

# Nonsolvent Water in Human Erythrocytes

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**ABSTRACT** From the ability of a concentrated suspension of human erythrocytes to regulate the pH of unbuffered, anisotonic, external media it is possible to calculate the fractional cell volume in which chloride is dissolved. The difference between this volume and the total cell water gives the nonsolvent water (for chloride) of the cell. Nonsolvent water is less than 3% of the isotonic cell volume. The quantity of nonsolvent water per cell may increase as the cells shrink in hypertonic solutions.

A significant fraction of intracellular water appears to be in a form different from bulk water; and much of this water is bound to proteins (for reviews, see Dick, 1965; Bernal, 1966; Hechter, 1965). Of pertinence to red cells, several lines of evidence indicate that hemoglobin in solution binds about 0.3 g H<sub>2</sub>O/g protein (Adair and Adair, 1936; Schwann, 1965), which amounts to about 14% of the total cell volume or 20% of the total cell water.

These observations have been used to explain the departures from "ideality" of erythrocyte volume changes in anisotonic media. Since the cells are about 70% water by volume, and yet only about 55% of the cell appears to respond to changes in tonicity (Ponder, 1948), the difference has been ascribed to bound water which is not available for solution of the principal solutes (LeFevre, 1964; Savitz et al., 1964).

There is, however, other evidence which makes it difficult to accept this conclusion. For example, measurements of Miller (1964) showed that glucose and fructose dissolve in *all* of the cell water over a wide range of cell volumes. Moreover, if all of the known constituents of the cell were dissolved in only 80% of the cell water, the calculated internal osmotic pressure would be significantly higher than that of the medium; to reconcile the difference, it is necessary to make further assumptions, such as binding or sequestering of cell solutes, or reduced activity coefficients of solutes, or a high internal hydrostatic pressure, etc. There is no compelling evidence to give quantitative support to such assumptions.

This paper presents a study of nonsolvent water in human erythrocytes, with nonsolvent water being defined as that amount of water which is not

available for solution of chloride. First, some of the properties of erythrocytes in the media used are established: total water content is determined, and the volume responses to changes in osmolality are measured. The nonsolvent water is then estimated from an equation which relates nonsolvent water to the buffering effects of a concentrated suspension of cells in an unbuffered medium. The conclusion is drawn from the data that the nonsolvent water for chloride is small, at most 3% of the isotonic cell volume. This nonsolvent water volume appears to be variable with the concentration of the medium.

#### MATERIALS AND METHODS

Freshly drawn and heparinized blood from healthy medical students was washed in five changes of medium; the white cells were removed on the first and second washes.

Washing and suspending media were either (a) NaCl buffered to pH 7.38 with  $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  (NaCl-phosphate solution) or (b) NaCl plus sucrose. In order to maintain a constant relationship between the chloride concentration and the freezing point depression in all media (see equation 5), media were always made up by mixing 4 volumes of a NaCl solution of a given freezing point depression and 1 volume of either phosphate buffer or sucrose solution with the same freezing point depression.

Water activity of the various solutions is expressed in terms of milliosmolality (milliosmolal or milliosmols/kg  $\text{H}_2\text{O}$ ), symbolized  $\pi$ , where a 1000 milliosmolal solution has a freezing point of  $-1.86^\circ\text{C}$ . Freezing points of the solutions were determined with a Fiske osmometer (Fiske Associates Inc., Bethel, Conn.). The standard medium, referred to here as isotonic, had a freezing point depression corresponding to 305 milliosmols/kg  $\text{H}_2\text{O}$ .

pH was determined with a Radiometer pH meter, model 4, with the capillary glass electrode and calomel electrode thermostatted to  $25^\circ\text{C}$  (Radiometer Co., Copenhagen, Denmark).

Relative cell volumes were determined from the ratio of hematocrit to hemoglobin concentration of a given suspension. For hematocrits duplicate samples of the suspension were drawn up into constant-bore capillary tubing and spun for 15 min in an International microcapillary centrifuge, model MB. After centrifugation, the tubes were placed on a holder and read under low power on a Zeiss microscope (Carl Zeiss, Inc., New York). The bottom and top of the sample and the top of the packed cell column were in turn aligned with a hairline in the eyepiece, and their positions read from the Vernier scale on the mechanical stage. Duplicate readings usually agreed to within less than 0.5%. The trapped plasma, labeled with radioiodinated serum albumin, was found to vary only from 0.8% in the most hypotonic solution to 1.1% in the most hypertonic. Consequently, a trapped plasma correction of 1.0% was made for all hematocrits. Hemoglobin was measured by the optical density at  $540\text{ m}\mu$  15 min after the appropriate dilution of the cell suspension was made. Since the hemoglobin determinations were used only for relative values within each experiment, the optical density measurements were not converted into absolute hemoglobin concentrations.

