EFFECT OF HYDRATION ON THE WATER CONTENT OF HUMAN ERYTHROCYTES

R. L. LEVIN, E. G. CRAVALHO, *and* C. E. HUGGINS

From the Cryogenic Engineering Laboratory, Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and the Department of Surgery, Harvard Medical School, and Low Temperature Surgical Unit, Massachusetts General Hospital, Boston, Massachusetts 02214. Dr. Levin's present address is the Biophysical Laboratory, Harvard Medical School, Boston, Massachusetts 02115.

ABSTRACT An ideal, *hydrated,* nondilute pseudobinary salt-protein-water solution model of the RBC intracellular solution has been developed to describe the osmotic behavior of human erythrocytes during freezing and thawing. Because of the hydration of intracellular solutes (mostly cell proteins), our analytical results predict that at least 16.65% of the isotonic cell water content will be retained within RBCs placed in hypertonic solutions. These findings are consistent not only with the experimental measurements of the amount of isotonic cell water retained within RBCs subjected to nonisotonic extracellular solutions (20–32%) but also with the experimental evidence that all of the water within RBCs is solvent water. By modeling the RBC intracellular solution as a hydrated salt-protein-water solution, no anomalous osmotic behavior is apparent.

INTRODUCTION

Several theories have been proposed to explain the freezing damage of cells (see Meryman [1] for review). However, only one of these theories, namely Mazur's two-factor model of freezing injury (2, 3), is generally applicable to widely disparate cell types. Mazur's working hypothesis is that:

(*i*) Slowly cooled cells are killed by relatively long exposure to major alterations produced in the intracellular and extracellular solutions by the conversion of water to ice. These alterations, or solution effects, include the "high" concentration of solutes (4, 5), stresses in the cell membrane produced by cell water loss (6), changes in pH, and the dehydration and precipitation of solutes.

(*ii*) Rapidly cooled cells are killed by the formation of intracellular ice during cooling and its subsequent growth by recrystallization during warming, especially during slow warming.

Of course in any real physical situation, these two factors overlap, resulting in a bell-shaped survival signature curve (percent survival vs. cooling rate).

In recent years, however, these qualitative statements regarding cell survival have been, for the most part, successfully expressed quantitatively. The resulting models

for the kinetics of the cell water loss during freezing (7–10) are not only interesting but also useful since they characterize the response of cells to the thermodynamic events associated with the freezing process in terms of certain cell parameters, most notably the water conductivity of the cell membrane and the surface area to volume ratio of the cell. With the aid of these models and numerical values for the cell parameters, it is now possible to predict the transport of cell water during freezing. More recently, these models have been coupled with models for the kinetics of ice nucleation to quantify from a theoretical point of view the conditions under which ice will form intracellularly (11).

At the present time, however, a large quantitative disparity exists between these theoretical predictions and available experimental measurements of the transition cooling rate, e.g. the cooling rate above which the probability of intracellular ice nucleation for a given cell type is virtually 100%, and the intracellular water content of cells during freezing. Specifically, these models predict a transition cooling rate for red blood cells between 2×10^3 (11) and 3×10^3°C/min (7) which is in direct conflict with the experimental results of Diller et al. (12) who observed that the frequency of intracellular freezing increased from 0% at −840°C/min to 100% at −850°C/min. Furthermore, these models indicate that more than 95% of the water contained in RBCs under isotonic conditions is transferable under an osmotic pressure gradient during freezing; whereas experimental results indicate that at least 20% (Savitz et al. [13] and Farrant and Woolgar [14]), if not 80% (Watson [15], of the isotonic cell water volume is retained within RBCs during cooling. We are thus left with the apparent anomalous osmotic behavior of the red cell water content under certain conditions. Even though the osmotic pressure difference across the membrane appears to be sufficient to drive the intracellular water out of RBCs, it seems that no such water transport occurs and that a substantial fraction of the initial cell water is retained within RBCs.

The present analytical investigation develops an ideal, *hydrated,* nondilute salt-protein-water solution model of the RBC intracellular solution which successfully explains in quantitative terms the large disparity which exists between presently available theoretical predictions and experimental measurements of the transition cooling rate and intracellular water content of human erythrocytes.

LIST OF SYMBOLS

A_c	Cell surface area
a	Activity
B	Cooling rate
c	Molar concentration
E_k	Cell membrane conductivity activation energy
F	Faraday constant (96,486.9 C/mol)
G	Free energy
h	Hydration number
J	Flux
$\tilde{k}$	Cell membrane conductivity
$\tilde{k}_{T_g}$	Cell membrane conductivity at temperature T_g
L_p	Hydraulic permeability
L_w	Latent heat of fusion of water
m	Molality
N	Number of molecules
p	Hydrostatic pressure
R	Universal gas constant (8.314 J/mol °K or 1.987 cal/mol °K)

r	Position	x	Mole fraction
s	Solute	z	Valence
T	Temperature	γ	Activity coefficient
T_g	Reference temperature (293.15° K)	η	Viscosity
T_o	Freezing point (273.15° K)	ν	Number of species per molecule
t	Time	π	Osmotic pressure
V	Volume	Φ	Osmotic coefficient
V_c	Cell volume	ϕ	Volume fraction
$\bar{v}$	Apparent molar volume	μ	Chemical potential
w	Water		

Subscripts

I	Ice
i	ith process or species
j	jth process or species
m	Solute mixture
o	Initial or isotonic
P	Protein salt
S	Inorganic salt
s	Solute
w	Water

