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THE EFFECT OF CHANGING EXTRACELLULAR OSMOLALITY ON WATER TRANSPORT IN THE HUMAN RED BLOOD CELL AS MEASURED BY THE CELL WATER RESIDENCE TIME AND THE ACTIVATION ENERGY OF WATER TRANSPORT

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Summary

A pulse NMR technique employing low extracellular Mn^{2+} concentrations has been used in following the effect of variations in extracellular osmolality on water transport through the human red blood cell membrane. We report results including the effect of osmolality on the cell water lifetime (τ_a) and, for the first time, the effect on the proton spin-spin relaxation of the intracellular water (T_{2a}) and the activation energy for the water transport process. Current results are encouraging in correlating the effects seen in this study with suspected membrane functional changes occurring in both in vivo and in vitro aging and during in vitro preservation attempts.

Introduction

The effect of medium osmolality on water transport through membrane systems is of primary interest in the understanding of membrane function. As early as 1927, Lucke' and McCutcheon [1] found that the permeability of single cells depends on the osmolality of the suspending solution. Many investigators have studied water transport using measurements of cell shrinkage or swelling under the influence of an osmotic gradient using either the method of per cent hemolysis as a function of time [2—4] or 90° light scattering in a rapid reaction constant-flow apparatus [5].

The dependence of the osmotic permeability coefficient, L_p, on medium osmolality has also been intensively studied. Rich et al. [6,9] Sha'afi et al. [7] and Blum and Forster [8] have all interpreted their findings as indicating that L_p depends on the osmolality of the suspending medium. However, in later

studies, Farmer and Macey [10,11] and Sha'afi and Gary-Bobo [12] interpret both their data and those of the previous groups as showing that there is no effect induced on L_p by medium osmolality or cell volume. They conclude rather that there is an apparent rectification of water flow through the membrane, L_p depending instead on whether the water is flowing into or out of the cell as the cell alternately swells or shrinks under an osmotic gradient.

In all these previously discussed studies, water transport is followed under non-equilibrium conditions during response to an osmotic gradient induced by sudden changes in the extracellular osmolality. However, the results of these studies have been difficult to interpret in some cases as can be observed in the above-mentioned discussion of the dependence of the osmotic permeability coefficient (L_p) on osmolality. Data interpretation is somewhat easier in experiments conducted at osmotic equilibrium [13]. The only non-NMR equilibrium method is the isotopic tracer technique [14—16]. This method involves an elaborate flow tube apparatus and an experimentally difficult technique of mixing and sampling involving about 50 ml of blood [17]. The tracer technique is also complicated by the problems introduced by the presence of unstirred layers adjacent to the membrane [18]. For these reasons routine application of this technique is somewhat hindered.

Several NMR studies have been performed on the effect of medium osmolality on water transport [13,19,20]. Outhred and Conlon [19] found that the product of the diffusional water permeability (P_w) and cell surface area is independent of cell volume using the impermeable solutes NaCl and sucrose. Chien and Macey [20] present results indicating that the diffusional water permeability is independent of osmolality using the permeable solutes, urea, methanol, ethanol and glycerol. However, in both cases, high concentrations of extracellular $MnCl_2$ were used (45—53 mM), concentrations which have been shown in this laboratory to produce serious systematic errors [21].

Fabry and Eisenstadt [13] have recently published results of the effect of medium osmolality on the cell water lifetime and the diffusional water permeability. Results were reported both on cells suspended in their original plasma with alterations in osmolalities and on washed cells resuspended in human serum albumin KCl/NaCl solutions of varying osmolality. The results of their study as discussed later are in good qualitative agreement with those reported here, although the lack of reproducibility in their samples suspended in the original plasma causes concern.

We present in this paper additional data on the effect of changes in extracellular osmolality on the cell water lifetime from samples exclusively produced using the original plasma. In all cases, the results obtained were very reproducible without the requirement of resuspending in a human serum albumin solution. In addition, we present for the first time results on the dependence of the proton spin-spin relaxation time of the intracellular water (T_{2a}) and the activation energy of water transport on extracellular osmolality. These results are related to characteristics seen during both in vivo and in vitro cell aging and in presently used in vitro cell preservation techniques.