The volume of cell water was determined by drying suspensions to minimum weight under conditions described in part 1 of Results. The densities of the suspensions having

been determined from the weight of known volumes, the volume of cell water was calculated from an equation which assumes that the partial specific volume of intracellular water is 1.00:

$$V_{\text{H}_2\text{O}} = \frac{\rho_s}{\rho_w H} \left( \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \right)_s - \frac{W_m}{H} (1 - H) \quad (1)$$

where  $V_{\text{H}_2\text{O}}$  is the water volume of the cells expressed as a fraction of the cell volume,  $\rho_s$  is the density of the suspension,  $\rho_w$  is the density of water (= 1.00 g/ml),  $H$  is the corrected hematocrit of the suspension, and  $W_m$  is the fractional water content by volume of the medium.

For chloride determinations, suspensions of high hematocrit were diluted in 6 volumes of distilled water to lyse the cells. 1 volume of 50% TCA was added and proteins were allowed to precipitate 30 min in the cold. Chloride in the supernatant was titrated on the Buchler-Cotlove chloridometer (Buchler Instruments, Inc., Fort Lee, N.J.). Corrections were made for the volume of protein which had been precipitated out. From the total chloride concentration of the suspension, the hematocrit, and the known chloride concentration of the medium, it was possible to calculate the chloride concentration in the cells,  $[\text{Cl}]_c$ . The method was checked by suspending a sample of cells in isotonic NaCl-phosphate at several hematocrits, varying from 0.46 to 0.88. The values of  $[\text{Cl}]_c$  determined in these suspensions agreed to within 0.3 meq/liter cells.

All experiments were carried out at room temperature, approximately 25°C.

Cell volumes and subdivisions of cell volumes are expressed as fractions of the isotonic volume. The following symbols are used:

$V^o$  = volume of cells in isotonic medium, taken as unity;

$V$  = volume of cells in any medium;

$V_{\text{H}_2\text{O}}^o$  = total water volume of cells in isotonic media;  $V_x^o$  = volume of cell solids;

$V_x^o \equiv V^o - V_{\text{H}_2\text{O}}^o$ ;

$b$  = "osmotically inactive volume," defined by equation (2) below;

$V_{\text{nsw}}^o$  = volume of nonsolvent water.

## RESULTS

### 1. Total Water Volume, $V_{\text{H}_2\text{O}}^o$

The total water volume,  $V_{\text{H}_2\text{O}}^o$ , of red cells equilibrated with the isotonic NaCl-phosphate buffer was determined by drying a concentrated cell suspension of known hematocrit to minimum weight;  $V_{\text{H}_2\text{O}}^o$  was calculated from equation (1). Since it has been suggested (LeFevre, 1964) that  $V_{\text{H}_2\text{O}}^o$  measurements depend on the conditions under which the samples are dried, the determinations were made by drying at 95°C, 70°C, and at room temperature in a vacuum desiccator. The results are given in Table I. Although there is no significant difference between the averages of all results obtained under the three conditions, in each individual blood sample a smaller water volume was consistently found in the desiccator-dried samples as compared

to the oven-dried samples. The reason for the difference is not certain. It was noted during the measurements, however, that after any given sample had maintained a constant minimum weight for about 24 hr, on subsequent days under continued drying conditions there was a slight gain in weight, possibly due to the oxidation of the dried residuum. Since the *rate* of drying is different under the three conditions, it is possible that competing reactions of weight loss due to evaporation of water and a small weight gain due to other processes give slightly erroneous results in all samples. We assume that the highest value of  $V_{\text{H}_2\text{O}}^{\circ}$ , corresponding to the greatest weight loss on drying, is the most likely; therefore, in the remainder of this paper,  $V_{\text{H}_2\text{O}}^{\circ}$  will be taken as 0.696, which is the mean calculated from samples dried at 95° C. Correspondingly, by definition, the volume of cell solids,  $V_x^{\circ}$ , will be taken as 0.304.