Superscripts

B	Bound
F	Free
h	Hydrated
I	Intracellular
O	Extracellular
$*$	Pure substance

DESCRIPTION OF PROBLEM

Cell Volume Regulation

Although most solutions of biological interest are complex salt-protein-water solutions, let us consider the case of an RBC intracellular solution consisting of a single un-ionized solvent, w (water), and a single solute, s.[1] For this case, the volume of the cell V_c is related to the volume occupied by the membrane and the intracellular components w and s such that:

$$V_c = V_{\text{memb}} + N_w\bar{v}_w + N_s\bar{v}_s, \tag{1}$$

where N_i and $\bar{v}_i$ are, respectively, the number of moles and partial molar volumes of the ith component inside the cell ($i = w,s$). For constant partial molar volumes and a cell membrane impermeable to the solute s but permeable to the solvent w

$$dV_c/dt = \cancelto{0}{(dV_{\text{memb}}/dt)} + (dN_w/dt)\bar{v}_w + \cancelto{0}{(dN_s/dt)\bar{v}_s},$$

or

$$dV_c/dt = \bar{v}_w(dN_w/dt) = -(\bar{v}_w \cdot J_w)A_c, \tag{2}$$

where A_c is the surface area of the cell and J_w is the net flow of water *out of* the cell per unit area of membrane per second (see Dick [17]). The flux J_w is given by an expres-

[1] Since the diffusion of ions of an electrolyte is restricted by the condition of electric neutrality, it is permissible to treat the partial volumes, concentrations, etc., as those of the electrolyte as a whole without considering the ν_s moles of ions per molecule as separate ionic quantities (see Robinson and Stokes [16], Chap. 2).

sion of the form (Katchalsky and Curran [18], Chap. 10)

$$J_w = [L_p/(\bar{v}_w)^2](\mu_w^I - \mu_w^O), \tag{3}$$

where μ_w^I and μ_w^O are, respectively, the chemical potential of the water at the internal and external surfaces of the cell membrane, and L_p is the hydraulic water permeability of the cell membrane. The chemical potential for water can be written as (see Robinson and Stokes [16], Chap. 2, or Katchalsky and Curran [18], Chap. 5)

$$\mu_w = \mu_w^*(T) + \bar{v}_w p + RT \ln a_w, \tag{4}$$

where p is the hydrostatic pressure and a_w is the activity of the water in the solution. Since it is certainly reasonable to assume that there are no significant temperature gradients within the small cell suspension under consideration (Mansoori [10]), we can let $\mu_w^*(T)|^I = \mu_w^*(T)|^O$. Furthermore, we can assume that the cell suspension is isobaric with $p^I = p^O$ (see Papanek [19], pp. 31–32). Consequently, Eq. 3 takes the form

$$J_w = (\tilde{k}/\bar{v}_w)(\ln a_w^I - \ln a_w^O), \tag{5}$$

where we have defined $\tilde{k}$ as the cell membrane water conductivity:

$$\tilde{k} = L_p RT/\bar{v}_w. \tag{6}$$

According to Eq. 2, the volume of the cell decreases as water leaves the cell during the freezing process and increases as water enters the cell during the thawing process. Since we are assuming that the cell membrane is impermeable to solutes, the total amount of solute within the cell is constant

$$N_s = (1/\bar{v}_s) \int_{V_c} \phi_s(r, t)\, dV_c = \text{constant}, \tag{7}$$

or

$$\phi_{s_0} \cdot V_{c_0} = \int_{V_c} \phi_s(r, t)\, dV_c, \tag{8}$$

where ϕ_{s_0} and V_{c_0} are, respectively, the solute volume fraction and the cell volume under isotonic conditions, and ϕ_s is the volume fraction of solute within the cell at any time t during the cooling process. Furthermore, if we assume that the composition of the intracellular solution remains uniform, with no concentration polarization of solutes taking place within the intracellular solution,[2] then $\phi_s(r, t) = \phi_s(t)$. Hence,

[2]It should be noted that for cooling rates less than 100°C/min, little, if any concentration polarization of solutes takes place within RBCs because at these cooling rates all of the solutes can diffuse within the intracellular solution faster than they are being convectively deposited at the intracellular solution-membrane interface by the water leaving the cell. However, a significant degree of solute polarization occurs within RBCs being cooled at rates in excess of 500°C/min, although the amount of water retained by erythrocytes during cooling is not significantly affected by the concentration polarization phenomenon for cooling rates less than 5,000°C/min (20, 21). The effect of solute polarization on the amount of water retained by erythrocytes being cooled at subzero temperatures will be reported separately.

from Eq. 8

$$\phi_s(t) = (\phi_{s_0} \cdot V_{c_0})/V_c(t), \tag{9}$$

where from Eq. 2

$$V_c(t) = V_{c_0} - \int_0^t (\bar{v}_w \cdot J_w) A_c \mathrm{d}t, \tag{10}$$

such that $(\bar{v}_w J_w)|_{t=0} = 0$ because chemical equilibrium is assumed to prevail across the cell membrane at $t = 0$, that is, before extracellular nucleation occurs.

Solute Hydration

According to Stokes' law for the motion of particles in viscous liquid solutions, such as protein solutions, the volume fraction of the solute, ϕ_s, is the product of the solute concentration, c_s, and the "effective rigid molar volume" of a solute molecule, $\bar{v}_s^h$ (see Robinson and Stokes [16], Chap. 11):

$$\phi_s = c_s \bar{v}_s^h. \tag{11}$$

$\bar{v}_s^h$ represents the molar volume of the solute particle including any water of hydration which is held too firmly to participate in any viscous shearing process. In solutions of biological interest the water of hydration can be significant, ranging from 1 H_2O molecule/salt molecule to over 1,260 H_2O molecules/protein molecule (see below). Hence, the "effective" molar volume of the solute computed on a hydrated basis, $\bar{v}_s^h$, will be significantly larger than its apparent molar volume computed on an unhydrated basis, $\bar{v}_s$. For a salt molecule such as NaCl or KCl where $\bar{v}_{NaCl} = 17$ cm^3/mol and $\bar{v}_{KCl} = 27$ cm^3/mol, $\bar{v}_S^h = \bar{v}_S + \bar{v}_w \sim 2\,\bar{v}_S$ since $\bar{v}_w = 18$ cm^3/mol, whereas for a much larger protein molecule such as hemoglobin where $\bar{v}_{Hb} = 5.0 \times 10^4$ cm^3/mol, $\bar{v}_{Hb}^h \sim \frac{7}{5}\,\bar{v}_{Hb}$.

