

Ionic and Osmotic Equilibria of Human Red Blood Cells Treated with Nystatin

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ABSTRACT Human red blood cells have been incubated in the presence of nystatin, which allows Na and K, as well as Cl and pH to equilibrate rapidly when cell volume is set with external impermeant sucrose. The intracellular mean ionic activity coefficients, relative to values in the extracellular solution, for KCl and NaCl are 1.01 ± 0.02 and 0.99 ± 0.02 (SD, $n = 10$), respectively, and are independent of external pH, pH_o , and of $[\text{sucrose}]_o$. With nystatin the dependence of red cell volume on $[\text{sucrose}]_o$ deviates from ideal osmotic behavior by as much as a factor of three. A virial equation for the osmotic coefficient, ϕ , of human hemoglobin, Hb, accounts for the cell volumes, and is the same as that which describes Adair's measurements of ϕ_{Hb} for Hb isolated from sheep and ox bloods. In the presence of nystatin the slope of the acid-base titration curve of the cells is independent of cell volume, implying that the charge on impermeant cellular solutes is independent of Hb concentration at constant pH. By modifying the Jacobs-Stewart equations (1947. *J. Cell. Comp. Physiol.* **30**: 79–103) with the osmotic coefficients of Hb and of salts, a nonideal thermodynamic model has been devised which predicts equilibrium Donnan ratios and red cell volume from the composition of the extracellular solution and from certain parameters of the cells. In addition to accounting for the dependence of cell volume on osmotic pressure, the model also describes accurately the dependence of Donnan ratios and cell volumes on pH_o either in the presence or absence of nystatin.

INTRODUCTION

Gibbs-Donnan theory, as applied to human red blood cells (Warburg, 1922; Van Slyke et al., 1923; Jacobs and Stewart, 1947), allows intracellular permeant solute concentrations and cell volumes to be estimated with about 10% accuracy purely from knowledge of extracellular solute concentrations and the amounts and acid-base titration curves of impermeant cellular solutes (see Passow, 1964). Due to the low permeability of human red cells to Na and K, previous studies of Donnan equilibria have examined Cl and H ratios and water contents (e.g., Dill et al., 1937; Bromberg et al., 1965; Funder and Wieth, 1966*c*; Duhm, 1971, 1972; Gunn et al., 1973), while theoretical treatments of normal cells, using the "double-Donnan" approximation, have

regarded Na and K as effectively impermeant solutes. In 1947 Jacobs and Stewart gave an ideal theory of red cell ionic and osmotic equilibria, including the case of cells having high cation permeability (Jacobs and Willis, 1947; Jacobs, 1962) in which stable volumes result when internal osmotic pressure is balanced with extracellular impermeant solutes (see also Wilbrandt, 1948).

This paper is concerned with the degree to which the Jacobs-Stewart theory quantitatively predicts equilibrium cation distribution, as well as anion ratios and cell volume. We have utilized the polyene antibiotic, nystatin, which induces high conductance for both cations and anions in red cells (Cass and Dalmark, 1973; Dalmark, 1975), as well as in black lipid membranes (Cass et al., 1970). With stable volumes maintained by extracellular impermeant sucrose in the presence of nystatin, equilibration of Na, K, and Cl is rapid. The high volume fraction of Hb (about 25%) causes deviations from ideal Gibbs-Donnan theory (see Passow, 1964) which are examined in three types of experiments with human red blood cells: (a) the dependence of Na, K, and Cl Donnan ratios and water contents on pH_o in the presence of nystatin and sucrose; (b) the dependence of water contents on external osmotic pressure in the presence of nystatin; and (c) the dependence of Cl ratios and water contents on pH_o with red cells having normal cation permeability. In this paper we examine the extent to which these equilibrium salt and water distributions may be described in terms of the thermodynamics of a nonideal multicomponent solution (Guggenheim, 1967). With appropriate modifications of the Jacobs-Stewart equations, it is possible to separate and estimate experimentally the contributions of mean ionic activity coefficients and osmotic coefficients, and thus to extend the classical treatment of equilibrium salt and water distribution for human red blood cells. Preliminary reports of parts of this study have been presented previously (Freedman and Hoffman, 1977 a, b).

GLOSSARY OF SYMBOLS

Solute		Properties of <i>i</i> th solute (<i>cont.</i>)
Hb	Hemoglobin	γ_i Activity coefficient
P	Organic phosphate plus glutathione	$\gamma_{j,k}$ Intracellular mean ionic activity coefficient for salt (j^+k^-) relative to value in extracellular solution
<i>b, a</i>	Permeant ions of salt (b^+a^-)	
<i>B, A</i>	Impermeant ions of salt (B^+A^-)	
<i>s</i>	All intracellular solutes except Hb	Properties of multicomponent solution
<i>M</i>	Impermeant neutral solutes	N_T Total moles of solute
		V_w Volume of water
		V_h Volume of "nonosmotic" water
		<i>w</i> Cell water as fraction of isotonic water, (V_w/V_w^o)
Properties of <i>i</i> th solute		w' Grams of H ₂ O/gram of cells
n_i	Number of moles	w'' Grams of H ₂ O/gram of cell solids
c_i	Millimolal concentration	w_h "Nonosmotic" water as fraction of intracellular water, (V_h/V_w)
r_i	Ratio of intracellular to extracellular concentrations	
ϕ_i	Osmotic coefficient	
z_i	Charge	
$\bar{\mu}_i$	Electrochemical potential	

Properties of multicomponent solution (<i>cont.</i>)	Subscripts <i>c</i> and <i>o</i> indicate intracellular and extracellular
<i>C</i> Total solute concentration, (N_T/V_w)	Superscript <i>o</i> indicates value for cells in isotonic solution at $pH_o = 7.4$
<i>d</i> Density	
π Osmotic pressure	
Φ Osmotic coefficient of mixture	
<i>z</i> Average net charge of intracellular impermeant solutes	Other constants
<i>m</i> Buffering capacity of intracellular solution	<i>R</i> Gas constant
<i>pI</i> Isoelectric point of intracellular solutes	\mathcal{R} Ponder's "R"
<i>T</i> Absolute temperature	Units: All concentrations are [millimolal]. Other units are given in text or Appendix.

MATERIALS AND METHODS

Preparation, Equilibration, and Sampling of Red Cells

Human blood from healthy donors was drawn by venepuncture, transferred to heparinized containers, and immediately centrifuged at 12,000 *g* for 3–5 min at 0–4°C, followed by aspiration of the plasma and buffy coat. The red cells were then washed three or four times by centrifugation, each time resuspending in about 5 vol of a chilled solution containing 145 mM NaCl, 5 mM KCl, 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4 at 22°C), and finally resuspending to 5% hematocrit in ice-cold medium (2°C) containing *x* mM KCl, (150 – *x*) mM NaCl, 5 mM HEPES (pH 7.4 at 22°C), plus 15–260 mM sucrose. Nystatin (E.R. Squibb & Sons, Inc., New York) was then added from a stock solution (5 mg/ml MeOH) to a final concentration of 50 μ g/ml suspension. It is assumed that the methanolic solution of nystatin acts only to increase membrane permeability for K, Na, and Cl and does not otherwise perturb the resultant equilibrium distribution. After 10 min, the cells were washed in the desired medium by centrifugation for 3 min at 7,500 *g* at 0–4°C. For some experiments, ^{36}Cl (0.1 μ Ci/ml) was added to the final suspension. The pH was adjusted to a desired value by slowly adding 0.3 M NaOH or 0.3 M HCl with frequent swirling. After reequilibration at 25°C, the pH was remeasured.

At desired times or after 15 min for equilibration, samples were removed by a technique modified from Funder and Wieth (1966 *a*) for analysis of K, Na, and Cl distribution ratios and for water and hemoglobin contents. Aliquots (3–5 ml) of incubated suspensions were poured into 5-ml or 10-ml polypropylene syringe tubes (Pharmaseal Laboratories, Glendale, Calif.), from which the plungers had been removed and to which 0.4-ml polycarbonate microcentrifuge tubes (Arthur H. Thomas Co., Philadelphia, Pa.) had been attached in place of the syringe needle. The syringe tubes were sealed with Parafilm (American Can Co., Greenwich, Conn.), and a supporting cylindrical collar (7 mm i.d., 13 mm o.d., 42 mm long, cut from acrylic tubing) was placed around the microcentrifuge tube with a few millimeters of the tube protruding from the bottom of the collar to ensure a tight seal between the tube and syringe during centrifugation. Cells were sedimented by centrifuging at 3,000 rpm and 25°C for 5 min (Sorvall HG-4 rotor, DuPont Instruments—Sorvall, Newtown, Conn.) and the syringe tube containing most of the supernate was removed and saved for analysis. The microcentrifuge tubes were capped and the cells were recentrifuged for tighter packing at 12,000 *g* for 15 min. After cutting off the bottom 1–2

mm of the tube and also cutting just below the cell-supernate interface, the packed cell mass was ejected by blowing through tubing attached to one end.

Chemical Analyses

For analyses of K and Na (millimolal), duplicate or triplicate samples were taken from a cell suspension, and 0.1–0.2 ml packed cells or 0.1 ml supernate was diluted and analyzed in duplicate or triplicate with a flame photometer (National Instrument Laboratories, Fredericksburg, Va.), using 15 mM LiCl as an internal standard. Cellular Na and K were normalized to Hb by analyzing an aliquot from each lysate prepared for ion analysis using Drabkin's reagent and cyanmethemoglobin standards (Hycel, Inc., Houston, Tex.). For each sample analyzed for K and Na, parallel samples in duplicate or triplicate of the same cell suspension were analyzed for cell water content by drying the packed cells to constant weight at 80°C. Cellular Hb content was obtained in each experiment by weighing two or three of the packed cell aliquots prior to analysis of Na, K, and lysate Hb, with dry weights determined in parallel samples.

The pH of solutions and suspensions was measured with a model 26 pH meter using a GK 2321C combination electrode (Radiometer, The London Co., Cleveland, Ohio), or for titration experiments (Fig. 5) using a G297 capillary pH electrode and K497 calomel reference electrode (Radiometer E5021a).

CHLORIDE RATIOS For experiments in which Cl ratios were determined isotopically, samples were taken in duplicate or triplicate as described above. To 0.1-ml aliquots of the extracellular supernates were added 1 ml water and 1 ml cold 10% trichloroacetic acid (TCA). After centrifugation, 1 ml was counted with 10 ml aqueous counting scintillant (ACS, Amersham/Searle Corp., Arlington Heights, Ill.). The packed cells (0.1–0.2 ml) in the microcentrifuge tubes were added to 1 ml water. Aliquots (0.1 ml) of each lysate were then mixed with 9.9 ml Drabkin reagent for Hb analysis. After adding 1 ml cold 10% TCA to each lysate, followed by centrifugation, 1 ml of the supernate was counted with 10 ml ACS.