Methods

Blood sample preparation

Blood sample preparation was similar to that of previous studies [13,21]. Fresh blood samples were obtained from three healthy donors, two males approx. 20 years of age and one female, approx. 30 years of age. Samples were placed in Vacutainers anticoagulated with a 1% (v/v) heparin solution, the concentration of the heparin solution being 1000 U.S.P. units heparin/ml. The plasma Mn^{2+} concentration was in all cases 1.7 ± 0.2 mM [21]. All samples were used within 1.5 h of collection, and the exchange rate measurements were complete within 3 h after the addition of $MnCl_2$.

The addition of extracellular Mn^{2+} as a paramagnetic ion is crucial to the experimental method employed here as previously described [21]. Briefly, the presence of Mn^{2+} drastically shortens the relaxation time of the extracellular water, serving to lower the extracellular water T_2 value well below that of the intracellular water T_2 value. In this manner, the extracellular water is labelled and thereby differentiated from the intracellular water during the course of the Carr-Purcell-Meiboom-Gill experiment allowing the kinetics of the water transport process to be followed. Experiments measuring τ_a as a function of time at fixed temperature show it to be constant over the 3 h period during which water transport data are acquired, indicating that the amount of Mn^{2+} used does not significantly affect the water transport process.

The extracellular osmolality was varied by replacement of 0.5 ml of plasma with water or concentrated salt solution (NaCl/KCl in the ratio 27 : 1) in a total volume of 7 ml of whole blood. In all cases, a 3% human serum albumin level was maintained in the extracellular medium. This presence of human serum albumin both enhances the effect of Mn^{2+} on water relaxation and diminishes its interaction with the cellular membrane, decreasing the rate of Mn^{2+} entry into the cell interior. Cells were allowed to equilibrate after changes in extracellular osmolality for a period of at least 30 min before data acquisition began, based on methods used by previous investigators studying volume changes [22]. At no point were time dependent effects seen during the course of the experiment, indicating that cell equilibration at this new osmolality was complete. All blood pH values were 7.4 ± 0.1 at 23°C.

Packed cell samples for use in the measurement of the intracellular T_2 value (T_{2a}) were centrifuged at 1000 × g for 60 min with an estimated 3% extracellular trapped plasma volume [23]. This small extracellular volume of trapped plasma was not observed to affect the resulting T_{2a} measurements by more than 1% during computer simulations performed in this laboratory and therefore should not have produced significant errors in the experimental results.

NMR measurements

All NMR measurements were made using an experimental setup in this laboratory which has been previously described [21]. Measurements were made at 60 MHz using the standard Carr-Purcell-Meiboom-Gill sequence [24,25]. The typical spacing between 180° pulses was 0.150 ms. The sample temperature was controlled to ±0.1°C by a Model 72 Proportional Temperature Controller (Yellow Springs Instrument Co., Yellow Springs, WY) employing thermally

regulated N_2. Plasma osmolality measurements were made after the NMR experiment was complete using a Model 301 Vapor Pressure Osmometer (Hewlett Packard, Palo Alto, CA). The osmometer was calibrated using known standards immediately before each experimental determination.

Data analysis and calculations

All data were analyzed as previously reported [21] using the nonlinear regression program (Blumenstein, B., personal communication) incorporating the two-site exchange expressions originally derived by Hazelwood et al. [26] for the effect of the two site exchange on the Carr-Purcell-Meiboom-Gill decay. As will be discussed later, because of the necessity of measuring one parameter separately to complete the data analysis, the intracellular T_2 value (T_{2a}) was measured using well packed cells. It was found, as shown below, that T_{2a} varied as a function of the extracellular osmolality because of the respective cell swelling and shrinkage. As a result of this variation in T_{2a}, separate packed cell determinations were carried out after the exchange rate run on whole blood for each blood sample to complete the necessary data acquisition.