Thus the phenomenon of solute hydration is of considerable importance in any discussion of transport in concentrated solutions. Since in liquid solutions not only must mass be conserved ($\Sigma x_i = 1$) but also volume ($\Sigma \phi_i = 1$), transport processes such as diffusion can be considered to be basically "volume transfer processes" rather than mass transfer processes as they are in either gaseous or solid solutions. Consequently, 1 cm^3 of solvent molecules must in general move in the opposite direction to 1 cm^3 of solute molecules. If the solute particles carry with them a permanently attached layer of solvent molecules, these solvent molecules from a diffusion point of view will act as part of the diffusing *solute* entity. That is, if 1 mol of solute s is associated with h_s moles of "bound water" such that the number of "bound water" molecules in the solution, N_w^B, is equal to $h_s N_s$ where N_s is the total number of solute molecules in the solution, then the number of "free water" molecules will be

$$N_w^F = N_w - N_w^B = N_w - h_s N_s, \tag{12}$$

where N_w is the total number of solvent molecules in the solution. Let us define the solute mole fraction on a hydrated basis as the ratio of the total number of solute

particles, $\nu_s N_s$, to the total number of diffusing entities in the solution, $N_w^F + \nu_s N_s$:

$$x_s^h = \nu_s N_s/(N_w^F + \nu_s N_s) = \nu_s N_s/(N_w + [1 - (h_s/\nu_s)]\nu_s N_s). \quad (13)$$

Since $x_s^h + x_w^h = 1$, the water mole fraction computed on a hydrated basis is

$$x_w^h = N_w^F/(N_w^F + \nu_s N_s) = (N_w - h_s N_s)/(N_w + [1 - (h_s/\nu_s)]\nu_s N_s). \quad (14)$$

If we define x_w and x_s as the mole fractions of solvent and solute computed on an unhydrated basis such that $x_w + x_s = 1$ and

$$x_w = N_w/(N_w + \nu_s N_s), \quad x_s = \nu_s N_s/(N_w + \nu_s N_s), \quad (15)$$

then

$$x_w^h = (-(h_s/\nu_s) + [1 + (h_s/\nu_s)]x_w)/([1 - (h_s/\nu_s)] + (h_s/\nu_s)x_w),$$

$$x_s^h = x_s/[1 - (h_s/\nu_s)x_s], \quad (16)$$

Likewise let us define ϕ_w and ϕ_s as the volume fractions of the solvent and the solute computed on a *hydrated* basis:

$$\phi_w = N_w^F \bar{v}_w/(N_w^F \bar{v}_w + N_s \bar{v}_s^h), \quad \phi_s = N_s \bar{v}_s^h/(N_w^F \bar{v}_w + N_s \bar{v}_s^h). \quad (17)$$

Consequently, if we consider the solute to be hydrated, the chemical potential or activity of the solute considered as a solvated species will be different from its value when considered as an unsolvated species ($a_s^h = a_s(a_w)^{h_s/\nu_s}$, see Levin [20], Robinson and Stokes [16], and Prausnitz [22]), but the total Gibbs free energy, G, of a given amount of solution is fixed, regardless of the method used for expressing the composition of the solution. The chemical potential of the solvent, μ_w, defined by (16, 18)

$$\mu_w = (\partial G/\partial N_w)_{N_s, T, p} \quad (18)$$

is therefore likewise unaffected by the method of expressing x—it is the free energy gained on adding one mole of solvent to an infinite amount of solution, regardless of whether part of the added solvent actually combines with the solute or not. Hence, we find that the activity of the water within the solution can be defined as

$$a_w = \gamma_w x_w, \quad (19)$$

where γ_w is the water activity coefficient defined on an unhydrated basis, or as

$$a_w^h = \gamma_w^h x_w^h \quad (20)$$

where γ_w^h is the water activity coefficient defined on a hydrated basis (16, 22). Thermodynamics requires that

$$a_w^h \equiv a_w,$$

or

$$\gamma_w^h x_w^h = \gamma_w x_w. \quad (21)$$

Consequently, a solution which can be considered to be ideal on a hydrated basis (i.e. $\gamma_w^h \equiv \gamma_s^h \equiv 1$) will not necessarily be ideal on an unhydrated basis.

Note that if the solution is hydrated and the volume fraction of water approaches zero ($\phi_w \to 0$ or $x_w^h \to 0$), the mole fraction of water does not approach zero but a finite positive limit of

$$x_w \xrightarrow[\phi_w \to 0]{} h_s/\nu_s/(1 + h_s/\nu_s).$$

It follows then that a biological cell which is being frozen or placed in a hypertonic solution may come to osmotic equilibrium ($\mu_w^I - \mu_w^O = 0$) by *losing all of its (free) water*, i.e. $\phi_w \to 0$, and yet *not dehydrate*, i.e. $x_w > 0$ (see Fig. 1). This seemingly contradictory statement is based on the fact that according to the above formulation all the water in the cell is solvent water, even though only a fraction of the total water content, $N_w^F/N_w = (N_w - h_s N_s)/N_w < 1$, is free to diffuse and/or leave the cell under an osmotic pressure gradient. We shall return to this point later when we discuss the supposedly anomalous osmotic behavior of RBCs in hypertonic solutions.

