w does not depend on cell volume or hemoglobin content or concentration in the range found in normal cells, these parameters were calculated in the usual fashion from measurements on whole blood of hematocrit determination, red cell count, and hemoglobin concentration. Red cell counts were made with a Coulter counter, and the standard error, based on a triplicate determination, was 2.2 per cent. Hemoglobin was determined spectrophotometrically, using reagent and standard solution supplied by Hycel, Inc. Concentration measurements were made in duplicate and usually agreed within 0.1 gm per cent of hemoglobin. As expected, there was no significant correlation of any of these parameters with V'_w in the normal cells used in these experiments. The mean hemoglobin content of these cells, collected from young males, was 35.1 ± 0.5 gm/100 ml

cells, somewhat higher than the commonly used value for the normal hemoglobin content for cells from a random adult population, 33.5 gm/100 ml cells (15).

DISCUSSION

We have confirmed the finding of previous investigations that red cells neither swell nor shrink as much as would be expected from simple theory. The work of Dick and Lowenstein (1) using a different method has cast doubt on these previous observations. These investigators estimated the volume of individual red cells in hypotonic solutions using immersion refractometry and found that cell swelling agreed quite closely with that predicted by equation 7 using Adair's data for Φ_{Hb}. Our results do not fit equation 7 and cannot be explained by Adair's value for Φ_{Hb}, extrapolated by a polynomial equation. The difference between the results obtained by Dick and Lowenstein and the present ones, which are in agreement with those obtained by many previous investigators, may lie in the method of immersion refractometry. In this method the cell volume is calculated from the concentration of albumin solution which has exactly the same refractive index as the cells. From this albumin concentration, an intracellular solute concentration and, finally, an intracellular solvent volume is calculated. Dick and Lowenstein's results may conform to equation 7 because immersion refractometry measures V_w' rather than V_w. If this is indeed the case, their method may provide a means of measuring V_w' directly in a number of types of cells.

Maizels and McConaghey (5) have also found that shrinking of red cells in hypertonic solutions was less than that expected. They attributed the deviation to the rising osmotic coefficient of hemoglobin with increased concentration and calculated the osmotic coefficient required to account for the deviation. The osmotic coefficients required ranged from 2 in hypotonic solutions (a hemoglobin concentration of 6 mmole Hb/kg cell water) to 12 in their most hypertonic case (18 mmole Hb/kg cell water). These osmotic coefficients are considerably higher in the hypertonic range than the osmotic coefficients measured by Adair and extrapolated according to the polynomial equation. Nonetheless, Maizels and McConaghey's osmotic coefficients are considerably lower than the values of 9 to 18 which are required to account for our deviations.

Our experiments indicate that equation 7 does not fit the data using Adair's osmotic coefficients when extrapolated by a reasonably continuous function such as equation 8. It is possible that the extrapolation need not be continuous and that the osmotic behavior of hemoglobin in a human red cell is different from that in a solution of the ox hemoglobin at the same concentration. This possibility might be supported by the ease with which hemoglobin-S crystallizes in sickle cells (16). However, Bateman *et al.* (17) indicate from x-ray

diffraction studies that the hemoglobin in isotonic human red cells is randomly oriented, and that this orientation persists even when the cells are shrunken in a medium of 0.620 osmolal. Furthermore their diffraction data are in agreement with the results of Riley and Herbert (18) who studied the x-ray diffraction of human hemoglobin in solution over the same concentration range. Since the x-ray diffraction appears to be the same in shrunken cells as in hemoglobin solutions of the same concentration, there seems no reason to expect a jump in osmotic coefficient by a factor 3 to 5 times that found in free solution, as required to account for our data, nor to expect that the disparity may be attributed to a discontinuity in the osmotic coefficient of human hemoglobin.

It seems more probable that the water of hydration of the protein is not available for the dilution of salts, principally KCl. Since b is a constant independent of the volume of the cell, the non-solvent water is present in a constant amount, as would be the case if it were protein water of hydration. Perutz (4) has shown that horse methemoglobin contains a significant amount of bound non-solvent water. This bound water of hydration is equivalent in amount to a layer whose weight is 0.3 gm H_2O/gm anhydrous protein, in good agreement with the observation of Adair and Adair (19) that the water of hydration of human hemoglobin in solution may reach a volume of 0.34 gm H_2O/gm anhydrous protein. These results have been confirmed by Drabkin (20) who has determined that 0.339 gm of H_2O is bound to 1 gm of crystalline human hemoglobin and that this water is not available for the solution of phosphate in the medium. On the basis of 35 gm hemoglobin/100 ml cells, some 12 gm of H_2O would be present as water of hydration. Table II shows that the water content of our cells is 72 gm/100 ml cells. 20 per cent of this water or 14 gm/100 ml cells appears to behave as non-solvent water. Thus there is good agreement between the measured water of hydration and the amount of non-solvent water, which leads us to conclude that the apparent anomalies of the osmotic behavior of the red cell may be attributed to the non-solvent character of the water of hydration of intracellular hemoglobin. This agreement is consonant with the hypothesis put forward by Drabkin that the non-solvent water present in hemoglobin crystals is also present when hemoglobin is in solution in human red cells.

Dick (21) has summarized observations indicating that many types of cells, for example rat lymphocytes and mouse ascites tumor cells, exhibit similar anomalous osmotic properties. The explanation for this behavior may well be found in non-solvent water bound to proteins and other intracellular components. Since proteins are a normal component of a wide variety of cells, the present findings suggest that non-solvent water may play a part in the osmotic behavior of most tissues.

Preliminary studies on this problem were carried out by Peter Hinkle as part of his undergraduate thesis at Harvard University.

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