With this procedure, which was designed to facilitate the analysis of large numbers of samples while avoiding the inaccuracies of pipetting shrunken red cells, the chloride ratio, $r_{Cl} = [Cl]_c/[Cl]_o$, is given by

$$r_{Cl} = \frac{(CPM)_c V_A (Hb)_c}{21 (CPM)_o V_L (Hb)_L} \quad (1)$$

where $(CPM)_c$ and $(CPM)_o$ are the counts/minute per milliliter from the lysate and extracellular supernate, V_A is the volume of supernate after TCA precipitation of the lysate, V_L is the total volume of lysate, $(Hb)_L$ and $(Hb)_c$ refer to the lysate (gram of Hb/milliliter of lysate) and to the cell water (gram of Hb/milliliter of cell water), respectively, and 21 is the dilution factor for the supernates. From parallel weighed samples for analyses of cell water, w' (gram of water/gram of cells), and separate samples for Hb content (gram of Hb/gram dry weight), $(Hb)_c$ is given by (gram of Hb/gram dry weight) $(1 - w')/w'$. If V_c is the volume of cells of density d_c lysed in 1 ml water, then $V_L = 1 + V_c$, and the conservation of Hb during lysis implies that $(Hb)_c w' d_c V_c = (Hb)_L V_L$. Then V_L may be obtained as follows:

$$V_L = \frac{(Hb)_c w' d_c}{(Hb)_c w' d_c - (Hb)_L} \quad (2)$$

V_A , which includes terms for the added cell water as well as for the water removed for analysis of Hb, is given as follows:

$$V_A = 2 + w'd_cV_c - (1 + w'd_cV_c)(0.1/V_L). \quad (3)$$

Since the only significant change in cell mass in the experimental solutions employed is due to water movement, i.e., $d_w(V_c - V_c^o) = d_cV_c - d_c^oV_c^o$, where the density of water, d_w , is unity, then the cell density, d_c , is given by $d_c = 1 + (d_c^o - 1)(V_c^o/V_c)$. In isotonic solution the cell-density, d_c^o , is approximated by $(0.717 \text{ g H}_2\text{O/ml cells}) / (0.666 \text{ g H}_2\text{O/g cells}) = 1.08$ (Savitz et al., 1964; Funder and Wieth, 1966 *b*). If Ponder's (1948) value of 1.096 is taken to be the normal red cell density, then the values of r_{Cl} would be altered by less than 1%. The relation between the relative cell volume, V_c/V_c^o , and the measured water content, w' , is obtained by considering the cell volume to be the sum of a water volume, V_w , and a solid volume, V_{sol} , as given by $V_c/V_c^o = (V_w + V_{sol}) / (V_w^o + V_{sol})$. Substituting $V_w/V_w^o = (w'/1 - w')(1 - w'^o/w'^o)$ and taking $w'^o = 0.666 \text{ g H}_2\text{O/g cells}$, $V_w^o = 0.717 \text{ ml H}_2\text{O/ml cells}$, and $V_{sol} = 0.283 \text{ ml solids/ml cells}$, we obtain $V_c/V_c^o = 0.359 (w'/1 - w') + 0.283$. Thus, V_A and V_L are specified by the measurements of $(Hb)_c$, $(Hb)_L$, and w' , enabling computation of r_{Cl} (Eq. 1).

TRAPPED MEDIUM The Donnan ratio, r' , corrected for intercellular trapped medium, ϵ (milliliter/gram of packed cells), is related to the uncorrected Donnan ratio, r , by $r' = (rw'^o - \epsilon) / (w'^o - \epsilon)$. Since the values for r in this study ranged from 0.78 to 1.76, and since $w'^o = 0.666 \text{ g H}_2\text{O/g packed cells}$ (Funder and Wieth, 1966 *b*), the maximum correction with $\epsilon = 0.025$ would be 1.7%. This small correction for r , which does not affect any of the conclusions, was omitted.

The relative cell water contents, w (gram of cell H₂O/gram of isotonic cell H₂O) are related to measured water contents, w' (gram of H₂O/gram of packed cells), by,

$$w = \left(\frac{w' - \epsilon}{1 - w' + \epsilon} \right) \left(\frac{1 - w'^o + \epsilon}{w'^o - \epsilon} \right), \quad (4)$$

so that upon substitution for w'^o and ϵ and rearrangement,

$$w' = \frac{w}{w + 0.560} + 0.025. \quad (5)$$

Eq. 5 was used to compare values of w , as predicted by Eqs. 7 or 8, with measured uncorrected water contents, w' .

CHARGE OF IMPERMEANT CELLULAR SOLUTES In order to determine the charge, z , of impermeant cellular solutes, of which Hb and organic phosphates are the most important, red cells were incubated in the presence of nystatin with cell volume set by external sucrose as described above. The suspensions (5% hematocrit) were divided into portions (10–33 ml) for titration by dropwise addition of $0.299 \pm 0.001 \text{ N}$ (SD, $n = 5$) NaOH or $0.301 \pm 0.001 \text{ N}$ (SD, $n = 3$) HCl with rapid stirring. The normalities of the NaOH and HCl were checked against a standard solution of 0.3 N KHPthalate (Skoog and West, 1963). To obtain a measure of z (equivalent/mole of Hb), relative to its value at the initial pH of the suspension, the number of equivalents of added H or OH (equivalent/liter of suspension) bound by the cell-free medium was subtracted from the number of equivalents bound by the cell suspension, and the difference normalized to [Hb] (moles/liter of suspension). Between pH 6.5 and 8.5 the amount of free acid and base in the unbuffered medium was negligible. A control experiment established that titration of a buffered cell suspension was additive with respect to titration of an unbuffered cell suspension and of medium buffered with 5 mM HEPES. Intracellular pH, pH_c , was calculated from Donnan ratios (see Appendix, Eq. A12 *b*). The small uncertainty of calculating pH_c by this method, estimated

to be ± 0.02 pH units depending on which ion is used, is comparable to the uncertainty of electrode measurements in concentrated lysates (Salling and Siggard-Andersen, 1971). The relative charge at each pH_c was then converted to an absolute charge, using the isoelectric point, $\text{pI} = 6.78 \pm 0.06$ (SD, $n = 4$) at 25°C (see Results), and then plotted vs. pH_c (Fig. 5 A) and $[\text{Hb}]$ (Fig. 5 B).

THEORY

The equilibrium data are analyzed in terms of a model in which the inside of the human red blood cell is a multicomponent solution which normally contains average concentrations (millimolar) of 140 K and 10 Na (Funder and Wieth, 1966 *b*), 7.3 Hb (Wintrobe, 1934; Wintrobe et al., 1974), 9 organic phosphate (Bartlett, 1959; Beutler, 1971; Jacobasch et al., 1974), and 3.4 glutathione (Beutler, 1971).¹ Deviations from ideal Donnan theory are described by introducing activity and osmotic coefficients into the equations which Jacobs and Stewart (1947) utilized to express the relative water contents and the concentration ratios of permeant solutes as a function of certain cell parameters and of the composition of the extracellular medium (see Appendix for derivation and Glossary for list of symbols). The resultant equations for predicting the ratios of intracellular to extracellular concentrations, r_a and r_b , where a and b refer to permeant monovalent anions and cations, respectively, are as follows:

$$r_a = \frac{R_a E + \sqrt{Q(D/\gamma_{j,k})^2 + [(\Phi_o R_a)^2 - Q(\phi_s/\gamma_{j,k})^2]E^2}}{D + \phi_s E}, \quad (6 a)$$

$$r_b = \frac{1}{\gamma_{j,k}^2 r_a}, \quad (6 b)$$

where $R_a = C_o/2[a]_o$, $Q = [b]_o/[a]_o$, $E = [B]_i^c + z[\text{Hb}]^o$ and $D = \phi_{\text{Hb}}[\text{Hb}]^o + \phi_s([B]_i^c + [P]_i^o)$. Eqs. 6 *a* and 6 *b* are generalizations of Eqs. 9 and 5 of Jacobs and Stewart's (1947) ideal theory, but with additional terms included for the intracellular mean ionic activity coefficient, $\gamma_{j,k}$, for a particular salt (j^+k^-) relative to its value in the extracellular solution; the osmotic coefficient of Hb, ϕ_{Hb} ; the average osmotic coefficient of the mixture of all intracellular solutes except Hb, ϕ_s ; and the average osmotic coefficient of the extracellular solutes, Φ_o .

To predict relative water contents, w , of cells having normal cation permeability, it is convenient to use

$$w = (D + \phi_s E)/\Phi_o C_o. \quad (7)$$

For cells having high cation permeability after treatment with nystatin, we use

$$w = \frac{D}{\Phi_o[M]_o - [a]_o(\phi_s r_a + \frac{\phi_s}{\gamma_{j,k}^2 r_a} - 2\Phi_o)}. \quad (8)$$

Since the average charge, z , on intracellular impermeant solutes depends on pH_c (Eq. A11), and since H^+ is also in Donnan equilibrium (Eq. A12), the dependence of r_a on

¹ The sum of the normal internal solute osmolalities assumed in our model is 96% of the normal plasma value of 289 mosM (Waymouth, 1970). An osmotic discrepancy of 15% noted by Drabkin (1950) is corrected by more precise analytical data and a more complete listing of components.

pH_o is a transcendental function which requires an iterative numerical computation. An additional complexity arises from the dependence of ϕ_{Hb} on $[Hb]$ (Eq. A6), which in turn depends on the water content, w . An iterative calculation (see Appendix) was therefore designed to yield for a given extracellular medium at a given pH_o an output consisting of the concentration ratios of permeant solutes, the absolute and relative cell water content, the osmotic coefficients of Hb and salts, the charge on impermeant cell solutes, the intracellular pH, and the membrane potential.

This model of the red cell interior neglects a number of interactions either for simplicity or for lack of specific information. The osmotic pressure due to organic phosphates is uncertain since the dependence on pH and temperature of the binding of ATP and 2,3-diphosphoglycerate to hemoglobin at physiological $[Hb]$ and ionic strength is not known. Our analysis refers to cells washed free of HCO_3^- but in equilibrium with air, and hence with nearly completely oxygenated Hb, and does not at this stage include the effects of variable partial pressures of O_2 and CO_2 . We have also neglected the effect of ionic strength on intracellular buffering, since 34% Hb introduces complexities which are beyond the scope of this paper. The effects which are neglected probably do not appreciably affect the predictions for the experiments reported.

RESULTS

It will first be shown that human red blood cells, which are rendered highly permeable to cations with nystatin, have solutes and solvent in equilibrium, and that Donnan ratios and water contents are stable for the duration of the experiments, a necessary condition for comparison of data with predictions based on Gibbs-Donnan theory. The dependence of equilibrium Na, K, and Cl ratios on pH_o will then be described and analyzed to yield intracellular relative mean ionic activity coefficients. After finding that cell volumes are nonideal both when pH_o and external osmotic pressure are varied, evidence will be described indicating that under the conditions studied, Hb charge is independent of $[Hb]$ at constant pH. Nonideal osmotic behavior is then explained with the osmotic coefficients of Hb and of salts, with the magnitude of the parameters consistent with those expected for protein and salt solutions.