On the basis of the physiochemical properties of the RBC intracellular solution species (see below), we will model the RBC intracellular solution as a hydrated nondilute solution. The chemical potential of the water within the RBC intracellular solution is then given by an expression of the form

$$\mu_w = \mu_w^*(T, p) + RT \ln(\gamma_w^h x_w^h), \tag{22}$$

where the solute and water volume fractions are computed on a hydrated basis (see Eq. 17). This assumption is supported by the experimental observations of Savitz et al. (13) who found that the osmotic behavior of human erythrocytes (RBCs shrunken in hypertonic solutions) is described by an expression of the form

$$\pi = \Phi RT \nu_s N_s / V_w^h, \tag{23}$$

where π is the osmotic pressure, Φ is the osmotic coefficient, and $V_w^h = N_w^F \cdot \bar{v}_w$ is the volume occupied by the "free" water in the intracellular solution. Eqs. 22 and 23 are equivalent:

$$\ln a_w = \ln \gamma_w^h x_w^h \equiv \Phi \ln x_w^h = \Phi \ln(1 - x_s^h). \tag{24}$$

If the solution can be considered dilute on a molar basis ($x_s^h/x_w^h = \nu_s N_s/N_w^F \ll 1$), then the right-hand side of the above equation can be expanded:

$$\ln a_w \sim -\Phi[x_s^h - \tfrac{1}{2}(x_s^{h2}) + \cdots] = -\Phi[\nu_s N_s/(N_w^F + \nu_s N_s^o)], \tag{25}$$

or[3]

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[3]It should be noted that Eq. 26 for $\ln a_w$ is not equivalent to the more prevalent form (16, 23)

$$\ln a_w = (-18/10^3) \Sigma \Phi_i \nu_i m_i, \tag{29}$$

where m_i is the molality of species i defined as the number of moles of species i per kilogram (or liter) of (total) water. This expression is valid only for the case of a solution which can be considered to be dilute on both a molar ($x_s/x_w \ll 1$) and volume ($\phi_s/\phi_w \ll 1$) basis. Consequently, it is not applicable to human RBCs even under isotonic conditions (see below).

$$\ln a_w \sim -\Phi(\nu_s N_s / N_w^F). \tag{26}$$

The osmotic pressure is defined such that (see Robinson and Stokes [16], Chap. 2, or Katchalsky and Curran [18], Chap. 5 and 10)

$$\pi = -(RT/\bar{v}_w) \ln a_w. \tag{27}$$

Then

$$\pi = \Phi RT(\nu_s N_s / N_w^F \bar{v}_w), \tag{28}$$

which is the same expression empirically arrived at by Savitz et al. (13).

The only additional thermodynamic assumption used in deriving these relationships has been that the value of h_s is unchanged as the composition of the solution is varied. This means that in applying the equations to actual solutions, we are limited to cases for which there are plenty of solvent molecules to go round among the solute particles and cases for which the forces between solute and solvent are at least approximately of a saturable nature. While the forces between ions and water molecules are at least mainly electrostatic, and therefore are not strictly saturable in the same way as chemical binding forces, there is strong reason to believe that water molecules in direct contact with ions are subject to much greater forces of attraction than subsequent layers,[4] and there is a geometrical limit to the number of such closest molecules (Robinson and Stokes [16], Chap. 9). Consequently, we may reasonably expect the assumption of an h value independent of concentration to apply up to moderately high concentrations.

APPLICATION OF THE MODEL TO HUMAN ERYTHROCYTES

The model presented above is applicable to a wide variety of physical situations, but for the purposes of illustration we have applied the analysis to determine the behavior of human red blood cells during cooling at constant rates of change of temperature. This situation is one of considerable clinical importance in the reversible freezing of blood for long-term storage. However, before presenting the results of this application of the theory, some remarks concerning the physical and geometrical characteristics of human red blood cells are in order since they play such a key role in the model.

Human Erythrocyte Geometry

Measurements of the diameter, surface area, and volume of individual red blood cells have been reported by several investigators using different techniques (15, 25–27). Canham and Burton (26) have shown that for one person, the surface area can vary from 105 to 180 μm^2 and the volume from 70 to 155 μm^3. In view of these variations, we have chosen to use the following average values for the surface area and volume of a RBC:

$$A_c = 135\ \mu m^2, \qquad V_c = 100\ \mu m^3. \tag{30}$$

[4]The free energy of interaction between an ion and a single water molecule is some 40 kcal/mol, as opposed to a value of some 2 kcal/mol for a hydrogen bonded water molecule (Stein [24], p. 91).

The shape of an erythrocyte may be described as a biconcave disc, but for simplicity, we have chosen to represent the erythrocyte as a simple disc-shaped cell with a constant surface area. This simplifying assumption is supported by the experimental observations of Rand and Burton (25) and Evans and Fung (27).

Human Erythrocyte Composition

The human erythrocyte is a cell without a nucleus and contains a complex aqueous solution of hemoglobin, nonhemoglobin proteins, lipids, glucose, electrolytes (mainly K^+, Na^+, Cl^-, HCO_3^-, phosphates) and other components with approximately 97% of its volume occupied by water and hemoglobin (26, 28–32). The volume of intracellular water alone is 72% of the total volume of the cell even though not all of this water is available for transport during freezing and thawing.

In the computation it will be assumed that under isotonic conditions the amount of protein present within a RBC is 5.20×10^{-16} mol ($\approx$34 g Hb/100 ml cell) and the amount of salt present within a cell is 1.080×10^{-14} mol with $\nu_{salt} = 2.0$ (300 mosmol/liter H_2O). It will be assumed that the properties of the protein can be approximated by the properties of hemoglobin which has a hydration number of 1,260 mol H_2O/mol Hb ($\sim$34 g H_2O/g Hb) (33–36) and an apparent molar volume (hydrated) of 7.0×10^4 cm^3/mol (37). Since intracellularly the amount of K^+ is much greater than the amount of Na^+ ($N_K/N_{Na} \sim 7.8$) and Cl^- is the dominant univalent anion, the properties of the salt will be approximated by the properties of KCl in an aqueous solution of equivalent molality. Hence, the salt will be assumed to have a hydration number of 1.0 mol H_2O/mol salt (16), an apparent molar volume (unhydrated) of 27.50 cm^3/mol (16, 38), and a hydrated apparent molar volume of 45.50 cm^3/mol (16). On the basis of the large binding energies associated with the hydration of salts (Stein (24), p. 21) and proteins, the hydration numbers of the salt and the protein will be considered to be constant and independent of the composition and temperature of the solution. Furthermore, since solutions of hemoglobin in the physiological pH range do not show any specific binding of sodium, potassium or other small electrolytes 39–41), we shall assume that there are no small ions bound to the proteins in the solution, i.e. no K^+—Hb^{---} association.