Results

For each osmolality, a complete study of the T_{2a} value of intracellular water in well packed cells was performed consisting of 10 equally spaced temperatures in the range 4—37°C. Fig. 1 shows the effect of variations in the external osmolality on the intracellular T_2 value (T_{2a}) for one normal individual. Packed cell determinations of T_{2a} from the other two normal individuals studied produced similar results. The data set falling in the range intermediate between the other two data sets represents results obtained from measurement of the T_{2a}-temperature relationship of packed cells originally suspended in isotonic, unaltered plasma. The upper plot presents data for the same T_{2a} measurement from cells suspended originally in plasma made hypotonic, the lower from cells in plasma made hypertonic. As discussed in a previous report from this labora-

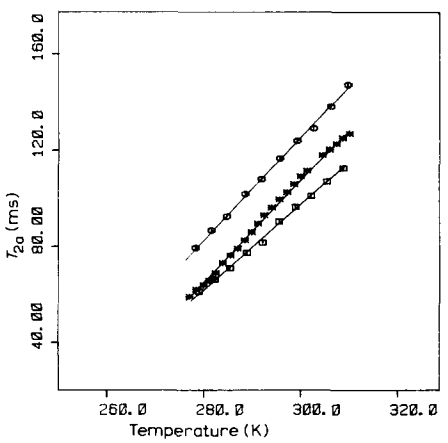
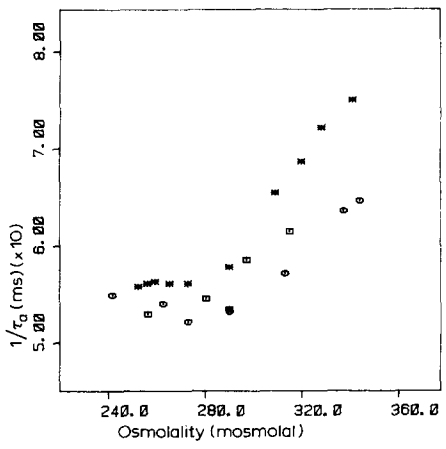


Fig. 1. Effect of changes in extracellular osmolality on T_{2a} as a function of temperature. (★) Isotonic, 290 mosmolal; (○) hypotonic, 256 mosmolal; (□) hypertonic, 313 mosmolal.



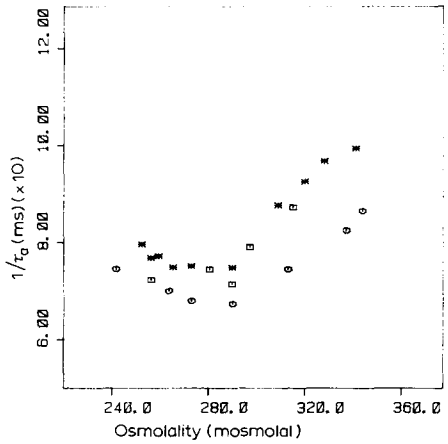


Fig. 2. (Cell residence time)$^{-1}$ (τ_a^{-1}) as a function of extracellular osmolality for three normal individuals (the values for each individual are denoted by ★, ○ and □, respectively) at 25°C. Age of blood 1.5 h; 1.7 mM $MnCl_2$ added.

Fig. 3. (Cell residence time)$^{-1}$ (τ_a^{-1}) as a function of extracellular osmolality for three normal individuals (the values for each individual are denoted as in Fig. 2), at 37°C. Age of blood 1.5 h; 1.7 mM $MnCl_2$ added.

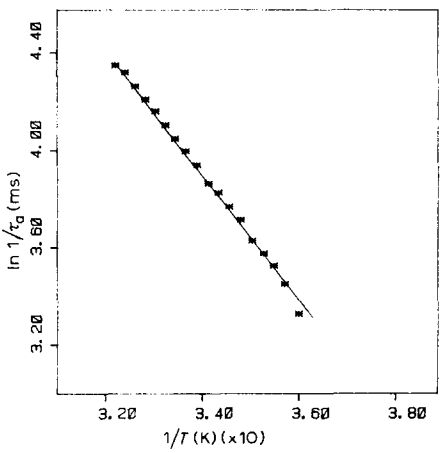
TABLE I

ACTIVATION ENERGY AS A FUNCTION OF EXTRACELLULAR OSMOLALITY

Age of blood 1.5 h; 1.7 mM $MnCl_2$ added.