Attainment of Equilibrium

As shown by the data in Fig. 1 A, the volume of human red cells which have high cation permeability in the presence of nystatin may be set with impermeant external sucrose. The water contents are stable at varied pH_o for 1–2 h either during incubation at 2°C (Fig. 1 A) or at 25°C after pH adjustment at 2°C (Fig. 1 B). The concentration ratios of Na and K are also stable between pH 6.5 and 7.9 for at least 1 h at 25°C (Table I). When the pH is adjusted with HCl or NaOH in the cold to minimize hemolysis, and the cells are subsequently equilibrated at 25°C, the data in Table I and Fig. 2 show that new equilibrium water contents and cation ratios are attained within 10 min (Table I and Fig. 2). These results establish for our experimental conditions the occurrence of rapid net cation exchanges similar to those reported by Cass and Dalmark (1973), and that these shifts lead to new stable, equilibrium Donnan ratios and cell volumes.

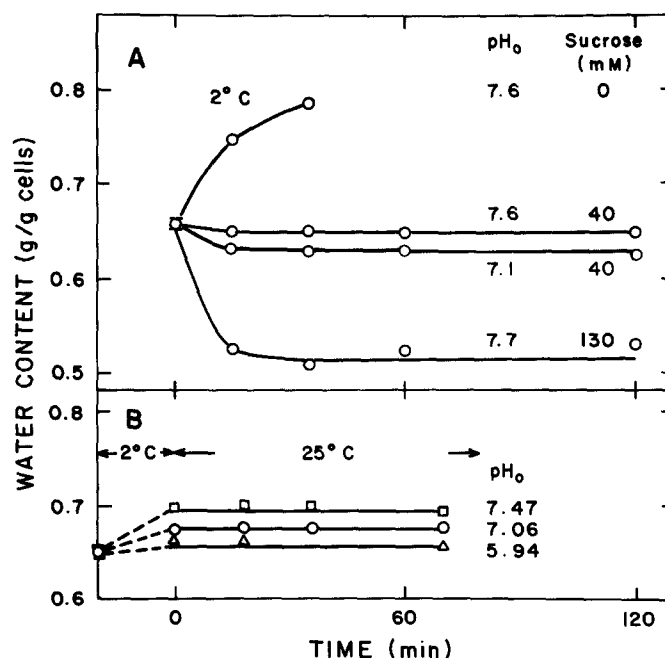


FIGURE 1. Stability of water contents of cation-permeable human red blood cells at varying [sucrose]_o and pH_o. Experiment A. Incubation at 2°C. Cells were washed four times by centrifugation and resuspension in medium containing 145 mM NaCl and 5 mM KCl, after which the water content, w' , was found to be 0.657 ± 0.002 g H₂O/g cells (SD, $n = 3$), as shown at zero time. The cells were then added to 5% final hematocrit to four media at 2°C containing 145 mM NaCl, 5 mM KCl, nystatin, and the indicated [sucrose]_o. The pH was adjusted at 2°C to the values shown. At the times indicated, samples were taken for analysis of w' . Considerable hemolysis occurred in the absence of sucrose, but the supernates were clear in all other samples. Between 80 and 100 min, the pH was remeasured and found to be constant (± 0.05) in the unlysed samples. Experiment B. Incubation at 25°C after pH adjustment at 2°C. After the cells were washed as described in experiment A, w' was 0.649 ± 0.001 g H₂O/g cells (SD, $n = 3$) as shown on the ordinate. Three 5% hematocrit suspensions were then prepared in media as described in experiment A but with 34 mM [sucrose]_o, and the pH_o was adjusted at 2°C. The suspensions were shifted to 25°C (shown as zero time), and samples were taken for analysis of w' after 1 min and at the two or three subsequent times indicated. The pH_o was remeasured at 25°C (values shown) and found to be 0.28 ± 0.07 units (SD, $n = 4$) lower than at 2°C (values not shown).

Donnan Ratios of Na, K, and Cl

A comparison of Na, K, and Cl distribution ratios with both K_o and Na_o at 72 mM shows little difference between K and Na (Table II). The ratio r_K/r_{Na} is 0.96 ± 0.02 (SD, $n = 10$) with a 99% confidence interval of ± 0.02 for data pooled from two experiments with points at various pH_o and [sucrose]_o (Table III). The ratios of Cl_o to Cl_c, or $1/r_{Cl}$, are very close to the cation ratios (Table

II), $r_{\text{Na}^+\text{rCl}^-}$ being 1.03 ± 0.04 (SD, $n = 10$), and $r_{\text{K}^+\text{rCl}^-}$ being 0.98 ± 0.04 (SD, $n = 10$), with 99% confidence intervals of 0.04 (Table III). When ionic activity coefficients are included in the expressions for the electrochemical potentials of the salts, the product of the cation concentration ratio and the anion

TABLE I
STABILITY OF Na AND K CONCENTRATION RATIOS IN RED CELLS TREATED WITH NYSTATIN

Expt.	pH _o	Time <i>min</i>	r_{Na}	r_{K}
A	7.40	0	1.25 ± 0.02	1.42 ± 0.01
		60	1.27 ± 0.03	1.41 ± 0.05
	6.51	5	1.13 ± 0.00	1.16 ± 0.08
		25	1.14 ± 0.01	1.28 ± 0.02
		55	1.09 ± 0.02	1.16 ± 0.05
	7.85	10	1.43 ± 0.01	1.51 ± 0.10
		30	1.38 ± 0.08	1.50 ± 0.11
		60	1.36 ± 0.03	1.49 ± 0.07
	B	7.21	0	1.20 ± 0.04
140			1.18 ± 0.03	1.42 ± 0.08
6.50		60	1.02 ± 0.02	1.22 ± 0.04
		140	1.03 ± 0.01	1.29 ± 0.05
C	6.50	60	1.02 ± 0.02	1.18 ± 0.02
		140	1.02 ± 0.02	1.21 ± 0.01

Experiment A. Human red cells prepared as described in Materials and Methods were incubated for 10 min at 5% hematocrit in medium containing 145 mM NaCl, 5 mM KCl, 37 mM sucrose, 5 mM HEPES (pH 7.4 at 22°C), and nystatin at 2°C. After centrifugation at 7,500 *g* for 3 min at 0–4°C, the cells were resuspended in the same medium and then recentrifuged, followed by resuspension to 5% hematocrit in medium without nystatin. The suspension was divided into three portions, equilibrated at 25°C, and sampled for analysis of Na and K (time 0). The pH was then adjusted to the indicated values, and samples were taken for analysis of Na and K at the times shown. Hemolysis was less than 0.2%. In all samples, the measured K_o was 5.3 ± 0.3 mM and Na_o was 143 ± 2 mM (SD, $n = 24$). [Hb] was 0.88 ± 0.02 g/g solids (SD, $n = 3$).

Experiment B. Similar to A but with final incubation for longer times. The measured K_o was 5.0 ± 0.1 mM and Na_o was 141 ± 2 mM (SD, $n = 18$). [Hb] was 0.896 ± 0.009 g/g solids (SD, $n = 3$).

Experiment C. Same cells as in B but with pH adjusted in medium containing nystatin. All values shown for experiments A, B, and C are means and standard deviations for triplicate samples, with both cells and supernates analyzed.

concentration ratio is a direct measure of the intracellular mean ionic activity coefficient, $\gamma_{j,k}$, for a particular salt (j^+k^-) relative to its value in the extracellular solution (Eq. A10; see Donnan and Guggenheim, 1932; Overbeek, 1956). The values obtained for $\gamma_{\text{K,Cl}}$ and $\gamma_{\text{Na,Cl}}$ appear not to deviate significantly from unity or to vary with cell volume, as set by external sucrose (Table II),

contrary to a suggestion of Mazzioti et al. (1976) that reduced intracellular ionic activity coefficients account for nonideal osmotic behavior of red cells. Since $\gamma_{K,Cl}$ and $\gamma_{Na,Cl}$ are also independent of pH_o , the data were pooled, yielding 0.99 ± 0.02 (SD, $n = 10$) for $\gamma_{Na,Cl}$ and 1.01 ± 0.02 (SD, $n = 10$) for $\gamma_{K,Cl}$, with a 99% confidence interval of ± 0.02 for both salts (Table III). Incidentally, from the K and Cl ratios at the $[K]_o$ at which addition of valinomycin gives no change in the fluorescence of the potential-sensitive cyanine dye, diS-C₃(5) (Hoffman and Laris, 1974), it may also be estimated that $\gamma_{K,Cl} = 1.03 \pm 0.02$.

In four experiments in which equilibrium Na and K concentration ratios

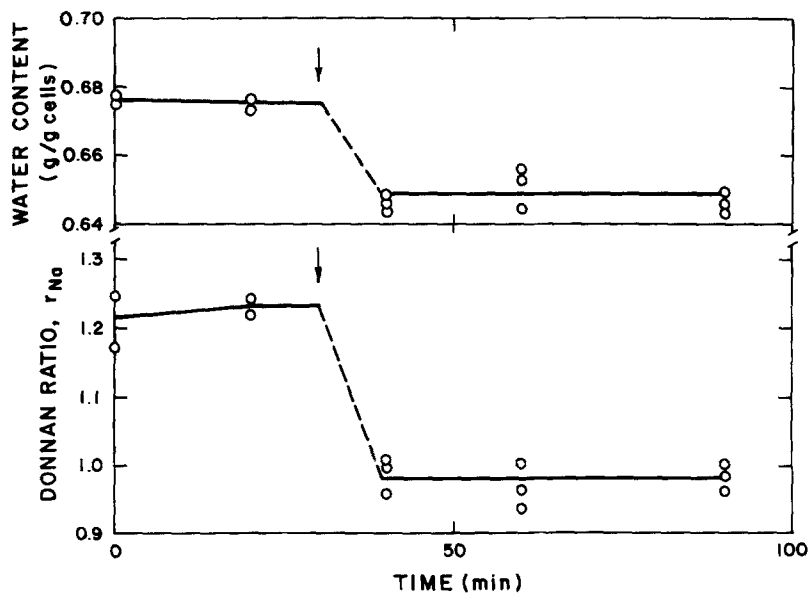


FIGURE 2. Effect of pH in shifting cell-to-medium Na distribution ratio, r_{Na} , and cell water content. The protocol was as described in the legend to Table I with incubation at 5% hematocrit and 25°C in medium containing 145 mM NaCl, 5 mM KCl, 5 mM HEPES, 37 mM sucrose, and nystatin. The arrows indicate the time at which HCl (0.3 M) was added to shift the pH from 7.4 to 6.5. $[Hb]$ was 0.89 ± 0.03 g/g solids (SD, $n = 2$).

and water contents were determined as a function of pH_o , the results may be described empirically by the following linear regressions: $r_{Na} = 0.195 pH_o - 0.254$ (Fig. 3), and $r_K = 0.164 pH_o + 0.124$ (data not shown). The predictions of both the ideal (Fig. 3, dotted line) and the nonideal (Fig. 3, solid line) forms of the Jacobs-Stewart theory agree well with the measured cation ratios. In these experiments at 5 mM K_o and 145 mM Na_o , an apparent second-order cation selectivity was evident, r_K/r_{Na} being 1.15 ± 0.06 (SD, $n = 17$), with a 99% confidence interval of ± 0.04 . In another experiment of a similar type, in which $[sucrose]_o$ ranged from 15 to 150 mM, the apparent K/Na selectivity increased from 1.07 to 1.29 as the cell water content decreased from

0.75 to 0.50 g H₂O/g cells. The apparent second-order K/Na selectivity was not seen when both K_o and Na_o were 75 mM (Tables II and III), and was not pursued further.