TABLE I  
TOTAL ERYTHROCYTE WATER ( $V_{\text{H}_2\text{O}}^{\circ}$ ) AS DETERMINED BY DRYING  
TO MINIMUM WEIGHT UNDER THREE SETS OF CONDITIONS

Conditions	Blood donor					Mean $V_{\text{H}_2\text{O}}^{\circ}$ ( $\pm$ sd)
	E. R.	M. Sa.	M. Sm.	J. K.	D. A.	
95° C	0.703	0.691	0.686	0.697	0.697	0.696 $\pm$ 0.005
	0.702	0.694		0.699	0.699	
70° C	0.693	0.685	—	0.689	0.691	0.690 $\pm$ 0.004
	0.697	0.685		0.691	0.690	
Vacuum desiccator, Room temperature	0.658	0.680	—	0.685	0.690	0.679 $\pm$ 0.013
	0.659	0.682		0.685	0.692	

It is to be emphasized that these values were obtained with cells equilibrated with the standard NaCl-phosphate medium, and do not necessarily represent the water and solid contents of the cells in their own plasma.

## 2. "Osmotically Inactive Volume," $b$

The well-known "perfect osmometer" equation given below (equation 2) is based on the assumption that the isotonic volume,  $V^{\circ}$ , of erythrocytes may be subdivided into two components, an "osmotically inactive volume,"  $b$ , and an "osmotically active volume," ( $V^{\circ} - b$ ) (Lucké and McCutcheon, 1932). By this definition, the former is a fraction of the volume which remains constant regardless of the osmolality of the medium, whereas the latter varies inversely with the osmolality. Hence the volume at any osmolality may be expressed

$$V = \frac{\pi^{\circ}}{\pi} (V^{\circ} - b) + b \quad (2)$$

where  $V$  is the cell volume in medium of osmolality  $\pi$ ,  $V^o$  is the cell volume in isotonic medium of osmolality  $\pi^o$ , and  $b$  is constant. Since  $\pi^o$  and  $(V^o - b)$  are all constant in this formulation,  $b$  may be determined by plotting  $V$  against  $1/\pi$  and extrapolating to the ordinate. Fig. 1 is a graph of such an experiment in which  $b$  was found to be 0.48. (In this experiment, the pH of the suspension was adjusted to 7.37–7.38 at all osmolalities.) The mean of 15 such determinations of  $b$  in NaCl-phosphate media was  $0.476 \pm 0.025$  (standard deviation). The “osmotically active volume,”  $(V^o - b)$ , was therefore 0.524.

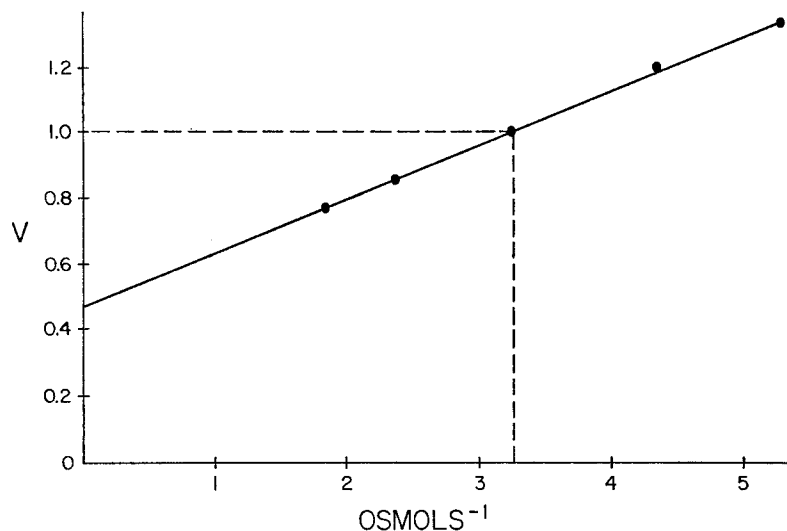


FIGURE 1. Cell volume as a function of the reciprocal of osmolality of the NaCl-phosphate medium. Volume of cells in isotonic medium taken as 1.0 (broken line).

The fact that the volume of cell water is distinctly greater than the “osmotically active volume” [in the present experiments  $V_{\text{H}_2\text{O}}^o$  exceeds  $(V^o - b)$  by some 17% of the isotonic volume] has been used as an argument that much of the cell water is nonsolvent water (see Dick, 1965, for review). Although this conclusion is not compatible with the data given below, nevertheless, equation (2) is an excellent and useful empirical description of the erythrocyte volume responses to anisotonic media.

