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K-Permeabilized Human Red Cells Lose an Alkaline, Hypertonic Fluid Containing Excess K over Diffusible Anions

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Summary. Experiments were performed to test specific predictions of an integrated red cell model developed by Lew and Bookchin [Lew, V.L., Bookchin, R.M. *J. Membrane Biol.* **92**:57–74 (1986)], that K-permeabilized human red cells suspended in low-K media would dehydrate and lose an alkaline, hypertonic fluid with excess K over accompanying anions, and that cell dehydration would precede medium alkalinization. Red cells were suspended at about 30% hematocrit in an initially K-free Na-saline and permeabilized to K by the addition of valinomycin. The results showed that by the time a quasi-steady state had been reached the cells had lost the equivalent of a hypertonic fluid containing about 180 mM KCl (SCN) and 10 mM KOH, and that cell dehydration did precede alkalinization of the medium, in good agreement with the theoretical predictions. Since these experiments critically test the interaction between transport, pH and volume regulatory functions in the human red cell, the observed agreement validates the basic assumptions and structure of the integrated model. The functional implications of these results are discussed.

Key Words K permeability · volume regulation · pH regulation · red cells

Introduction

A basic mathematical model was recently developed by Lew and Bookchin [24] which integrates present knowledge about the nonideal osmotic behavior of hemoglobin, the proton-titration properties of the impermeant cell buffers and the ion transport characteristics of the human red cell membrane. The computing strategy of the model permits prediction of the time course of changes in red cell volume, pH and composition in a manner that allows direct comparison with experimental results. A surprising prediction of this model was that the fluid and electrolyte loss from K-permeabilized cells, suspended in low-K media, would be alkaline and hypertonic to the medium, with an excess of K over accompanying anions, and that cell dehydration would precede medium alkalinization. It is important to investigate this prediction because it represents a critical test of the integrated model and also because, if it were true, it could account for some of the hitherto unexplained properties of dehydrated sickle cell anemia and other abnormal red cells [2, 8, 13, 14, 21] as well as for apparent K : H exchanges described in amphibian red cells [4, 5, 6].

The present experiments were designed to characterize the changes in normal human red cell composition following K permeabilization and to test, at a semiquantitative level, the predictions of the model as described above.

Materials and Methods

Experimental Design

Preliminary simulations were performed, using the integrated red cell model [24], to find the optimal experimental conditions for a critical comparison between theory and experiment. Maximal cell shrinkage and acidification are predicted at infinite cell dilution, but in order to measure any changes in medium osmolarity resulting from ion and water efflux from the red cells, high hematocrits are required. On the other hand, the higher the hematocrit, the larger the increase in external K concentration, which reduces the extent of all other changes. A compromise was chosen with the cells suspended at about 30% hematocrit in initially K-free media buffered to pH 7.4–7.6 with 10 mM HEPES-Na. Under these conditions, alterations in hematocrit, medium osmolarity, pH, K and Na concentrations, and in cell Na and K contents, could be measured with sufficient precision to detect the predicted changes. These quantities could then be used to estimate the composition of the fluid lost from the cells, as shown below.

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Experimental Procedure

Valinomycin is only soluble in solvents which, when added to the cell suspension, would increase medium osmolarity substantially more than was expected from the K-permeabilizing effect of valinomycin, which the model predicted to be about 6 ideal mosmol/liter. Therefore, in order to detect the small predicted differences with little error, it was necessary either to add identical amounts of solvent to simultaneous control and test conditions (experiments II and III) or else make a second calibrating addition of valinomycin solution in the steady state (experiment I). Valinomycin was dissolved in DMSO to give 10 and 100 mM stock solutions. DMSO was preferred to other solvents because, with its higher molecular weight, the relative increase in osmolarity would be smaller. Preliminary experiments showed that at the high hematocrits used here, valinomycin concentrations of 30–40 μM were required to dehydrate the cells in 20–30 min. Addition of valinomycin solution to give this concentration increased medium osmolarity by about 50 ideal mosmol/liter when using the 10-mM stock (experiment I) or by about 5 ideal mosmol/liter when using the 100-mM stock (experiments II and III).