Cell Volume

Compared to the marked pH dependence of the water contents of red cells with normal cation permeability (Jacobs and Parpart, 1931), the water contents, w' , of red cells with high cation permeability are much less sensitive to pH_o, averaging 0.662 ± 0.016 g H₂O/g cells (SD, $n = 16$) between pH 6.5 and 8.8 at 37 mM sucrose (Fig. 3; cf. Fig. 6). The insensitivity of cell volume to pH_o exhibited by cells equilibrated in the presence of nystatin is due in

TABLE II
EQUILIBRIUM DONNAN RATIOS AND WATER CONTENTS AT
VARIED pH_o AND [SUCROSE]_o

Expt.	pH	[Sucrose] _o	w	r_{Na}	r_K	$1/r_{Cl}$	$\gamma_{Na, Cl}$	$\gamma_{K, Cl}$
A	6.38	37	0.84	0.92 ± 0.02	0.85 ± 0.02	0.92 ± 0.02	1.00	1.03
	7.22	37	0.92	1.13 ± 0.03	1.09 ± 0.04	1.20 ± 0.04	1.03	1.05
	7.88	37	1.06	1.40 ± 0.06	1.29 ± 0.06	1.36 ± 0.03	0.99	1.03
B	6.49	15	1.25	0.92 ± 0.03	0.91 ± 0.01	0.92 ± 0.03	0.99	1.01
	6.43	32	0.90	0.94 ± 0.02	0.90 ± 0.02	0.89 ± 0.00	0.98	1.00
	6.55	150	0.51	0.91 ± 0.02	0.88 ± 0.02	0.86 ± 0.00	0.98	0.99
	7.28	15	1.42	1.17 ± 0.03	1.14 ± 0.02	1.08 ± 0.02	0.96	0.97
	7.27	32	1.00	1.18 ± 0.02	1.14 ± 0.02	1.19 ± 0.11	1.00	1.01
	7.29	150	0.52	1.26 ± 0.02	1.17 ± 0.03	1.19 ± 0.00	0.97	1.02
	8.42	150	0.62	1.81 ± 0.06	1.76 ± 0.08	1.68 ± 0.02	0.97	1.00

Experiment A. Red cells were prepared as described in Materials and Methods and equilibrated for 15 min at 2°C with medium made up to contain 75 mM KCl, 75 mM NaCl, 5 mM HEPES (pH 7.4 at 22°C), 37 mM sucrose, and nystatin. The cells were then washed twice by centrifugation (3 min, 7,500 g, 0–4°C) and resuspension in the same medium containing nystatin. After pH adjustment and 15 min at 25°C, the cells and supernates were sampled in triplicate and analyzed to obtain K, Na, and Cl ratios. Hemolysis was $0.5 \pm 0.3\%$ (SD, $n=3$). The measured K_o was 72 ± 2 mM, and Na_o was 71 ± 1 mM (SD, $n=8$). [Hb] was 0.852 ± 0.003 g/g solids (SD, $n=3$).

Experiment B. The same procedure as in A was employed, but with [sucrose]_o varied as indicated to compare the Donnan ratios at different cell volumes. Hemolysis was $0.5 \pm 0.2\%$ (SD, $n=7$). The measured K_o was 76 ± 1 mM, and Na_o was 78 ± 2 mM (SD, $n=27$). [Hb] was 0.862 ± 0.008 g/g solids (SD, $n=2$). Values shown are means and standard deviations for triplicate samples for r_{Na} and r_K and duplicate samples for r_{Cl} , with both cells and supernate analyzed. $\gamma_{Na, Cl}$ and $\gamma_{K, Cl}$ were calculated by Eq. A10 (see Appendix).

part to the fact that the Cl shifts dependent on pH are nearly osmotically balanced by cation shifts in the opposite direction. In these cells, the water contents are systematically larger than predicted for an ideal osmometer (i.e., $\phi_{Hb} = \phi_s = \Phi_o = 1$), the maximum deviation being 26% at pH 6.8 (Fig. 3, dotted line). When corrections for osmotic coefficients of Hb and of salts are included in the theory, as described in the Appendix, the average relative deviation between the measured and predicted water contents is 2.7% (Fig. 3, solid line). This deviation is consistent with the assumptions of the theory and with the experimental uncertainty, which was sufficient to preclude detection of the expected shallow minimum at the isoelectric point (Table V).

TABLE III
RELATIVE MEAN IONIC ACTIVITY COEFFICIENTS FROM
EQUILIBRIUM Na, K, AND Cl DISTRIBUTION RATIOS

	$r_{K/r_{Na}}$	$r_{Na/r_{Cl}}$	$r_{K/r_{Cl}}$	$\gamma_{Na, Cl}$	$\gamma_{K, Cl}$
Mean	0.96	1.03	0.98	0.99	1.01
Standard deviation	0.02	0.04	0.04	0.02	0.02
Number	10	10	10	10	10
99% Confidence interval	0.02	0.04	0.04	0.02	0.02

Values of r_K , r_{Na} , and r_{Cl} are from Table II. $\gamma_{Na, Cl}$ and $\gamma_{K, Cl}$ were calculated by Eq. A10 (see Appendix).

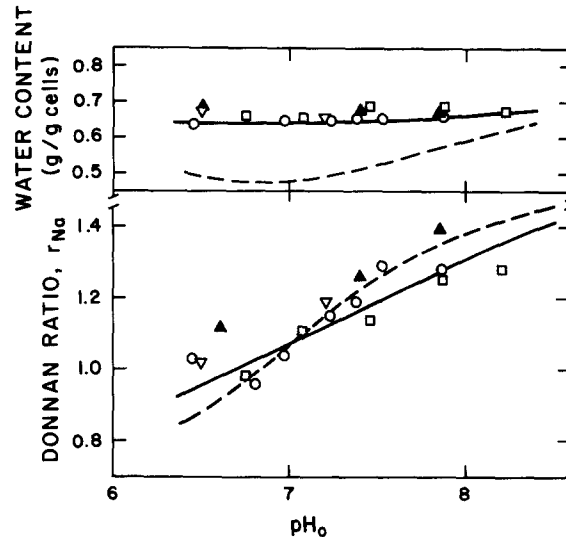


FIGURE 3. Effect of pH on equilibrium Na distribution ratios and water contents of red cells in the presence of nystatin. The different symbols represent data from four experiments. Experiments A (○) and B (□). Red cells prepared as described in Materials and Methods were incubated at 5% hematocrit for 10 min at 2°C in medium containing 145 mM NaCl, 5 mM KCl, 37 mM sucrose, 5 mM HEPES (pH 7.4 at 22°C), and nystatin, and then divided into portions (10 ml). The cells in each portion were then washed twice by centrifugation at 7,500 *g* for 3 min at 0–4°C and resuspension to 5% hematocrit in the same medium with nystatin. The resuspension media were previously titrated so as to obtain final suspension pH's between 6.5 and 8.8. After equilibration of the suspensions for 15 min at 25°C, samples were analyzed for Na and K concentration ratios and water contents. The measured K_o was 5.2 ± 0.1 mM, and Na_o was 145 ± 3 mM (SD, $n = 16$). Experiments C (▲) and D (▼). Average values taken from time-courses of Table I. The dotted lines are predictions of the unmodified ideal Jacobs-Stewart theory, which assumes solute activity and osmotic coefficients equal to unity. The solid lines are predictions of the nonideal thermodynamic model, which includes the osmotic coefficients of hemoglobin and of salts (see Discussion and Appendix).

When the dependence on external osmotic pressure of the volume of cells equilibrated in the presence of nystatin was examined by increasing $[\text{sucrose}]_o$ from 26 to 260 mM at pH 7.02, w' decreased monotonically to 0.45 g $\text{H}_2\text{O}/\text{g}$ cells (Fig. 4). It would be expected that cation-permeable red cells

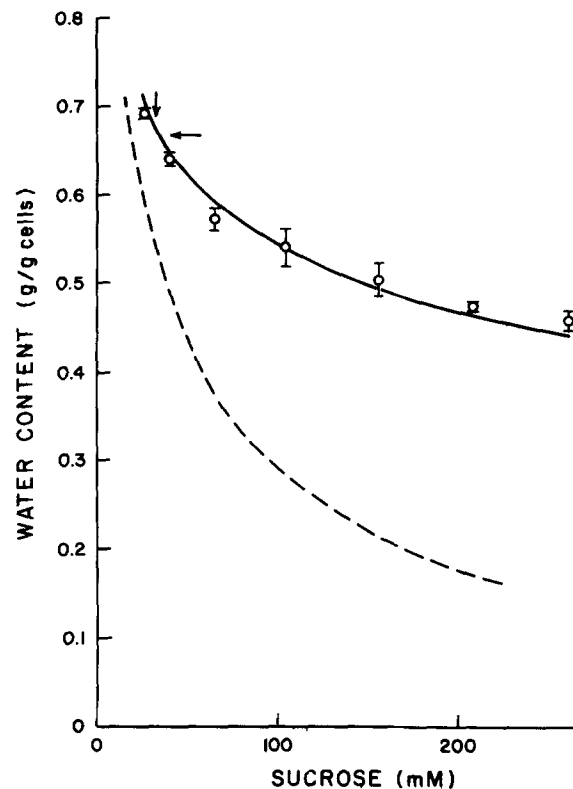


FIGURE 4. Effect of $[\text{sucrose}]_o$ on equilibrium water contents of red cells in the presence of nystatin. Cells were prepared as described in Materials and Methods and incubated at 5% hematocrit for 10 min at 2°C in medium-containing 145 mM NaCl, 5 mM KCl, 37 mM sucrose, 5 mM HEPES, and nystatin. The pH was adjusted to 7.0 at 25°C and the suspension was divided into seven portions. The cells in each portion were washed twice by centrifugation (7,500 g , 3 min, $0-4^\circ\text{C}$) and resuspension to 5% hematocrit in media containing 145 mM NaCl, 5 mM KCl, and sucrose at 26, 39, 65, 104, 156, 208, or 260 mM (pH 7.0). After 10 min at 25°C the pH_o was 7.03 ± 0.03 (SD, $n = 7$) and triplicate samples were analyzed for water contents. Points with error bars represent means and standard deviations. Arrows indicate that normal water contents, w^o (0.666 g $\text{H}_2\text{O}/\text{g}$ cells), were obtained with 33 mM sucrose for these cells. The dotted line is the prediction of the ideal Jacobs-Stewart theory considering all osmotic and activity coefficients to equal unity. The solid line shows that the nonideal thermodynamic model, which includes the osmotic coefficients of Hb and of salts, predicts values which agree with the data with an average deviation given by $(\text{predicted-observed})/\text{observed} = 2.4 \pm 0.9\%$ (SD, $n = 7$). See Appendix for method of calculating theoretical lines.