### 3. Chloride Movement at Various Osmolalities

One of the assumptions underlying equation (2) is that there is no movement of solute into or out of the cells in anisotonic media. This assumption was in part validated by the demonstration of Savitz et al. (1964) that the volume changes were completely reversible as the cells were returned from nonisotonic to isotonic media. However, this fact does not rule out the reversible

gain or loss of solute as the cells shrink and swell. With washed cells in the artificial media used here, the only osmotically important solute which might reasonably be expected to undergo such reversible changes is the chloride ion (Jacobs and Stewart, 1947). Therefore, the chloride content of the cells was measured in isotonic (= 305 milliosmolal), hypotonic (= 185 milliosmolal), and hypertonic (= 420 milliosmolal) solutions. Since the chloride content, rather than concentration, per cell was of interest in these experiments, the results were calculated as the amount of chloride per unit of hemoglobin, i.e., meq Cl<sup>-</sup>/OD<sub>640</sub> m $\mu$ . If there were no change in the amount of cell chloride, the Cl<sup>-</sup>/Hb ratio would be constant at all osmolalities. For comparing the results within any given experiment, the Cl<sup>-</sup>/Hb ratio at  $\pi = 185$  milliosmolal and  $\pi = 420$  milliosmolal are expressed as fractions of the Cl<sup>-</sup>/Hb ratio at  $\pi = 305$  milliosmolal. Again, if there were no chloride shift, this final ratio would be 1.0 at all osmolalities. From Table II it may be seen that

TABLE II  
CHANGES IN AMOUNTS OF INTRACELLULAR  
CHLORIDE ( $[\text{Cl}^-]_i/[\text{Hb}]$ ) AND OF THE  $[\text{Cl}^-]_i/[\text{Cl}^-]_o$  RATIO (=  $r$ )  
IN ANISOTONIC BUFFERED MEDIA

Experiment	$([\text{Cl}^-]_i/[\text{Hb}])_{\pi=185}$	$([\text{Cl}^-]_i/[\text{Hb}])_{\pi=420}$	$r_{\pi=185}$	$r_{\pi=305}$	$r_{\pi=420}$	$b$
	$([\text{Cl}^-]_i/[\text{Hb}])_{\pi=305}$	$([\text{Cl}^-]_i/[\text{Hb}])_{\pi=305}$				
1	0.99	1.01	0.88	0.79	0.71	0.43
2	0.97	1.01	0.86	0.79	0.73	0.49
3	0.97	1.04	0.79	0.73	0.70	0.50
4	0.94	1.01	0.81	0.80	0.75	0.47

cells (reversibly) lose about 3% of their chloride in the hypotonic medium and gain 1–2% in the hypertonic medium. Cells equilibrated with the isotonic NaCl-phosphate medium have a chloride concentration 65–70 meq/liter cells. Hence the cells lose about 2 meq/liter of osmotically active solute in the hypotonic medium, and therefore do not swell as much as they would otherwise; conversely, because of the gain of about 1.5 meq Cl<sup>-</sup>/liter in the hypertonic medium, they do not shrink as much as they would in the absence of a chloride shift. In other words, this gain and loss of solute contribute to the experimentally determined value of  $b$ . Table II includes  $b$  values for the individual chloride experiments. Even in this small series, high  $b$  values are found when the chloride movement is greatest.

If appropriate corrections are made for the chloride movement, the mean value of  $b$  is reduced in fact very little, i.e., from 0.476 to about 0.45. It will be pointed out in the discussion that this small change is a consequence of the experiments having been done in buffered media which leads to a change in extracellular pH as the volume is changed; it is the buffering of this in-

tracellular pH change by hemoglobin which leads to the chloride shift. In unbuffered anisotonic media, the change in intracellular pH is negligibly small (see below). The value of  $b$  should therefore be smaller if the measurement is made with cells suspended in NaCl-sucrose rather than NaCl-phosphate. In eight experiments comparing the  $b$  values determined in the two types of solution, this prediction was consistently borne out, the average in NaCl-sucrose being  $0.444 \pm 0.025$ . Although the difference between 0.476 and 0.444 is not large, it should not be considered negligible (see Discussion).

#### 4. *Nonsolvent Water*, $V_{nsw}^{\circ}$

When volume changes occur in anisotonic media without the cells gaining or losing solute, as is the case in NaCl-sucrose media, the value of  $b$  still substantially exceeds the volume of cell solids ( $b - V_x^{\circ} = 0.14 V^{\circ}$ ). The question remains whether this discrepancy represents nonsolvent water; formally stated, is it true that  $b = V_x^{\circ} + V_{nsw}^{\circ}$ ? This question is approached here, defining  $V_{nsw}^{\circ}$  as that fraction of cell water which is not available for the solution of chloride, by relating these parameters to a readily measurable variable, the pH of a cell suspension.