Red cells from 3-week-old bank blood (experiment I) or from fresh, heparinized blood (experiments II and III) were washed four times with 5–8 volumes of a K-free solution containing (in mM): NaCl, 70; NaSCN, 70; and HEPES-Na, 10; (pH 7.45). The reason for using SCN anions was that K-permeabilized cells were shown to dehydrate at least 20 times faster in low-K media when Cl was partially replaced by SCN [25]. This allowed quasi-steady states to be attained at room temperature (20–23°C) within 20–30 min of valinomycin addition. The washed cells were suspended at about 30% hematocrit in the same medium and incubated at room temperature with magnetic stirring. Hemolysis varied from 0.5 to about 3% in different experiments and was minimally increased by valinomycin relative to DMSO controls. The contribution of lysis to the measured valinomycin-induced changes was therefore considered negligible.

The pH in the suspension was continuously monitored and recorded using a Radiometer GK2321C combined pH electrode. DMSO, with or without valinomycin, was added using syringes with reproducibility better than 1%. Samples for all measurements other than pH were taken either in quasi-steady state, as established by the pH record (experiments I and II), or at the indicated times (experiment III, Fig. 2). In experiment III, the cell suspension was divided into two. One half was incubated in the presence and the other half in the absence of the anion transport inhibitor SITS (4-acetamido-4′-isothiocyano-2,2′-stilbene disulphonic acid) [3] at a concentration of 300 μM in the suspension. This concentration was expected to inhibit anion transport by about 80%. SITS was used to test the prediction that partial inhibition of anion exchange would further delay the pH changes induced by K permeabilization relative to the decrease in hematocrit or increase in medium potassium concentration.

Processing of Samples

Samples were prepared for measurement of hematocrit, hemoglobin, cell and medium Na and K contents, and medium osmolarity.

Hematocrits were measured by centrifuging glass capillaries containing about 50 μl of cell suspension in a microhematocrit centrifuge. After reading each hematocrit, the part of the glass capillary containing packed cells was broken off and the cell column was lysed in 1 ml of distilled water. No attempt was made to correct for trapped fluid, or for differences in trapped fluid, from samples taken before and after cell shrinkage. Hemoglobin concentrations in samples of cell lysate and suspension were determined by the cyanmethemoglobin method. A high sensitivity EEL flame photometer was used for Na/K determination in the lysates. The Na and K contents of the cells were expressed in mmol per 340 g hemoglobin (experiment I), or per measured mean cell hemoglobin concentration (MCHC) of 346 and 343 g/liter red cells, respectively, in experiments II and III.

To measure the osmolarity and Na/K contents of the medium, samples of the suspension were centrifuged for 2 min at 14,000 × g in a microfuge and the supernatant was removed. Na and K concentrations were determined in 4 to 10 replicate samples of the supernatant using a Corning 435 flame photometer. Medium osmolarity was measured in 0.2-ml samples using an Advanced Digimatic osmometer model 3DII. The measured osmolarity is reported in mM units, for direct comparison with the predictions of the model, uncorrected for nonideality. In experiments II and III, DMSO was added to the control and to the test suspensions. Therefore, the change in osmolarity due to the effects of valinomycin was simply the difference between the measured osmolarities in tests and controls. In experiment I a second identical volume of valinomycin in DMSO was added during steady state. The increase in osmolarity that followed was subtracted from the change in osmolarity caused by the first addition. The difference was recorded as the change in osmolarity caused by K permeabilization.

Analysis of Results

In this section we provide a glossary of symbols used throughout the paper and also the equations applied to describe the composition of the valinomycin-induced cell effluent. The nomenclature used here follows that used by Lew and Bookchin [24]. Additional symbols were needed to define the mean content and osmolarity of the cell effluent. For the purpose of these calculations the cell effluent was considered a hypothetical fluid which, if it had been added to the original suspending medium in the correct proportions, would have altered its composition as predicted or measured for K-permeabilized red cells in quasi-steady state. The calculations assume the effluent to contain only KCl, KSCN and KOH, and neglect the minor Na ion shifts between cells and medium.