would lose much more water in hypertonic solutions than normal cells with effective cation impermeability since the concentration of intracellular impermeant solutes would be 20 millimolal instead of 170 millimolal. At 200 mM [sucrose]_o, the ideal condition of equality of total intracellular and extracellular concentrations would be achieved in red cells in the presence of nystatin if w' fell to 0.18 g H₂O/g cells (Fig. 4, dotted line); however, the equilibrium water contents were higher by a factor of 3. The difference between actual and predicted cell volumes (Figs. 3 and 4) is much larger than the 10% deviations from ideality previously found with human red cells of normal cation permeability (e.g., Savitz et al., 1964). As shown by the solid line in Fig. 4, the discrepancy for cells treated with nystatin can be explained by utilizing a virial equation (Eq. A6) for ϕ_{Hb} . The virial equation for ϕ_{Hb} of human Hb which accurately predicts cell volumes in the experiments shown in both Figs. 3 and 4 is the same as that which describes measurements on Hb from the blood of sheep (Adair, 1928, 1929) and oxen (Adair's data reported by Hoffman, 1958, and by Dick, 1967; see Appendix and Fig. 7).

The predictions of ideal osmotic theory (Figs. 3 and 4, dotted lines) were based on the assumption of equality of total intracellular and extracellular solute concentrations, and neglected deviations of osmotic coefficients from unity (Jacobs and Stewart, 1947; Eq. A3). This ideal assumption appears to apply to red cells of normal volume (Table IV, columns 3 and 4; Footnote 1), at least within the experimental uncertainty of summing the concentrations. However, in medium containing nystatin and 150 mM [sucrose]_o, in which the relative cell water content, w , is 0.52, the total cellular solute concentration, C_c , is less than C_o by 110 mM (Table IV, columns 5 and 6). In these cells [Hb] has risen from 7.3 to 14 millimolal, and when multiplied by an osmotic coefficient, ϕ_{Hb} , of 7.0 (Eq. A6), the cellular protein contributes 91 milliosmolal, accounting for 75% of the discrepancy. Using an osmotic coefficient, ϕ_s , for internal and external salts equal to 0.93 (see Appendix) brings the ratio of internal to external osmolalities, $\Phi_c C_c / \Phi_o C_o$, to within 4% of unity for cells of shrunken, swollen, or normal volumes (Table IV).

Charge of Impermeant Cellular Solutes

In an attempt to explain osmotic anomalies of red cells, Gary-Bobo and Solomon (1968; 1971) proposed that the charge on Hb decreases with increasing [Hb] on both sides of the isoelectric point, causing Cl shifts which modify osmotic responses. However Gary-Bobo and Solomon's Cl measurements were inconsistent with Cook's earlier (1967) data, and neither Cook nor Dalmark (1975) were able to account for osmotic deviations from measured Cl shifts. Therefore it was of interest to test whether the equivalent charge on cellular impermeant solutes, primarily Hb and organic phosphates, changes with cell volume and [Hb] in nystatin-treated cells which exhibit such large osmotic deviations (Fig. 4). The charge on intracellular impermeant solutes, relative to the value at the initial pH of a suspension, is readily determined in titration experiments from the amount of added HCl or NaOH, since the amount of free acid or base in the extracellular medium is negligible between pH 6 and

8, and since the buffering capacity of the medium is easily measured. Fig. 5 shows the relative charge for cells with [Hb] set at 4.7, 6.4, and 12.1 millimolar by varying cell volume with [sucrose]_o at 15, 32, and 150 mM. The charge on impermeant cell solutes with resultant relative cell volumes, w , of 1.6, 1.0, and 0.5 fell on the same curve when plotted vs. pH_c (Fig. 5 A) or pH_o (not shown), indicating that the slope of the titration curve (equivalent/mole Hb/pH_c) is independent of cell volume and [Hb]. These results indicate that when red cells are titrated at virtually constant cell volume (see Fig. 3) in the presence of nystatin, Hb charge is independent of [Hb] at constant pH (Fig. 5 B).²

TABLE IV
OSMOTIC BALANCE IN RED CELLS TREATED WITH NYSTATIN

[Sucrose] _o , mM	15		32		150	
pH _o	6.49	7.28	6.43	7.27	6.55	7.29
w' (gram of H ₂ O/gram of cells)	0.713	0.738	0.642	0.666	0.504	0.509
w	1.23	1.39	0.902	1.00	0.515	0.525
[Na] _c + [K] _c + [Cl] _c	304	317	310	305	312	313
[P]	10	9	14	12	24	24
[Hb]	6	6	8	7	14	14
Millimolar internal solutes, C_c	320	331	332	324	350	351
Millimolar external solutes, C_o	324	324	341	341	459	459
C_c/C_o	0.99	1.02	0.97	0.95	0.76	0.76
ϕ_{Hb}	2.1	1.9	3.0	2.7	7.0	7.0
Milliosmolar internal, $\Phi_c C_c$	305	313	325	314	410	411
Milliosmolar external, $\Phi_o C_o$	301	301	317	317	427	427
$\Phi_c C_c / \Phi_o C_o$	1.01	1.04	1.03	0.99	0.96	0.96

The values shown above are from the results of experiment B of Table II. The relative water content, w , was estimated from Eq. 4 (see Materials and Methods) using $w' = 0.666$ g H₂O/g cells and assuming $\epsilon = 0.025$. [P] was estimated from $12.4/w$ and [Hb] from $7.3/w$ (see Theory). C_c is the sum of internal solute concentrations (millimolar) including Na, K, Cl, P, and Hb. C_o is the sum of external solute concentrations from measured [K]_o and [Na]_o (see footnote to Table II, expt. B), using [Cl]_o = 150, [HEPES]_o = 5, and [sucrose]_o as indicated. ϕ_{Hb} was calculated from Eq. A6 and $\Phi_c C_c$ from Eq. A5 with $\phi_s = 0.93$ (see Appendix). Φ_o was also taken as 0.93.

Determination of the Isoelectric Point, pI

According to ideal Donnan theory, the pI of impermeant cellular solutes is the pH at which the Cl ratio is unity, at least when intracellular and extracellular cation concentrations are equal. The differences in the Donnan ratios of Cl, Na, and K (Table II) lead to an uncertainty in pI of only 0.1–0.2 units, depending on which ratio is chosen for consideration. According to

² Our finding that the charge on Hb is independent of Hb concentration in nystatin-treated red cells (Freedman and Hoffman, 1977 *b*) has been confirmed recently for cells with normal permeability (Hladky and Rink, 1978) and for Hb solutions (Gros et al., 1978).

nonideal theory, it follows as a special case of the generalized equation (Eqs. 6 *a* and 6 *b*) for the concentration ratio r , where $z = 0$ at pI, that $r_b = r_a = 1/\gamma_{j,k}$. Thus when r_b and r_a are plotted vs. pH_o (not shown), the crossover point ($r_b = r_a$) is located at pI on the abscissa and at $1/\gamma_{j,k}$ on the ordinate. By this method of analyzing the data in Table II, pI is 6.78 ± 0.06 (SD,

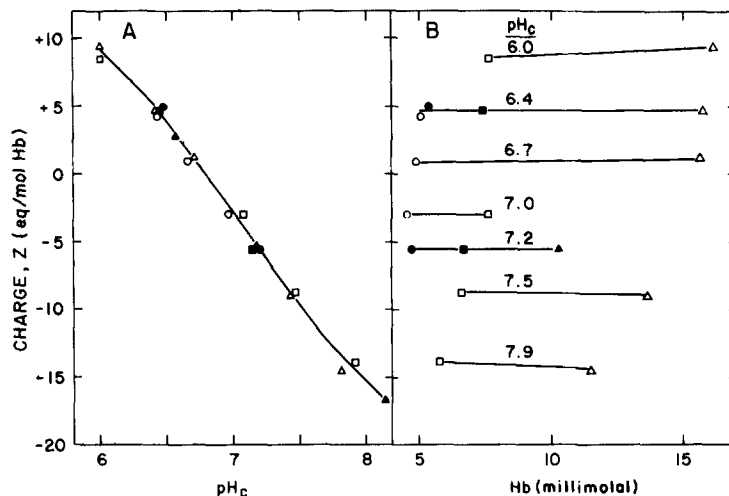


FIGURE 5. Charge on impermeant red cell solutes at varying cell volume. (A) Cell suspensions were titrated with HCl and NaOH in the presence of nystatin as described in Materials and Methods, with relative cell volumes, w , set at 1.33 ± 0.09 , 0.95 ± 0.06 , and 0.52 ± 0.01 (SD, $n = 6$) by varying $[\text{sucrose}]_o$ at (○) 15 mM, (□) 32 mM, and (Δ) 150 mM. Solid symbols refer to buffered suspensions described in expt. B of Table II, in which up to $35.7 \mu\text{eq H}^+$ or $69.0 \mu\text{eq OH}^-$ were added per 10 ml suspension to vary pH_o from an initial value of 7.28 ± 0.01 (SD, $n = 3$) to final values ranging between 6.43 and 8.42. The 5 mM HEPES-buffered cell-free medium was titrated with up to $14.4 \mu\text{eq H}^+$ or $27.0 \mu\text{eq OH}^-$ per 10 ml to vary pH in the same range, yielding a titration curve (equivalent bound/mole HEPES vs. pH) which allowed computation of the amount of added charge bound by the cells. For example, at $\text{pH}_o = 6.43$ and 32 mM sucrose, HEPES binding was $15.0 \mu\text{eq H}^+$ per 10 ml, leaving $22.5 \mu\text{eq}$ per 10 ml bound by the cells. Since $[\text{Hb}]$ for this suspension was determined to be $2.2 \pm 0.1 \mu\text{mol}/10 \text{ ml}$ suspension (SD, $n = 2$), the relative charge for this sample was $+10.2$. The pH_c was estimated from the pH_o and the measured Na distribution ratios (Eq. A12 *b*). Each value for relative charge was then adjusted to absolute charge by subtracting an amount (5.5) which adjusted the titration curve to give $z = 0$ at pH 6.78 (see Results). The open symbols refer to a similar experiment conducted with unbuffered suspensions in which up to $31.5 \mu\text{eq H}^+$ and $21 \mu\text{eq OH}^-$ were added to vary pH_o between 5.97 and 8.10. (Corrections for H^+ and OH^- bound by the unbuffered cell-free medium were unnecessary.) Linear regression gives $z = -12.6 (\text{pH}_c - 6.77)$ at 25°C . (B) The same data for absolute charge (equivalent/mol Hb) as shown in A is plotted vs. $[\text{Hb}]$. The horizontal lines show that the charge on impermeant cell solutes does not change with $[\text{Hb}]$ between 5 and 16 millimolal at constant values of pH_c between 6.0 and 7.9.