A. THEORETICAL. Assume that  $[H^+]$ ,  $[OH^-]$ , and  $[Cl^-]$  are all in thermodynamic equilibrium across the cell membrane and that the ratio of activity coefficients in cell and medium water are equal for each (Glynn, 1957). Then

$$\frac{[H^+]_i}{[H^+]_o} = \frac{[OH^-]_o}{[OH^-]_i} = \frac{[Cl^-]_o}{[Cl^-]_i} = \exp\left(\frac{EF}{RT}\right) \quad (3)$$

where the subscripts  $i$  and  $o$  refer to intracellular and extracellular concentrations respectively,  $E$  is the membrane potential, and  $R$  and  $T$  have their usual meanings. If the cells are suspended at high hematocrit in a medium containing no buffers, the pH of the medium will be regulated by the intracellular buffers and the membrane potential. Since cation permeabilities are so low in human erythrocytes and since chloride is the only diffusible anion in significant concentration, the membrane potential will be established by the chloride concentration ratio. If the tonicity is altered, the pH of the medium will change in accord with changes in the chloride ratio and the changes, if any, in  $[H^+]_i$ . A formal statement of these relationships may be developed from equation (3), from which

$$pH_o = pH_i + \log \frac{[Cl^-]_o}{[Cl^-]_i} \quad (4)$$

Since the media were made up so that the external chloride always bore a constant relationship to the osmolality,

$$[Cl^-]_o = k\pi \quad (5)$$

The cell volume is related to  $\pi$  by equation (2), which may be restated

$$\pi = \pi^{\circ}(V^{\circ} - b) \left( \frac{1}{V - b} \right). \quad (2 a)$$

$\pi^{\circ}$  and  $(V^{\circ} - b)$  in equation (2 a) are constant, as is  $k$  in equation (5). If these constants are collected into a new constant  $c$ , then the two equations may be combined to give

$$[\text{Cl}^{-}]_o = \frac{c}{(V - b)}. \quad (6)$$

For internal chloride, the concentration is equal to  $N_{\text{Cl}}$  meq dissolved in the volume of solvent water. The latter consists of the entire cell volume  $V$ , excluding both the volume of cell solids,  $V_x^{\circ}$ , and any volume of nonsolvent water,  $V_{nsw}^{\circ}$ , that may be present. Hence,

$$[\text{Cl}^{-}]_i = \frac{N_{\text{Cl}}}{V - (V_x^{\circ} + V_{nsw}^{\circ})}. \quad (7)$$

Substituting (6) and (7) into (4)

$$\text{pH}_o = \text{pH}_i + \log c - \log N_{\text{Cl}} + \log \frac{V - (V_x^{\circ} + V_{nsw}^{\circ})}{V - b}. \quad (8)$$

In a previous section, it was shown that  $N_{\text{Cl}}$  is virtually constant with volume in unbuffered media. Since only changes in pH will be considered,

$$\Delta \text{pH}_o = \Delta \text{pH}_i + \Delta \log \frac{V - (V_x^{\circ} + V_{nsw}^{\circ})}{V - b}. \quad (9)$$

If the intracellular pH is constant at all volumes, the first term on the right-hand side vanishes. If the "osmotically inactive volume" is composed of and equal to the volume of cell solids plus the volume of nonsolvent water, the second term on the right-hand side will always be 0, and  $\text{pH}_o$  will be constant at all values of  $V$ . Alternatively, if the chloride is dissolved in a volume other than the "osmotically inactive volume," there will be a systematic variation in  $\text{pH}_o$  with  $V$ .

B. EXPERIMENTAL. *i.*  $\Delta \text{pH}_i$  with  $\Delta V$  Extracellular buffers were removed by washing cells five times at 4° C in 180 milliosmolal KCl-sucrose. To one 4 ml sample of packed cells from the last wash were added 2 ml 180 milliosmolal KCl-sucrose, and to another 4 ml sample were added 2 ml 855 milliosmolal KCl-sucrose. The second sample had a final osmolality of about 470 milliosmols/kg H<sub>2</sub>O. The samples were then spun down in the cold,



and all of the supernatant plus the top half of the packed cell column were removed. The remaining cells were lysed by freezing and thawing twice, and the pH of the lysate was measured at 25° C. Measurements on these extremely viscous solutions with the capillary electrode were not entirely satisfactory. Duplicate measurements on four pairs of lysates gave a difference of  $0.008 \pm 0.004$  pH unit, with the 470 milliosmolal sample more acid than the 180 milliosmolal sample. These results are in the direction expected from the data of Cohn, Green, and Blanchard (1937) on the titration curves of horse carboxyhemoglobin, and from the known effects of ionic strength on the titration curves of proteins generally (see Tanford, 1961).