Because the model for the phenomenon of solute polarization developed above deals only with a two component solution, we have defined a pseudobinary mixture such that the number of moles of a fictitious solution species, m, present within the cell is equal to the number of moles of protein plus salt present within the cell:

$$N_m = N_P + \nu_S N_S, \qquad N_m = 2.212 \times 10^{-14} \text{ mol}. \tag{31}$$

The fictitious solute is assumed to have a hydration number and a hydrated apparent molar volume which is a weighted average of those corresponding values for the protein and the salt:

$$\bar{v}_m^h = (N_S \bar{v}_S^h + N_P \bar{v}_P^h)/N_m, \qquad \bar{v}_m^h = 1{,}668 \text{ cm}^3/\text{mol}, \tag{32}$$

$$h_m = (h_S N_S + h_P N_P)/N_m, \qquad h_m = 30.11 \text{ mol } H_2O/\text{mol}, \tag{33}$$

where it has been assumed that $\nu_m = 1.0$.

Since water occupies 72% of the cell volume, the total number of water molecules present within the cell under isotonic conditions is

$$N_{w_o} = 4.00 \times 10^{-12} \text{ mol}, \tag{34}$$

where it has been assumed that the partial molar volume of water is 18.0 cm^3/mol. For a solute hydration number of 30.11 and 2.212×10^{-14} mol of solute within the cell, the total number of "bound water" molecules is

$$N_w^B = h_m N_m,$$

or

$$N_w^B = 6.660 \times 10^{-13} \text{ mol}, \qquad N_w^B/N_w = 0.1665. \tag{35}$$

Consequently, the number of "free water" molecules present within the cell under isotonic conditions is

$$N_w^F = N_w - N_w^B, \qquad N_w^F = 3.334 \times 10^{-12} \text{ mol}. \tag{36}$$

It should be noted that while the mole fraction of water under isotonic conditions, computed on either a hydrated or an unhydrated basis, is greater than 0.993, the volume fraction of water is less than 0.62, and the RBC intracellular solution cannot be considered dilute on a "volume" basis. From the diffusion, i.e. volume-transfer point of view, the intracellular RBC solution is not dilute even under isotonic conditions. Therefore, we cannot make any simplifying assumptions like Eq. 29 regarding the dilution of the RBC intracellular solution. Instead, we shall assume that the RBC intracellular solution is an ideal,[5] *hydrated,* nondilute pseudobinary solution of salts, proteins, and water. The variation of the mole fraction of water within the cell as computed on an unhydrated and hydrated basis is plotted as a function of the intracellular water volume fraction in Fig. 1.

Water Permeability of the Erythrocyte Membrane

The temperature dependence of the water conductivity of the RBC membrane is given by (19, 42–44)

$$\tilde{k} = \tilde{k}_{T_g} \exp\{-E_k/R\,[(1/T) - (1/T_g)]\}, \tag{37}$$

where $\tilde{k}_{T_g}$ corresponds to the effective cell water conductivity at $T_g = 293.15$°K (20°C)

[5]This assumption does not yield results which are substantially different from those obtained for the case of considering the RBC intracellular solution as a non-ideal, hydrated, nondilute salt-protein-water mixture (20). We shall be reporting later the effects of non-ideal solution behavior.

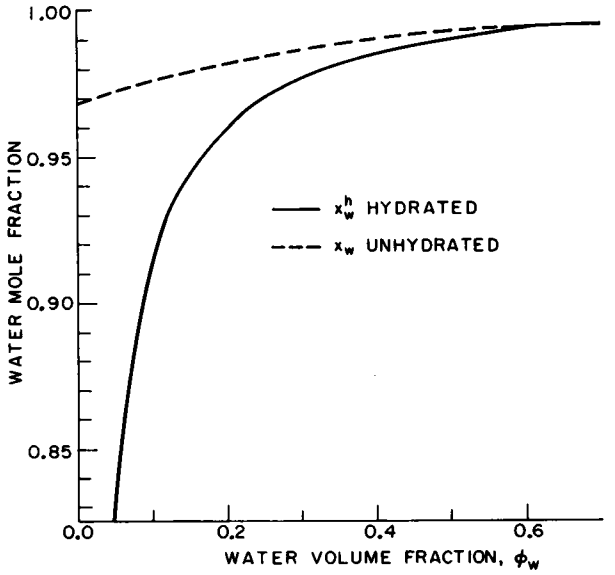

FIGURE 1 Comparison of the concentration dependence of a hydrated and an unhydrated mole fraction.

and E_k is the apparent activation energy for the permeation process:

$$\tilde{k}_{T_g} = 17.3 \times 10^{-3}\,\text{cm/s},$$
$$E_k = 16.34\,\text{J/mol}\,(3.90\,\text{kcal/mol}).$$

Composition of the Extracellular Medium

In order to evaluate Eq. 5 values are needed for a_w^o, the activity of the water at the extracellular solution-membrane interface. By assuming that during the freezing process (*i*) solid solution consisting of *pure ice* forms only in the extracellular medium; (*ii*) once ice forms extracellularly, chemical equilibrium prevails between the solid and liquid phases in the extracellular medium; (*iii*) the composition of the liquid phase of the extracellular medium is uniform, with no polarization of solutes taking place at either the external surface of the cell membrane or at the liquid-solid interfaces.

a_w^o can be shown to vary with temperature such that (20)

$$\partial \ln a_w^o / \partial T = L_w / RT^2, \tag{38}$$

where L_w is the latent heat of fusion of water at the temperature and pressure of the solution. Using Kirchoff's Law and the equations given by Dorsey (45) for the temperature dependence of the heat capacities, one obtains the following expression for the variation of $\ln a_w^o$ with temperature:

$$\ln a_w^o = 3.736 \times 10^3[(1/T) - (1/T_o)] + 36.18 \cdot \ln(T/T_o) + 0.1024(T_o - T) + 5.435 \times 10^{-5}(T^2 - T_o^2), \tag{39}$$

where $T_o = 273.15°\text{K}$ (20).