Normal individual	Osmolality (mosmolal)	Activation energy (kcal/mol)
No. 1		
	256.2	5.23
	259.4	5.10
	265.5	4.92
	282.9	4.77
	290.0 *	4.74
	304.6	4.92
	305.5	4.98
	311.0	5.15
	313.0	5.23
No. 2		
	241.7	5.24
	262.2	4.92
	273.0	4.70
	290.0 *	4.45
	311.1	4.77
	337.4	5.11
	344.1	5.21
No. 3		
	256.5	5.35
	282.9	5.10
	290.0 *	4.99
	297.2	5.16
	315.3	5.40

* Isotonic environment.



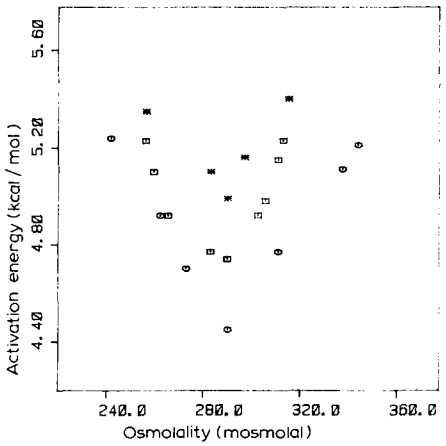


Fig. 4. Activation energy of the water exchange process as determined by the slope of the temperature dependence of the cell water residence time (τ_a). Age of blood 1.5 h; 1.7 mM $MnCl_2$ added.

Fig. 5. Activation energy of the exchange process as a function of external osmolality for three normal individuals (the values for each individual are denoted as in Fig. 2). Age of blood 1.5 h; 1.7 mM $MnCl_2$ added.

tory [21], the exponential decay obtained from the Carr-Purcell-Meiboom-Gill experiment affords only three pieces of information upon fitting, whereas the Hazelwood model for two-site exchange requires four independent values. This necessitates the independent measurement of the intracellular T_{2a} value as the fourth parameter required. As a result of this importance of determining an accurate value for T_{2a} as a function of temperature and the obvious dependence of this relationship on the extracellular medium osmolality, separate determinations of the T_{2a}-temperature curve were made for each osmolality studied for each of the three participating normals to provide the additional necessary information to facilitate the τ_a determination.

Figs. 2 and 3 present data obtained from the study of the cell water residence time, τ_a, as a function of extracellular osmolality at 25 and 37°C. Although τ_a is the actual measured parameter, $1/\tau_a$ has the units (time^{-1}) and therefore that of a rate constant for the water transport process. As can be seen, the results indicate that although the values for τ_a differ slightly at similar osmolalities for the three normal cases, the general trend of the effect of external osmolality on the value of τ_a is very consistent. At both 25 and 37°C, τ_a is constant or decreases slightly in hypotonic solution and increases significantly in hypertonic solution.

During the course of each osmolality experiment, the temperature dependence of $1/\tau_a$ and thus the activation energy for the exchange process were determined. Fig. 4 shows a plot of ln $1/\tau_a$ vs. $1/T$ for the 256.2 mosM activation energy determination of normal individual No. 1 consisting of 18 points spaced equally along the range 4—37°C. Such results were typical and were obtained for each activation energy measurement. Table I and Fig. 5 show the effect of changes in external osmolality on the activation energy for the water exchange process. Again, it must be noted that although the values obtained

for a given external osmolality vary among the three individuals studied, the general trend observed is the same in all cases, the value for the activation energy rising significantly as the external plasma osmolality is raised or lowered.

Discussion

The variation of the T_{2a}-temperature dependence with medium osmolality is shown in Fig. 1. This has not been previously reported, possibly because T_{2a} has not been involved in prior NMR studies of transport in red blood cells. The behavior shown in the figure seems reasonable in relation to the variation of erythrocyte volume with external osmolality. As the external osmolality increases, the cell volume decreases, concentrating the intracellular hemoglobin thus making the relaxation mechanisms present more effective and the respective T_{2a} values smaller. Conversely, in a hypotonic environment, the red cell swells, diluting the intracellular hemoglobin concentration, thus leading to larger T_{2a} values.