$n = 4$) at 25°C, in good agreement with Dalmark (1975). This value for pI appears not to change appreciably with cell volume or to depend on whether KCl or NaCl is considered.

DISCUSSION

A main conclusion reported in this paper is that a nonideal thermodynamic generalization of the ideal Jacobs-Stewart (1947) theory accounts better for equilibrium salt and water distribution in human red blood cells than has previously been possible (Figs. 3, 4, and 6). We also find that Hb charge is constant with varying $[Hb]$ at constant pH (Fig. 5) in cells exhibiting large osmotic deviations (Figs. 3 and 4) after treatment with nystatin. Even though normal red cell $[Hb]$ is 45 g/100 ml water, with spacings between the surfaces of neighboring Hb tetramers of 10–20 Å, the ideal Jacobs-Stewart theory agrees well with measured Donnan ratios for cells having both cations and anions at equilibrium (Fig. 3, bottom dotted line), primarily because the intracellular mean ionic activity coefficients for KCl and NaCl are within 2% of the values in the extracellular solution (Tables II and III). The major improvement is due to inclusion of the osmotic coefficient of Hb and of salts, resulting in better agreement between predicted and measured water contents (Figs. 3 and 4).

The nonideal thermodynamic model also predicts the dependence on pH_o of Cl ratios and water contents in dilute suspensions of red cells with normal cation permeability (data of Gunn et al., 1973, their Fig. 3). Good agreement between data and theory is found (Fig. 6) using the same general equations with the same parameters as were used for cells treated with nystatin. Inclusion of terms for nonidealities in the theory (Fig. 6, top solid line) leads to improvements in the predicted water contents in shrunken cells at alkaline pH, where ϕ_{Hb} is relatively more important than in cells of normal volume. Table V summarizes how the various equilibrium parameters behave according to the model as a function of pH_o in red blood cells having either high or low cation permeability. Note that at the isoelectric point, where $z = 0$, E_{Cl} is zero for cation-permeable cells but is displaced to -0.5 mV for cation-impermeable cells. This small potential is due to the unequal internal and external impermeant cation concentrations—the “double-Donnan” effect. The predictive success of the nonideal thermodynamic model for cells with an intact membrane supports the view that the properties determined in cation permeable cells are relevant to cells in more normal physiological conditions.

Both the Van Slyke et al. theory (1923) and the Jacobs and Stewart (1947) formulation assume that the charge on impermeant cell solutes varies only with pH_c (Eq. A11) and is not an explicit function of cell volume or $[Hb]$, and this assumption agrees with the results shown in Fig. 5. In Gary-Bobo and Solomon's analysis (1968), the calculated charge on Hb decreased with increasing $[Hb]$ on both sides of pI (their Fig. 8) by an amount which reduced the slope of the titration curve of shrunken cells by a factor of 3 (their Fig. 9), in contrast to the invariant slope seen in the direct titrations of nystatin-treated red cells set at different cell volumes with sucrose (Fig. 5 A). However, as water leaves the cells during shrinkage at constant pH_o , the concentrations

of Na, K, and Hb all increase proportionately, causing a small shift of r_{Cl} , and consequently of pH_c and of z . For cation-impermeable cells, Donnan theory predicts that the direction of the change of pH_c is independent of whether the initial pH is above or below pI , and that the shift of r_{Cl} should be larger for shrinkage by sucrose than by NaCl, an example of the "double-Donnan" effect. For normal human red cells equilibrated with sucrose such that the

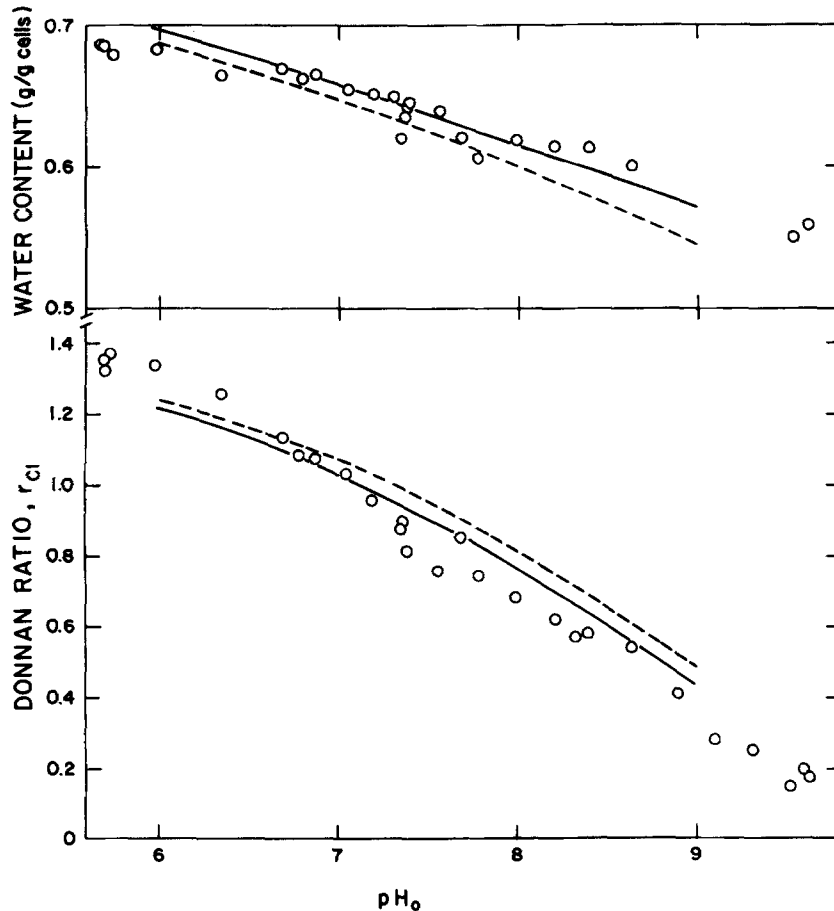


FIGURE 6. Equilibrium pH dependence of Cl distribution ratios and water contents of human red cells with normal low cation permeability. Data from Gunn et al. (1973, their Fig. 3) for cells suspended in 145 mM NaCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM D-glucose, and 27 mM glycylglycine at 0°C. The solid lines are predictions of the nonideal thermodynamic model (see Appendix), and the dotted lines are predictions of the ideal Jacobs-Stewart theory. The water contents, w' (gram of H_2O /gram of cells), were converted from the reported units, w'' (gram of H_2O /gram of solids), using $w' = w''/(1 + w'')$. Since the water contents were reported with corrections for trapped medium, they were compared with predicted relative water contents, w , using $w' = w/(w + 0.560)$ (see Eq. 5).

relative volumes decrease from 1.2 to 0.9, the nonideal thermodynamic model predicts that the membrane potential should increase by 8 mV, in good agreement with the 11-mV depolarization estimated from equilibrium sulphate ratios by Castranova et al. (1979). This change for cation impermeable cells is larger than the hyperpolarization of 3 mV at pH 7.3 and the depolarization of 2 mV at pH 6.5, as predicted and observed for cation

TABLE V
EQUILIBRIUM PROPERTIES OF HUMAN RED BLOOD CELLS,
AS DESCRIBED BY THE NONIDEAL THERMODYNAMIC MODEL

pH _o	pH _c	<i>z</i>	<i>r</i> _{Cl}	<i>E</i> _{Cl}	φ _{Hb}	<i>w</i> '	<i>w</i>
<i>mV</i>							
A. Cation-impermeable cells							
6.00	6.05	+11.5	1.21	+4.5	2.1	.697	1.29
6.40	6.43	+7.7	1.16	+3.4	2.3	.681	1.20
6.80	6.80	+4.0	1.08	+1.8	2.5	.666	1.12
7.20	7.16	+0.4	0.99	-0.3	2.7	.650	1.04
7.40	7.34	-1.4	0.94	-1.5	2.8	.642	1.00
7.60	7.51	-3.1	0.88	-2.9	2.9	.633	0.97
8.00	7.85	-6.5	0.77	-6.2	3.2	.616	0.90
8.40	8.17	-9.7	0.64	-10.4	3.5	.598	0.83
8.80	8.47	-12.7	0.50	-16.1	3.9	.580	0.77
B. Cation-permeable cells							
6.00	6.05	+7.5	1.21	+4.9	2.9	.656	0.96
6.40	6.41	+3.9	1.11	+2.7	3.1	.642	0.90
6.80	6.77	+0.3	1.01	+0.2	3.2	.639	0.89
7.20	7.13	-3.3	0.92	-2.2	3.2	.644	0.91
7.40	7.31	-5.1	0.88	-3.4	3.1	.649	0.93
7.60	7.49	-6.9	0.84	-4.5	3.0	.654	0.95
8.00	7.86	-10.6	0.78	-6.4	2.7	.672	1.03
8.40	8.23	-14.3	0.74	-7.8	2.5	.692	1.12
8.80	8.62	-18.2	0.71	-8.9	2.2	.714	1.24

Shown above as a function of varying pH_o are computer-generated equilibrium parameters for human red cells (A) having normal low cation permeability and (B) having high cation permeability after treatment with nystatin. The program inputs included the composition of the suspending solutions as given in Fig. 6 for normal cells (at 0°C) and in Fig. 3 for cation-permeable cells (at 25°C), and also the average values for the cell contents (see Theory). The assumptions and details of the calculation are described in the Appendix. The program outputs included intracellular pH, pH_c; charge, *z*, on impermeant cell solutes; Cl Donnan ratio, *r*_{Cl}; membrane potential, *E*_{Cl} (mV); osmotic coefficient of Hb, φ_{Hb}; cell water content, *w*' (grams of H₂O/gram of cells); and the water content, *w*, relative to its value for cells in isotonic solution.

permeable cells in the presence of nystatin and sucrose with relative volumes decreasing from 1.4 to 0.5 (Table II). In cation-permeable cells, the direction of the small shift of *r*_{Cl} does change on opposite sides of pI, as expected for the "single-Donnan" system. Potential-sensitive fluorescent dyes also show small changes in equilibrium potential (Freedman and Hoffman, 1977 *b*, 1978; cf. Hladky and Rink, 1978) which are consistent with the Gibbs-Donnan theory. Thus the nonideal thermodynamic model accounts quantitatively for shifts in

the Donnan ratio with changes of cell volume, and for the patterns that red cells in the presence of nystatin have water contents which are less sensitive to pH_o but more sensitive to π_o than cells of normal cation permeability, as well as for the dependence on pH of Donnan ratios in both types of cells.