Since the difference of 0.008 pH unit is small but apparently real, this variation in  $\text{pH}_i$  was assumed to be linear with cell volume, and was applied as a correction factor in the experiments described below.

*ii.  $\Delta\text{pH}_o$  with  $\Delta V$*  Red cells were washed six times at 4° C in 180 milliosmolal NaCl-sucrose. Preparation in the cold was necessary to minimize the production of acid metabolites. During washing, the hematocrit was maintained at 20% or more to prevent overloading the cell buffers with NaCl, which is very slightly acidic. The external plasma buffers were diluted at least  $10^5$ -fold by the washing. The final concentrated cell suspension (hematocrit above 90%) was kept in an ice bath. To 2 volumes of this suspension were added 1 volume of NaCl-sucrose solution of sufficient concentration to bring the final osmolality to approximately (a) 185, (b) 305, or (c) 470 milliosmols/kg  $\text{H}_2\text{O}$ . Hematocrits,  $\text{OD}_{540\text{ m}\mu}$  readings, suspension pH, and osmolalities of the supernatants were determined after the cells had come into equilibrium with their new environment at 25° C. The time to reach this equilibrium was considered to be the time required for the pH readings to stabilize, or about 3–5 min. Measurements were made in triplicate at each osmolality.

Since the pH excursion within any one experiment was usually not greater than 0.09 pH unit, the meter was recalibrated after every third reading with a standard buffer. In the interval between calibrations, no instrument drift greater than 0.002 pH unit was ever encountered.

Fig. 2 depicts the data from a representative experiment. For these cells,  $b$  in NaCl-sucrose was determined to be 0.462. The figure includes four drawn curves, calculated from equation (9), which have been fitted to the point indicated by the cross. Curve *a* is calculated from the assumptions that  $\text{pH}_i$  is constant and that  $b = V_x^\circ + V_{nsw}^\circ$ , i.e., the nonsolvent water comprises 16% of the isotonic cell volume. This horizontal line clearly does not fit the data. In curve *b*, the same assumption is made with regard to  $V_{nsw}^\circ$ , but the correction is made for  $\Delta\text{pH}_i$ . Note that this correction shifts the calculated line further away from the data. Curves *c* and *d* include the  $\Delta\text{pH}_i$  correction and assume  $V_{nsw}^\circ$  to be 0.02 and 0.05 respectively.  $V_{nsw}^\circ = 0.02$  gives the best

fit to the data in the hypotonic range, while  $V_{nsw}^{\circ} = 0.05$  gives the best fit in the hypertonic range. This disparity was observed in all six repetitions of the experiment and led to the trial assumption that the nonsolvent water is not a constant amount of the cell volume, expressible as a fixed fraction of the isotonic volume, but rather is an amount of water which varies with cell volume.

As a test of this assumption, the data of each experiment were grouped into hypotonic, isotonic, and hypertonic classes, and the pH and cell volume

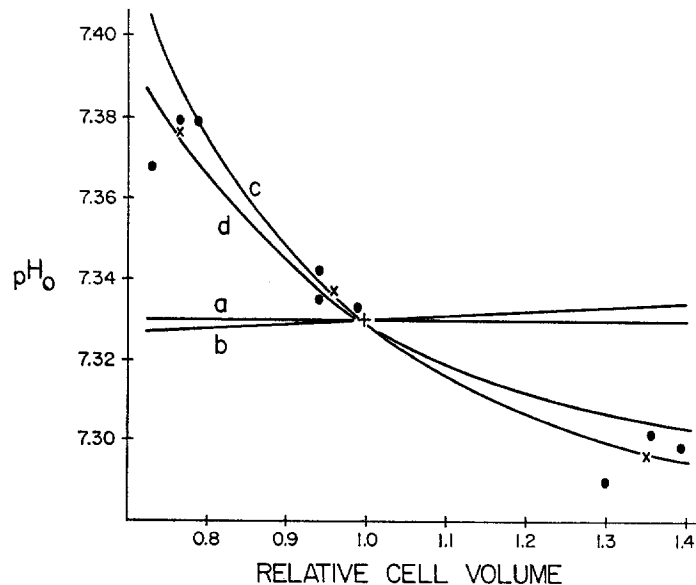


FIGURE 2. pH of cell suspensions in NaCl-sucrose media as a function of cell volume. Curves are fitted to the cross and calculated from equation (9) using the assumptions given in the text.