Our findings of the temperature dependence of the cell water lifetime (τ_a) on medium osmolality agree well qualitatively with those presented by Fabry and Eisenstadt [13]. Quantitative comparisons are to a large extent impossible, however, because of the requirement in their study of resuspension in human serum albumin KCl/NaCl to acquire reproducible results. This resuspension causes a substantial decrease in τ_a values across the whole osmolality range studied. Although the addition of physiological quantities of fibrinogen to the resuspension caused the values to rise once again to the previous level, few results of this type are presented in their report.

Our plot of τ_a vs. medium osmolality shows a very small decrease in the value of τ_a as the cell volume increases (as can be seen in several cases, τ_a actually appears to remain essentially constant in a hypotonic environment), with a correspondingly much larger decrease as the medium becomes increasingly hypertonic. Thus, we see that τ_a assumes a maximum value in an isotonic environment. The hypertonic behavior could be caused by the decreased intracellular volume occupied by the cell water and smaller distances available for water movement within the cell resulting in a shorter residence time within the cell before a given water molecule is exchanged. The small decline in τ_a values observed in some hypotonic experiments is more difficult to explain, perhaps arising from compensating changes in the surface area and cell volume. Such a possibility has been suggested elsewhere [19].

Also presented are figures showing the effect on the activation energy of water transport through the cell membrane produced by changes made in the extracellular medium osmolality. The most striking observation in each of the three normal individuals studied is that a minimum is observed in the activation energy value in normal isotonic plasma; as the plasma osmolality is both increased and decreased this value rises significantly. Although values for the activation energy in normal plasma differ from individual to individual (from 4.45 to 4.99 kcal/mol in this study), each individual's normal value is extremely reproducible (each determination within ±2% for all normal individuals studied in this laboratory), and the effect produced in each case by changes in

osmolality merely extends from different minima. As the cell volume begins to shrink, the prominent shape becomes increasingly echinocytic, the crenated cell surfaces perhaps possessing different membrane transport characteristics accompanied by an increase in the activation energy for water transport [13]. As cell volume increases in a hypotonic environment, changes may occur in the cell membrane prior to the actual lysing of the cells, producing increases in the determined value of the activation energy. Alternatively, the shape/volume changes discussed above in reference to changes in the cell water lifetime (τ_a) may contribute to the effects observed in the activation energy study. It is, of course, possible that no one cause alone is responsible for the results seen in this study.

It is known that in vivo aged human red blood cells exhibit decreased cell volumes and correspondingly increased corpuscular hemoglobin concentrations [27—30]. Increased osmotic fragility [31], along with a decrease in cell deformability [32], has also been observed in older in vivo cells. Studies have also been performed on erythrocytes incubated in vitro in glucose-free isotonic solution at physiological temperatures with the resultant shape change from discocyte to echinocyte [33,34]. Finally, in the area of cell preservation through freezing for the purpose of later thawing and transfusion, much difficulty has arisen because of the high rate of cell lysing during the initial 24 h period after thawing. This high rate of hemolysis has until recently restricted the shelf-life of the thawed blood to 24 h and produces a critical obstacle to the effective use of blood storage techniques [35].

Changes occurring in the membrane during the course of in vivo and in vitro aging will play an important role in the determination of cellular structure and function. It will be recalled from the discussion above that many of the cellular characteristics seen in in vivo and in vitro aging are also seen during the incubation of cells in hypotonic and hypertonic environments. These characteristics include decreased cell volume with increased intracellular hemoglobin concentration and echinocyte formation in the hypertonic environment and the increased rate of lysis in the hypotonic environment. As changes in the membrane are also responsible for these effects, osmolality studies may provide important insights as to changes occurring during cell aging in both the in vivo and in vitro states. Several investigators have already used osmotic studies to model effects seen in the preservation through freezing of various tissues such as human red blood cells and Chinese hamster ovary cells using non-NMR techniques [36—40]. Studies establishing the necessary correlation for such work in our laboratory between the behavior of aging cells at both physiological and freezing temperatures with that seen during these osmolality studies using our cell water lifetime and activation energy measurements obtained from the NMR experiment as probes and as possible criteria for success in maintaining cell viability during preservation are in progress at this time. It is hoped that this will enable us to use the results obtained from these osmolality measurements to give a greater understanding of membrane changes during the course of cell aging and also in the evaluation of various methods used to enhance cell viability as a function of time during preservation studies.

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