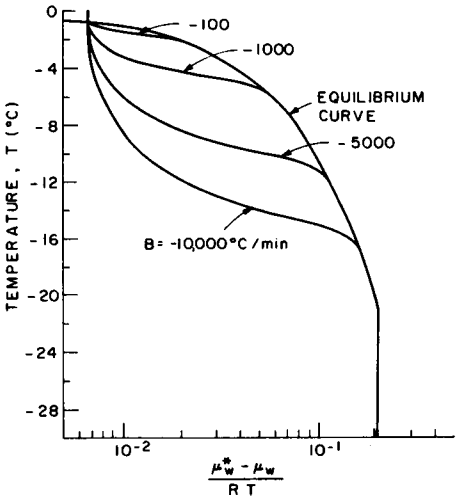

FIGURE 2 Volume flux of water out of RBCs cooled at different rates for the case of no solute polarization within the ideal, hydrated pseudobinary intracellular solutions.

The red cell in vivo is suspended in an aqueous solution containing primarily salts and proteins, but since it is common clinical practice to suspend packed red blood cells in normal saline solution with no significant damage to the cell, we shall assume that the extracellular medium is an initially isotonic sodium chloride aqueous solution (m_{NaCl} = 0.154 mol/liter H_2O) with a eutectic composition of 5.205 mol/liter H_2O for temperatures less than −21.12°C.

RESULTS

Fig. 2 shows the temperature dependence of the volume flux of water ($\bar{v}_w \cdot J_w$) out of the RBCs for several different cooling rates. It is evident that the volume flux of water leaving a cell is not constant but goes through a maximum that is a function of the cooling rate. For $B = -10{,}000$°C/min, $\bar{v}_w \cdot J_w \mid_{max} = 7.36 \times 10^{-4}$(cm^3/mol)·(mol/cm^2·s) at $T = -12.2$°C whereas for $B = -100$°C/min, $\bar{v}_w \cdot J_w \mid_{max} = 0.47 \times 10^{-4}$(cm^3/mol)·(mol/cm^2·s) at −1.50°C. The reason that the magnitude of the maximum water volume flux increases with faster cooling rates in spite of the fact that the cell membrane conductivity $\tilde{k}$ decreases with decreasing temperature, is that the difference in chemical potential of the water across the cell membrane, $\Delta\mu_w/RT = \ln a_w^i - \ln a_w^o$, is greater at a given temperature for the faster cooling rates (see Fig. 3). That is, at the higher cooling rates heat transfer dominates over mass transfer and a large volume of intracellular water remains within the cell at very low temperatures, thus increasing the probability of internal ice nucleation. At the lower cooling rates, however, mass transfer dominates over heat transfer and most of the intracellular water is able to leave the cell before very low temperatures are reached, thereby de-

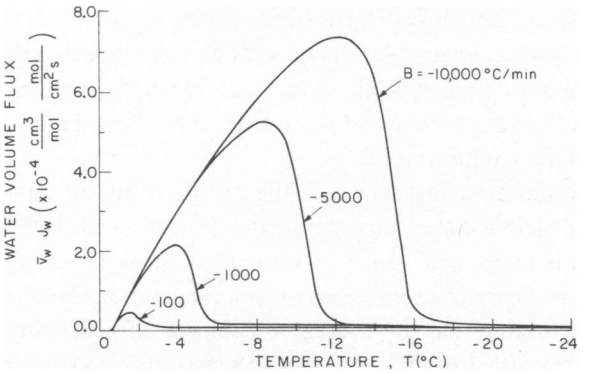

FIGURE 3 Thermodynamic states of the ideal, hydrated pseudobinary intracellular solutions of RBCs cooled at different rates for the case of no solute polarization. Locus of states of extracellular solution coincides with two-phase equilibrium states for all cooling rates.

creasing the probability of intracellular ice nucleation. Fig. 3 also indicates that the intracellular solution approaches equilibrium, with $\Delta\mu_w \rightarrow 0$ and $\bar{v}_w \cdot J_w \rightarrow 0$, at temperatures above the NaCl-H_2O eutectic temperature of -21.12°C even for cooling rates as fast as 10,000°C/min.

The amount of water present within an RBC cooled at various rates can be seen in Fig. 4. This figure shows that as a cell approaches equilibrium at subzero temperatures, $V_w/V_{w_o} \rightarrow 0.1907$ or $\phi_m \rightarrow 0.9550$. Although these results are qualitatively similar to those of both Silvares (8, 9) and Mazur (7) in that at a given subzero temperature more water remains within a cell for the faster cooling rates, quantitatively, they are completely different. In both Mazur's and Silvares's treatments, more than 95% of the initial intracellular water content of an RBC can leave the cell, whereas in our treatment at least 16.65% of the initial RBC water content must remain within a

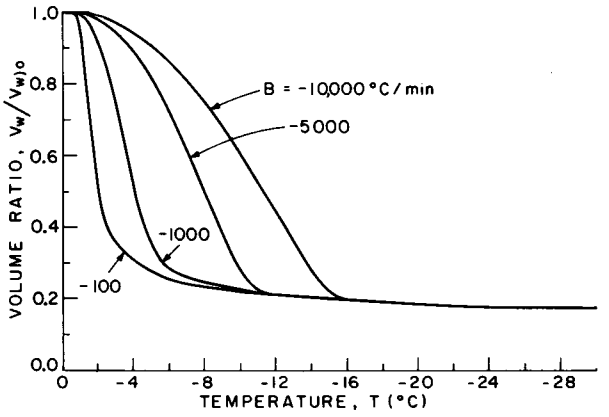

FIGURE 4 Volume of intracellular water of RBCs cooled at different rates for the case of no solute polarization within the ideal, hydrated pseudobinary intracellular solutions.

cell.[6] Since both Mazur and Silvares considered only unhydrated salt-water solution models of the RBC intracellular solution whereas we have modeled the RBC intracellular solution as a hydrated protein-salt-water mixture with 16.65% of the water under isotonic conditions serving as water of hydration, the difference in the resulting final volumes must be due to hydration effects.