values for each group were averaged, e.g.,  $\bar{x}$ 's for the experiment in Fig. 2.  $V_{nsw}^{\circ}$  was calculated from equation (9) by comparing first the hypotonic and isotonic data, and then the hypertonic and isotonic data. The computations give an intermediate value for  $V_{nsw}^{\circ}$  over the range for which each calculation was made. The results are listed in Table III. One experiment is given in parentheses because, in that case,  $b$  had not been determined and an assumed value had to be chosen for the calculation.

In the hypotonic range, the pH of the suspension changes in a manner consistent with the assumption that there is virtually no nonsolvent water in the cell. In the hypertonic range, on the other hand,  $V_{nsw}^{\circ}$  is larger in every experiment and, in the five complete experiments, has an average value of nearly 5% of the isotonic volume.

## DISCUSSION

1. *Comparisons with Earlier Data*

In the present experiments, the standard NaCl-phosphate medium had a freezing-point depression corresponding to 305 milliosmols/kg H<sub>2</sub>O. Red cells suspended in this medium had a mean water volume of 0.696, and a mean *b* value of 0.476. Values of *b* have been expressed by Ponder (1948) in terms of the factor *R*, where  $R = \frac{V^{\circ} - b}{V_{\text{H}_2\text{O}}^{\circ}}$  and represents the "departure

TABLE III  
VOLUME OF NONSOLVENT WATER ( $V_{nsw}^{\circ}$ ) IN  
HUMAN ERYTHROCYTES IN HYPOTONIC AND HYPERTONIC  
RANGES; CALCULATED FROM EQUATION (9)

Experiment	$V_{nsw}^{\circ}$	
	Hypotonic range	Hypertonic range
1	-0.003	0.046
2	0.020	0.072
3	-0.027	0.039
4	0.015	0.027
5	0.026	0.053
(6)	(0)	(0.077)
Mean of 1-5	0.006	0.047

from ideality" of erythrocyte volume/tonicity responses. In the work reported here  $R = 0.76$ .

Recently published values for the osmolality of human plasma are 282 milliosmols/kg H<sub>2</sub>O (Savitz et al., 1964) and 285 milliosmols/kg H<sub>2</sub>O (Williams et al., 1959). If one accepts that the change of cell volume in anisotonic media is due solely to water movement, then it may be calculated from the data in the previous paragraph that in a NaCl-phosphate medium of 282 milliosmols/kg H<sub>2</sub>O, the red cells used here would have a total water volume of 0.710, which is close to the values of 0.717 given by Savitz et al. and of 0.716 which may be calculated from the data of Keitel et al. (1956) for red cells in equilibrium with their own plasma.

Values of *b*, and therefore *R*, are expressed as fractions of the isotonic cell volume, and hence depend on the tonicity of the medium which is considered to be isotonic. Various authors have used "isotonic" media ranging from roughly 275 to 315 milliosmolal (see Dick 1965 for references). The value of *b* determined from the present data varies from less than 0.45 to nearly 0.49 depending on which osmolality in this range is taken as isotonic. *R* is

somewhat less sensitive to the choice of isotonicity, varying only from 0.75 to 0.77.

A second factor influencing  $b$  and  $R$  is the chloride shift, the extent of which again depends on the experimental method employed. In the experiments described in section 2 of Results and depicted in Fig. 1 above, cells were repeatedly washed in anisotonic media of pH 7.38. (This experiment is essentially the reciprocal of those described in section 4.) Since there was a change in chloride concentration ratio,  $[\text{Cl}^-]_i/[\text{Cl}^-]_o$ , with change in volume (Table II), and since the external pH was maintained constant by the repeated washing with the buffered medium, there is inevitably a change in internal pH. These pH changes are buffered by hemoglobin leading to well-known  $\text{OH}^- - \text{Cl}^-$  exchanges between cells and medium, which exchanges increase the amount of intracellular chloride in hypertonic media and decrease it in hypotonic media. The magnitude of the chloride shift depends upon the buffering capacity in the suspending media vs. the buffering capacity of the cells, whether the anisotonic media are made up by changing the concentrations of all solutes equally or just by changing concentration of NaCl, and on other similar factors.

These experimental variables are detailed here because it is possible to make quite precise volume measurements with red cells, and yet values of  $b$  and  $R$  can vary considerably simply because of the variable definitions of isotonicity and of appropriate pH control.