Activity Coefficients

The use of cation and anion concentration ratios to measure intracellular relative mean ionic activity coefficients, $\gamma_{j,k}$ (Eq. A9), is contained in the theory of nonideal solutions in equilibrium across semipermeable membranes (Donnan and Guggenheim, 1932) but has evidently not been previously applied to red cells, because cations as well as anions must be at equilibrium. That $\gamma_{K,Cl}$ and $\gamma_{Na,Cl}$ are both within 2% of unity (Tables II and III) indicates that Na and K, as well as Cl (Cook, 1967) and nonelectrolytes (Gary-Bobo, 1967) have normal solubility in virtually all of the water of cells of normal or shrunken volume. This result agrees with the generally held view that any interactions of these monovalent cations and anions with intracellular charged constituents do not appreciably perturb solute distribution. The evidence that Hb does not selectively bind Na, K, or Cl in dilute solution (Passow, 1964) is extended by the data of Table II to 12 millimolal or 58% [Hb], although some second-order effects are evident. The nearly ideal behavior of r_{Cl} (Table II and Fig. 6) is not inconsistent with interactions of Cl with Hb of a magnitude which may affect the oxygen dissociation and titration curves of Hb (Antonini et al., 1963, 1972; Chiancone et al., 1972). The normal solubility of Na, K, and Cl in the water of red cells treated with nystatin is inconsistent with a fraction of red cell water being at the same time "nonsolvent" for electrolytes and "solvent" for nonelectrolytes (cf. Gary-Bobo and Solomon, 1968).

"Nonosmotic" Water

In spite of the evidence that total intracellular and extracellular concentrations are nearly equal in normal human red cells (see Footnote 1), a fraction of water assumed to be unavailable for dissolving solutes, or "nonosmotic water", amounting to 15–20% of the isotonic water content has been postulated to account for the deviations from ideal Boyle-van't Hoff osmotic behavior (e.g., Savitz et al., 1964; Levin et al., 1976, 1977; for discussion, see Ponder, 1948, Chapter 3). The amount of nonosmotic water has frequently been obtained from a linear extrapolation of Eq. A4 to infinite osmotic pressure, implicitly assuming that there are no net solute movements concomitant with cell volume changes, or $N_T = N_T^o$, and no changes in the osmotic coefficients of any of the solutes, or $\Phi_c = \Phi_c^o$. With these assumptions Eq. A4 is equivalent to the Ponder equation with Ponder's " \mathcal{R} " = $(1 - V_h/V_w^o)$. But Adair's data (1928, 1929; Hoffman, 1958; Dick, 1967) implies that Φ_c is not constant, since it depends on the relative cell volume, w , through the dependence of ϕ_{Hb} on [Hb] (Fig. 7, Eqs. A5 and A6). Therefore, a linear extrapolation neglecting changes in Φ_c at best overestimates the amount of nonosmotic water. A more serious problem is that subtraction of nonosmotic water, V_h , in the osmotic pressure equation (Eq. A1) evidently imparts the wrong limiting behavior to

the expression (Eq. A4) for relative cell volumes in that at infinite π_o , a finite water fraction V_h/V_w^o , is predicted to remain in the cells. The notion of nonosmotic water being unable to dissolve cellular solutes is not only contrary to the evidence for electrolytes (Tables II and III; Cook, 1967) and nonelectrolytes (Gary-Bobo, 1967), but also it ascribes a specific molecular interpretation to a complex phenomenon to which many molecular effects may be contributing simultaneously. In lieu of quantitative knowledge of these effects, we prefer to express osmotic pressures using the osmotic coefficient, Φ , a parameter which is both independent of mechanism and which allows for

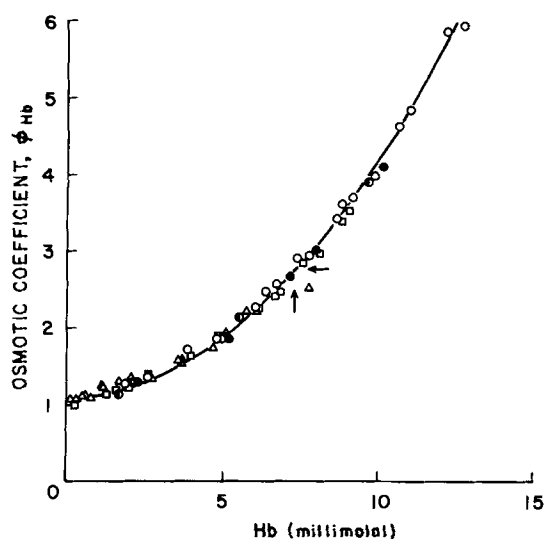


FIGURE 7. Osmotic coefficient of hemoglobin, ϕ_{Hb} , as determined by Adair. Triangles are for sheep HbCO at pH 7.8 and ionic strength, μ , of 0.29 (Adair, 1928, 1929). Squares are data for ox HbCO at pH 7.4 and $\mu = 0.1$ obtained from Adair by Hoffman (1958). Circles are Adair's data for ox HbCO up to 12.7 millimolal [Hb] (reported by Dick, 1967), for the following values of pH and μ : (●) pH 7.32, $\mu = 0.15$; (●) pH 6.96, $\mu = 0.1$; (○) pH 7.43, $\mu = 0.10$. Linear regression of $(\phi_{\text{Hb}} - 1)/[\text{Hb}]$ vs. $[\text{Hb}]$ gives the virial equation (Eq. A6), $\phi_{\text{Hb}} = 1 + 0.0645[\text{Hb}] + 0.0258[\text{Hb}]^2$. Arrows indicate that at a normal [Hb] of 7.3 millimolal, ϕ_{Hb} is 2.85.

nonlinear osmotic behavior at high π_o . Thus by setting nonosmotic water, V_h , equal to zero, and retaining the osmotic coefficients of Hb and of salts (see Appendix), we found that the measured water contents for red cells treated with nystatin over a wide range of π_o may be predicted from a virial equation (Eq. A6) for ϕ_{Hb} of human Hb. Ideal Jacobs-Stewart theory, which assumes that $\phi_{\text{Hb}} = \phi_s = \gamma_{j,k} = \Phi_o = 1$, predicted water contents too low by a factor of 3 (Fig. 4, dotted line), while including ϕ_{Hb} (Fig. 4, solid line) improved the prediction to within 3% of the data. Dick and Lowenstein (1958) used ϕ_{Hb} to describe the nonideal osmotic behavior of hypotonic suspensions of human

red cells, and Dick (1967) later suggested it might apply also to hypertonic suspensions (see also Williams et al., 1959), and this suggestion is borne out by the results of Fig. 4. Although it appears that the values for ϕ_{Hb} which predict human red cell osmotic behavior are identical to those measured for isolated sheep, oxen, and human Hb (Adair, 1928, 1929; Dick, 1967), this conclusion should be reassessed with additional measurements on human Hb using an osmometer with a shorter time constant than was available to Adair (1961), and using conditions which approximate the red cell interior.

Osmotic Coefficient of Non-Hb Solutes, ϕ_s and Φ_o

From Eq. A7 and the results of Fig. 3, the derived value for the average osmotic coefficient of intracellular salts, ϕ_s , is 0.93, equal to its value in 150 millimolal salt solutions (Handbook of Chemistry and Physics, 1977), indicating that ϕ_s , like $\gamma_{j,k}$, is close to ideal. The relation between ϕ_s and $\gamma_{j,k}$ could be determined by using the Gibbs-Duhem equation, for which purpose it would be necessary to know the dependence of both ϕ_s and $\gamma_{j,k}$ on the concentration of the salts. The effect of including the average osmotic coefficient of salts in the extracellular solution, $\Phi_o = 0.93$, is to remove a discrepancy between the predicted and measured water contents (Fig. 4) equivalent to 7% nonosmotic water (see Eq. A3).

Volume Stabilization of Cation-Permeable Cells

The colloid osmotic pressure of cells made highly permeable to cations with nystatin is no longer opposed by the effective impermeability to Na and K, and the cells swell (Fig. 1 A) and eventually hemolyze. By varying the concentration of impermeant solute in the medium, cell volume can be set at various levels (Figs. 1 and 4). The [sucrose]_o, or [M]_o, needed to maintain normal cell volume, or $w = 1$, referred to as the compensation point (Wilbrandt, 1948), is found by setting $w = 1$ in Eq. 7 and solving for [M]_o. In the special case where [a]_o = [b]_o, and at pI, where $r_a = 1/\gamma_{j,k}$, we have,

$$[M]_o = \frac{\phi_{\text{Hb}}[\text{Hb}]^o + \phi_s[\text{P}]^o + 2[a]_o \left(\frac{\phi_s}{\gamma_{j,k}} - \Phi_o \right)}{\Phi_o} \quad (9)$$

In ideal Donnan theory, where $\phi_{\text{Hb}} = \phi_s = \Phi_o = \gamma_{j,k} = 1$, Eq. 9 predicts normal water contents at only 19 mosM, considerably less than is found experimentally. When the theory is modified by letting $\phi_{\text{Hb}} = 2.85$, $\phi_s = \Phi_o = 0.93$, and $\gamma_{j,k} = 1.00$, then normal water contents are expected at 34 mM [sucrose]_o, in good agreement with the 33 mM obtained at pH 7.0 (Fig. 4) and with the data of Wilbrandt (1948).

Interpretation of ϕ_{Hb}

The increase of ϕ_{Hb} with [Hb] (Fig. 7) expresses the tendency of Hb to retain greater amounts of water than would an ideal solution of the same concentration at any given external osmotic pressure. Although this osmotic behavior

could also be expressed in terms of the activity coefficient of the solvent, interpretations at the molecular level are difficult to separate and quantitate. Recent calculations (see Richards, 1977) of the tortuosity of the surface of soluble proteins indicate that at 34% [Hb], the volume fraction of water in the first monolayer around the protein surface would be a substantial fraction (about 30%) of the total intracellular water—a property which differentiates the red cell interior from a dilute solution. With increasing [Hb] during osmotic shrinkage, both enthalpic and entropic effects are expected to contribute to nonideal cell water retention. An unfavorable enthalpy, or desolvation energy, results if Hb changes its conformation to fill the void left by the removal of solvent from the interstices of the tightly packed system. In addition to excluded volume effects and the nonideal entropy of mixing resulting from the difference in the sizes of the protein and solvent molecules (Dick, 1959), the restriction of translational and rotational motions of the protein associated with tight packing would also contribute to an unfavorable entropy increase during cell shrinkage. Until concentrated protein solutions are better understood, the molecular bases for the values of ϕ_{Hb} , ϕ_s , and $\gamma_{j,k}$ in red blood cells cannot be determined (cf. Overbeek, 1956). The extent of cytoplasmic influence on deviations of equilibrium salt and water distribution from the predictions of ideal Gibbs-Donnan theory are best expressed by the nonideal thermodynamics of a multicomponent solution containing a large volume fraction of protein.