Our present findings are consistent with the results of an alternative approach for the study of volumetric changes in erythrocytes (Savitz et al., [13] and Farrant and Woolgar [14]). This approach, which involves simulating freezing conditions by resuspending RBCs in hypertonic solutions of different osmolalities and using hematocrit measurements to compute the average magnitude of cell volume change, shows that at least 20–32% of the isotonic cell water volume is retained within RBCs. Clearly, these static room temperature experiments do not fully model the dynamics of the freezing process under all conditions, but their results are approached by red blood cells cooled at very slow rates for which the osmolality of the extracellular medium also varies slowly, with osmotic equilibrium established for each temperature (or osmolality). Hence their results provide an approximate lower bound to the amount of water retained within erythrocytes during cooling which is close to our assumed value of 16.65%.

Furthermore, the hydration model that we have developed is in accord with the work of Kwant and Seeman (46) and of Colombe and Macey (47). Both of these studies showed that resealed erythrocyte ghosts, freshly prepared by a one-step hemolysis technique with membranes resealed at 37°C and containing less than 5% of the hemoglobin present within the originally intact cells, behaved as "perfect osmometers." Consequently, according to Kwant and Seeman, the semipermeable properties of the erythrocyte membrane cannot account for the imperfect osmotic response of intact cells.

Finally, our results are consistent with the findings of Miller (48), Cook (49), and Gary-Bobo and Solomon (50) who found that all of the water within RBCs is "solvent water." We contend further that even though all of the water is solvent water from the point of view of the solutes, not all of this water is transferable under an osmotic pressure gradient. Admittedly, this is a fine distinction, but unlike many investigators, we believe that the concept of "solvent water" and "bound water" are not contradictory but complimentary concepts. The fact that a substantial fraction of the water within erythrocytes is "bound" to solutes (mostly to cell proteins) is consistent not only with the experimental evidence concerning the amount of isotonic cell water retained within intact RBCs (13, 14) and resealed erythrocyte ghosts (46, 47) subjected to non-isotonic extracellular solutions but also with the experimental evidence that all of the water within RBCs is solvent water (48–50) as the thermodynamic model de-

[6]Note: $V_w/V_{w_o} = 0.1665$ when there is no "free" water remaining within the cell $\phi_w = 0$. For RBCs suspended in an aqueous NaCl solution, equilibrium below the NaCl-H_2O eutectic temperature of −21.12°C requires that $\ln a_w^I = -0.20602$ or $\phi_m = 0.9550$ and $V_w/V_{w_o} = 0.1907$.

veloped above shows. Consequently, in our opinion, erythrocytes do not experience any anomalous osmotic behavior.

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REFERENCES

1. MERYMAN, H. T. 1966. Review of biological freezing. *In* Cryobiology. H. T. Meryman, editor. Academic Press, London. 1.
2. MAZUR, P., S. P. LEIBO, and E. H. T. CHU. 1972. A two-factor hypothesis of freezing injury. *Exp. Cell Res.* **71**:345.
3. MAZUR, P. 1965. Causes of injury in frozen and thawed cells. *Fed. Proc. Am. Soc. Exp. Biol.* **24**:S175.
4. LOVELOCK, J. E. 1953. The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. *Biochim. Biophys. Acta.* **11**:28.
5. LOVELOCK, J. E. 1953. The haemolysis of human red blood cells by freezing and thawing. *Biochim. Biophys. Acta.* **10**:414.
6. MERYMAN, H. T. 1968. Modified model for the mechanism of freezing injury in erythrocytes. *Nature (Lond.).* **218**:333.
7. MAZUR, P. 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J. Gen. Physiol.* **47**:347.
8. SILVARES, O. M. 1974. A thermodynamic model of water and ion transport across cell membranes during freezing and thawing: the human erythrocyte. Sc.D. Thesis, Dept. of Mechanical Engineering, MIT, Cambridge, Mass.
9. SILVARES, O. M., E. G. CRAVALHO, W. M. TOSCANO, and C. E. HUGGINS. 1975. The thermodynamics of water transport for biological cells during freezing. *Trans. ASME, J. Heat Transfer.* **98**:582.
10. MANSOORI, G. A. 1975. Kinetics of water loss from cells at subzero centigrade temperatures. *Cryobiology.* **12**:34.
11. TOSCANO, W. M., E. G. CRAVALHO, O. M. SILVARES, and C. E. HUGGINS. 1975. The thermodynamics of intracellular ice nucleation in the freezing of erythrocytes. *Trans. ASME. J. Heat Transfer.* **97**:326.
12. DILLER, K. R., E. G. CRAVALHO, and C. E. HUGGINS. 1976. An experimental study of intracellular freezing in erythrocytes. *Med. Biol. Eng.* **14**:321.
13. SAVITZ, D., V. W. SIDEL, and A. K. SOLOMON. 1964. Osmotic properties of human red cells. *J. Gen. Physiol.* **48**:79.
14. FARRANT, J., and A. E. WOOLGAR. 1972. Human red cells under hypertonic conditions; A model system for investigating damage. *Cryobiology.* 9:9, 131.
15. WATSON, W. W. 1974. Volumetric changes in human erythrocytes during freezing at constant cooling velocities. S.M. Thesis, Dept. of Mechanical Engineering, MIT, Cambridge, Mass.
16. ROBINSON, R. A., and R. H. STOKES. 1959. *Electrolyte Solutions.* 2nd edition. Butterworth & Co. Ltd., London.
17. DICK, D. A. T. 1966. Cell Water. Butterworth & Co. Ltd., London.
18. KATCHALSKY, A., and P. F. CURRAN. 1967. Nonequilibrium Thermodynamics in Biophysics. Harvard University Press, Cambridge, Mass.
19. PAPANEK, T. H. 1975. An apparatus to measure red cell permeability at low temperature. S.M. Thesis, Department of Mechanical Engineering, MIT, Cambridge, Mass.
20. LEVIN, R. L. 1976. Kinetics of water transport in biomaterials during freezing. Sc.D. Thesis, Department of Mechanical Engineering, MIT, Cambridge, Mass.
21. LEVIN, R. L., E. G. CRAVALHO, and C. E. HUGGINS. 1976. Diffusion transport in a liquid solution with a moving, semi-permeable boundary. *Trans. ASME. J. Heat Transfer.* (1976).
22. PRAUSNITZ, J. M. 1969. Molecular Thermodynamics of Fluid-Phase Equilibrium. Prentice Hall, Inc., Englewood Cliffs, N.J.