## 2. *Validity of $V_{n,w}^0$ Determinations by Measurement of $\text{pH}_o$*

There are several important assumptions underlying the derivation of equation (9) and the reliability of the results in Table III. (a) The assumption that the external medium is absolutely unbuffered is an unattainable ideal. Nevertheless, the original plasma buffers were diluted at least  $10^5$ -fold by the washing procedure, and hemolysis, which would release hemoglobin into the medium, was less than 0.1% in these experiments. The final hematocrit was always greater than 0.38, which means that the buffering capacity of the cells was enormously greater than any residual buffers in the medium. (b) In changing the cells' environment from 180 milliosmolal to 470 milliosmolal, 5 min might not be sufficient time for reequilibration. By the end of 5 min, the  $\text{pH}_o$  had reached a stable value (no detectable drift of the meter needle) which was maintained for at least 1 min before the sample was discarded. (c) In each experiment,  $b$  was determined and used in equation (9). The values of  $b$  were slightly higher than many of the previously reported values (Dick, 1965, p. 55). Factors affecting  $b$  values were discussed above. (d) Finally, the possibility that some of the cell chloride is bound to hemoglobin must be acknowledged. If the amount bound per hemoglobin molecule remains constant over the small range of ionic strengths studied here,

such bound chloride would have no effect on the relationships expressed in equations (8) and (9), since  $\Delta \log N_{Cl}$  would be 0 in any case. (It was because of uncertainties in the amount of bound chloride that only changes in pH, rather than absolute values, were considered in these experiments.) As the cells shrink in hypertonic solution, the internal concentrations of both hemoglobin and chloride increase in parallel. If there is any significant change in chloride binding under these conditions, one would expect more chloride to be bound at the higher ionic strength. This would lead to a loss of chloride from solution.

If any one of these four factors had a significant effect on the results, it would lead to an overestimate of  $V_{nsw}^o$ .

A fourth potential objection to the experiments lies in the possibility that  $\Delta(H^+)_{i/o} / (H^+)_{i/o} > \Delta(Cl^-)_{i/o} / (Cl^-)_{i/o}$ , whereas equation (9) is based on the assumption that these are equal. The extensive data of Bromberg et al. (1965) suggest that these terms might differ by about 8%, a discrepancy which would affect the value of  $V_{nsw}^o$  as calculated from equation (9) only in the third decimal place.

It is concluded, therefore, that the values for nonsolvent water in Table III are an upper estimate. For cells at their normal volume in an isotonic medium, the fraction of nonsolvent water lies between the "hypotonic" and "hypertonic" estimates in the table and thus has a *maximum* value of less than 3% of the total cell volume.

### 3. *Significance of b Measurements*

The concept that  $b$  represents the sum of two *constant* quantities, volume of cell solids plus volume of nonsolvent water, is based almost entirely on the excellence-of-fit of experimental data to the linear equation (2). However, the derivation of that equation assumes a constant osmotic coefficient for the cell solutes throughout the range of osmolalities measured, and no gain or loss of cell solutes. The change in osmotic coefficient of hemoglobin is now well-recognized, and has been thoroughly discussed by Dick (1965). The movement of  $Cl^-$ , when the experiments are performed in buffered media, has been demonstrated in the results above. Although these factors, even when taken together, are not large enough to account for the observed values of  $b$  and  $R$ , the very fact that these two assumptions underlying equation (2) are demonstrably not valid makes the linearity of the experimental data seem fortuitous.

It is possible that  $Cl^-$  may be dissolved to some extent in water "bound" to protein, and that such bound water is not necessarily nonsolvent water. In solutions of high ionic strength, such as the interior of red cells in hypertonic media, there may be selective solvation of the hemoglobin in that relatively more water than dissolved salts becomes associated with the hemo-

globin. This effect would account for an increasing osmotic coefficient of hemoglobin as cells are exposed to hypertonic media as well as an *increasing* amount of nonsolvent water. The data of Table III are consistent with such an interpretation.

The concept that nonsolvent water is not a constant but a variable dependent on ionic strength and hemoglobin concentration will account for both the high value of  $b$  (obtained by extrapolating volume/tonicity curves to infinite tonicity) and the finding that nonsolvent water is a very small fraction of cells in isotonic media.

I am indebted to Mr. Michael Mullarkey for his careful technical assistance.

This research was supported by Grant BG-3622 from the National Science Foundation.

Received for publication 11 July 1966.

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