APPENDIX

In order to improve the theory which predicts the concentrations of intracellular permeant solutes and the volume of human red blood cells as a function of the composition of the extracellular solution and of certain cell parameters, the equations for osmotic equilibrium and Donnan ratios are modified in this section by including osmotic and activity coefficients. These modified equations of constraint, when combined with equations for the electroneutrality condition and for the titration curve of the impermeant cell solutes, enable a nonideal generalization of the ideal Jacobs-Stewart (1947) theory for red cell ionic and osmotic equilibria. (See Glossary for definitions of symbols. Note that in the following equations the subscripts *c* and *o* refer to the intracellular and extracellular solutions, respectively.)

Osmotic Equilibrium

The osmotic pressure, π , of a multicomponent solution containing N_T moles of total solutes dissolved in V_w liters of water may be assumed to be given by

$$\pi = \frac{\Phi RT N_T}{V_w - V_h}, \quad (\text{A1})$$

where Φ is the osmotic coefficient of the mixture, R is the gas constant, and T is the absolute temperature. V_h is the volume of “nonosmotic” water, or water assumed to be unavailable for dissolving solutes, and has been included in Eq. A1 in order to relate our treatment to previous studies, but will later be neglected since interactions between solutes and water are already contained in Φ .

Similarly, the osmotic pressure, π_c , of cells in isotonic medium (denoted by superscript *o*) is given by

$$\pi_c^o = \frac{\Phi_c^o RT N_T^o}{V_w^o - V_h} \quad (\text{A2})$$

At low external [protein], V_h may be neglected in the medium. Now defining $w_h = V_h/V_w$ for the cytoplasmic solution, and equating π_c with π_o , we obtain,³

$$\Phi_o C_o = \frac{\Phi_c C_c}{(1 - w_h)}, \quad (\text{A3})$$

where C_o and C_c are the total extracellular and intracellular solute concentrations. For ideal dilute solutions where $\Phi = 1$, and $w_h = 0$, Eq. A-3 reduces to the commonly used ideal osmotic expression assumed by Jacobs and Stewart (1947).

Dividing π_c (from Eq. A1) by π_c^o (Eq. A2) yields upon rearrangement (with $\pi_c = \pi_o$) the cell water content relative to that of cells in isotonic medium, V_w/V_w^o , as follows:

$$\frac{V_w}{V_w^o} = \left(1 - \frac{V_h}{V_w^o}\right) \left(\frac{\Phi_c N_T}{\Phi_c^o N_T^o}\right) \frac{\pi_o^o}{\pi_o} + \frac{V_h}{V_w^o} \quad (\text{A4})$$

Eq. A4 expresses succinctly three ways of describing osmotic deviations: (a) by changes in net solute content concomitant with changes in cell volume, or $N_T \neq N_T^o$; (b) by the presence of nonosmotic water, or $V_h \neq 0$, and (c) by changes in osmotic coefficients during volume changes, or $\Phi_c \neq \Phi_c^o$.

The osmotic coefficient of the intracellular mixture, Φ_c , is the average of the osmotic coefficients, ϕ_i , of the components, n_i , of the solution weighted according to their respective mole fractions,

$$\Phi_c = \sum_i \frac{\phi_i [c_i]}{C_c} = \frac{1}{C_c} (\phi_{\text{Hb}}[\text{Hb}] + \phi_s[s]), \quad (\text{A5})$$

where ϕ_s is the osmotic coefficient for the mixture of all intracellular solutes except Hb. By combining the osmotic coefficients of Na, K, Cl and P into a single term, ϕ_s , it is unnecessary to make assumptions about their individual values.

The osmotic coefficient of Hb, ϕ_{Hb} , (Adair, 1928, 1929; Adair's data obtained by Hoffman, 1958; Adair's data reported by Dick, 1967; cf. McConaghey and Maizels, 1961) up to 12.7 millimolal [Hb] may be described by the following virial equation:

$$\phi_{\text{Hb}} = 1 + 0.0645[\text{Hb}] + 0.0258[\text{Hb}]^2, \quad (\text{A6})$$

where $[\text{Hb}] = [\text{Hb}]^o/w$. At a normal $[\text{Hb}]^o$ of 7.3 millimolal, ϕ_{Hb} is 2.85. As shown in Fig. 7, for [Hb] up to 7 millimolal, ϕ_{Hb} of sheep HbCO (Adair, 1928, 1929) agrees well with data for ox HbCO (obtained from Adair both by Hoffman, 1958, and by Dick, 1967). Adair (1928) also reported that for concentrations up to 5 g Hb/100 ml, ϕ_{Hb} of sheep Hb is quite close to that of human Hb.

To obtain ϕ_s , Eqs. A3 and A5 are combined and solved. With $w_h = 0$, we obtain

$$\phi_s = (\Phi_o C_o - \phi_{\text{Hb}}[\text{Hb}])/[s]. \quad (\text{A7})$$

Electroneutrality

The equation for electroneutrality is identical for ideal and nonideal solutions,

$$\sum_i z_i [c_i] = 0. \quad (\text{A8})$$

³ The hydrostatic pressure difference of 2.3 mm H₂O in normal and swollen cells, and zero in shrunken cells (Rand and Burton, 1964), can be neglected.

Donnan Ratios

Including ionic activity coefficients, γ_i , in the expressions for the electrochemical potentials, $\tilde{\mu}_i$, prior to equating $\tilde{\mu}_c = \tilde{\mu}_o$ for each univalent permeant salt (j^+k^-), leads to a generalized Donnan equation (Donnan and Guggenheim, 1932; Overbeek, 1956) given by

$$r_j r_k = \frac{[j]_c [k]_c}{[j]_o [k]_o} = \frac{(\gamma_j)_o (\gamma_k)_o}{(\gamma_j)_c (\gamma_k)_c} = \frac{1}{\gamma_{j,k}^2}, \quad (\text{A9})$$

where $r_j = [j]_c/[j]_o$ and $r_k = [k]_c/[k]_o$.

Therefore the mean ionic activity coefficient for a particular salt in the intracellular solution, $\gamma_{j,k}$, relative to its value in the extracellular solution is given by

$$\gamma_{j,k} = (r_j r_k)^{-1/2}. \quad (\text{A10})$$

Titration of Impermeant Cellular Solutes

Over a restricted range of pH, a linear approximation for the titration curve (e.g., Fig. 5 A) of impermeant cell solutes, chiefly Hb and intracellular phosphates, adequately represents the average net charge, z (equivalent/mole Hb), given by

$$z = m [\text{pH}_c - (\text{pI}_{T=0} - 0.016T)]. \quad (\text{A11})$$

The isoelectric point, pI, of the intracellular mixture depends on the relative amounts of intracellular phosphates and Hb, and decreases with increasing celsius temperature, T , as indicated. According to Dalmark (1975), $m = -10$ eq/mol Hb per pH and $\text{pI}_{T=0} = 7.2$.

Since the proton activity ratio, $\frac{[a_H]_o}{[a_H]_c} = 0.92 r_{\text{Cl}}$ (Fitzsimmons and Sendroy, 1961; Bromberg et al., 1965; Funder and Wieth, 1966 c), and since $r_{\text{Na}} r_{\text{Cl}} = 1.03$ (Table III), pH_c and pH_o are related as follows:

$$\text{pH}_c = \text{pH}_o + \log r_{\text{Cl}} - 0.034, \quad (\text{A12 a})$$

$$\text{pH}_c = \text{pH}_o - \log r_{\text{Na}} - 0.023. \quad (\text{A12 b})$$

Estimated differences of liquid junction potentials of saturated KCl bridges in plasma and hemolyzed packed red cells (Salling and Siggard-Andersen, 1971) would lower pH_c by an additional 0.013, a negligible amount for the present calculations and experiments. Since $\gamma_{\text{K,Cl}}$ and $\gamma_{\text{Na,Cl}}$ do not change with cell volume (Table II), it is probable that the factor 0.92 is also independent of cell volume.

Generalized Equations for the Donnan Ratio and Water Content

Following the same algebraic manipulations as Jacobs and Stewart (1947), but using the revised equations for nonideal solutions (Eqs. A3, A5, and A9 above), and assuming that nonosmotic water, w_h , equals zero (see Discussion), we arrive at a modified expression for the relative water content, w , given by

$$w = \frac{D}{\Phi_o C_o - \phi_s (r_a [a]_o + r_b [b]_o)} = \frac{E}{r_a [a]_o - r_b [b]_o}, \quad (\text{A13})$$

where $D = \phi_{\text{Hb}}[\text{Hb}]^o + \phi_s([B]_c^o + [\text{P}]^o)$ and $E = [B]_c^o + z[\text{Hb}]^o$. Solving Eqs. A13 for the Donnan ratio, r_a , to eliminate w gives the generalized expressions (Eqs. 6 a and 6 b) for r_a and r_b as a function of z , ϕ_{Hb} , ϕ_s , $\gamma_{j,k}$, Φ_o , and the concentrations of intracellular impermeant solutes and of extracellular solutes.

Method of Calculation

The dependence of r on pH_o , as determined by the simultaneous Eqs. 6, A11, and A12 yields a transcendental function. Since r also depends on ϕ_{Hb} which is a quadratic function of w (eq. A6) and since w may also contain r (Eq. 8), an iterative calculation was devised to predict the pH-dependence of both r and w . A computer program (Hewlett-Packard 9820A, Palo Alto, Calif.) was constructed such that after entering the composition of an extracellular solution, and the concentrations of impermeant cellular components, a provisional r was computed on the initial assumptions that $z = 0$, $\phi_{\text{Hb}} = 2.85$, and $\phi_s = 0.93$. This value for r yielded a pH_c (Eq. A12) which was used to estimate z (Eq. A11), and then r was recalculated (Eq. 1). The process was repeated until r converged to within ± 0.005 . Then r was used to compute the water content, w (by Eq. 7 or 8). Eqs. 7 or 8 and A6 upon combination yield a cubic equation for w , but with only one real positive root, which then allowed revision of ϕ_{Hb} (Eq. A6) and ϕ_s (Eq. A7). These were then used to repeat the iterative calculation of r . The iterations were continued until both r and w converged to within ± 0.005 and ± 0.02 , respectively.

We thank R.B. Gunn for providing us with tabulated data from previously published experiments for comparison with our model, and D.A.T. Dick for helpful suggestions and for tabulations of Adair's unpublished data. We also thank C. Miller for reading the manuscript. This research was supported by grants HL-09906, AM-17433, and GM-00167 from the U. S. Public Health Service. Dr. Freedman was a recipient of a Fellowship in Membrane Pathology/Physiology.

Received for publication 29 September 1978.

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