23. KATCHALSKY, A., and Z. ALEXANDROWICZ. 1963. On the additivity of osmotic properties of polyelectrolyte solutions. *J. Pol. Sci.* Part A. **1**:2093.
24. STEIN, W. D. 1967. The Movement of Molecules across Cell Membranes. Academic Press, Inc., New York.
25. RAND, R. P., and A. C. BURTON. 1963. Area and volume changes in hemolysis of single erythrocytes. *J. Cell Comp. Physiol.* **61**:245.
26. CANHAM, P. B., and A. C. BURTON. 1968. Distribution of size and shape in populations of normal human red cells. *Circ. Res.* **22**:405.
27. EVANS, E., and Y. C. FUNG. 1972. Improved measurements of the erythrocyte geometry. *Microvasc. Res.* **4**:335.
28. ALTMAN, P. A., ed. Blood and Other Body Fluids. 1961. Federation American Societies for Experimental Biology, Washington, D.C.
29. WILLIAMS, W. J., E. BEUTLER, A. J. ERSLEY, and R. W. RUNDLES. 1972. Hematology. McGraw-Hill, Inc., New York.
30. WOLF, A. V. 1966. Aqueous Solutions and Body Fluids. Harper and Row, New York, 1966.
31. BOLIS, L., and B. D. GOMPERTS. 1972. Red blood cells. *In* Transport and Accumulation in Biological Systems. E. J. Harris, editor. University Park Press, Baltimore.
32. BISHOP, C., and D. M. SURGENOR. 1964. The Red Blood Cell. Academic Press, Inc., New York.
33. ADAIR, G. S., and M. E. ADAIR. 1938. The densities of protein crystals and the hydration of proteins. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* **120**:422.
34. PERUTZ, M. F. 1946. The composition and swelling properties of haemoglobin crystals. *Trans. Faraday Soc.* **42B**:187.
35. DRABKIN, D. L. 1950. Hydration of macro-sized crystals of human haemoglobin and osmotic concentrations in red cells. *J. Biol. Chem.* **185**:231.
36. TANFORD, C. 1961. Physical Chemistry of Macromolecules. John Wiley & Sons, New York.
37. BRAGG, W. L., and M. F. PERUTZ. 1952. The external form of the haemoglobin molecule. *Acta Crystallogr.* **5**:277.
38. HARNED, H. S., and B. B. OWEN. 1958. The Physical Chemistry of Electrolytic Solutions. 3rd edition. Reinhold Publishing Co., New York.
39. MORRIS, R., and R. D. WRIGHT. 1954. On the interaction of hemoglobin with sodium and potassium. *Aust. J. Exp. Biol. Med. Sci.* **32**:669.
40. CARR, C. W. 1956. The binding of sodium and potassium ions in solutions of various proteins. *Arch. Biochem. Biophys.* **62**:476.
41. TANFORD, C., and Y. NOZAKI. 1966. Titration of the histidyl and α-amino group of hemoglobin. *J. Biol. Chem.* **241**:2832.
42. LEVIN, R. L., E. G. CRAVALHO, and C. E. HUGGINS. 1976. A membrane model describing the effect of temperature on the water conductivity of erythrocyte membranes at subzero temperatures. *Cryobiology.* **13**:415.
43. ZWOLINSKI, B. J., H. EYRING, and C. E. REESE. 1949. Diffusion and membrane permeability. *J. Phys. Chem.* **53**:1426.
44. MAZUR, P. 1965. The role of cell membranes in the freezing of yeast and other single cells. *N.Y. Acad. Sci.* **125**:658.
45. DORSEY, N. E. 1940. Properties of Ordinary Water Substance. American Chemical Society Monograph Series No. 81, Reinhold Publishing Co., New York.
46. KWANT, W. O., and P. SEEMAN. 1970. The erythrocyte ghost as a perfect osmometer. *J. Gen. Physiol.* **55**:208.
47. COLOMBE, B. W., and R. I. MACEY. 1974. Effects of calcium on potassium and water transport in human erythrocyte ghosts. *Biochim. Biophys. Acta.* **363**:226.
48. MILLER, D. M. 1964. Sugar uptake as a function of cell volume in human erythrocytes. *J. Physiol.* **170**:219.
49. COOK, J. S. 1967. Non-solvent water in human erythrocytes. *J. Gen. Physiol.* **50**:1311.
50. GARY-BOBO, C. M., and A. K. SOLOMON. 1968. Properties of hemoglobin solutions in red cells. *J. Gen. Physiol.* **52**:825, 852.