Glossary and Equations

Q_i: amount of solute i in one liter of original packed cells (mmol/loc; 1 loc is equivalent to 340 g Hb or to the measured MCHC expressed in g Hb per liter of packed cells).

C_i^m: concentration of solute i in extracellular medium (mM). i represents sodium (Na), potassium (K), the diffusible anions Cl and SCN (A), and the proton buffer HEPES (B).

C_H^m: concentration of proton ions in the medium (M).

pH^m: medium pH.

K_B: proton dissociation constant of HEPES ($10^{-7.55}$ M).

Ht: hematocrit, expressed as the fractional volume of cells in the suspension.

MCHC: mean corpuscular hemoglobin concentration (g Hb/100 ml red cells).

ΣC_i^m: medium osmolarity (mM).

$P_{K,A}^G$: diffusional K or A permeability (h^{-1})

k_{HA}: rate constant of H : A cotransporter representing the turn-

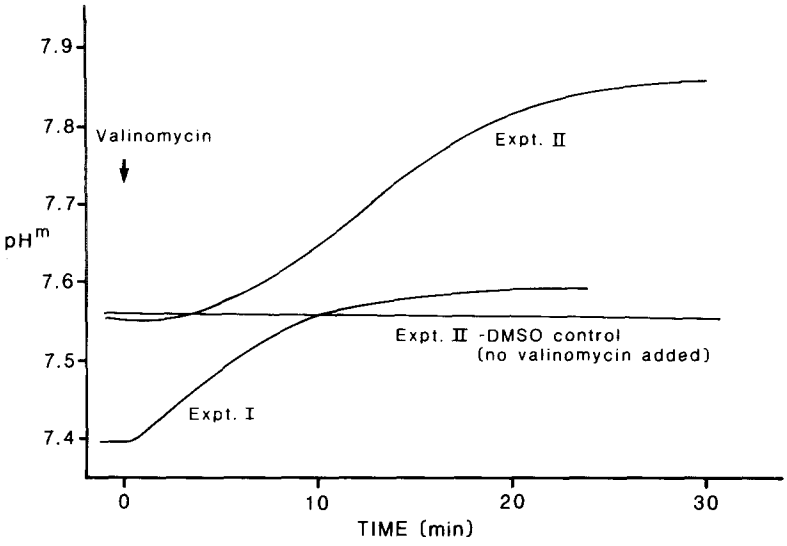


Fig. 1. Time course of pH changes in experiments I and II. The different protocols used in these experiments are detailed in Materials and Methods

over rate of the limiting step in Jacobs Stewart mechanism. Superscripts ^{o} or ^{f} on any variable denote its value either before valinomycin or after DMSO only (^{o}), or in the steady state following valinomycin (^{f}).

Definitions

$C_H^m = \exp_{10}(-\mathrm{pH}^m)$
$\Delta Ht = Ht^f - Ht^o$

Composition of the Valinomycin-Induced Cell Effluent

The symbols used to describe the composition of the valinomycin-induced cell effluent are:
$\bar{C}_K^E$, $\bar{C}_A^E$: mean concentration of K or of the diffusible anion (mM).
$\bar{C}_{OH}^E$: mean concentration of hydroxyl ions (mM).
$\Sigma\bar{C}_i^E$: mean osmolarity (mM).

The quantities reporting the mean, time averaged, composition of the valinomycin-induced cell effluent were calculated from computed or measured values using the equations below. The results are reported in Table 2.

$$\bar{C}_K^E = ((C_K^m)^f(1 - Ht^f) - (C_K^m)^o(1 - Ht^o))/\Delta Ht \quad (1)$$

$$\bar{C}_{OH}^E = -((C_B^m)^f((C_H^m)^f/(K_B + (C_H^m)^f))(1 - Ht^f) - (C_B^m)^o((C_H^m)^o/(K_B + (C_H^m)^o))(1 - Ht^o))/\Delta Ht \quad (2)$$

$$\bar{C}_A^E = \bar{C}_K^E - \bar{C}_{OH}^E \quad (3)$$

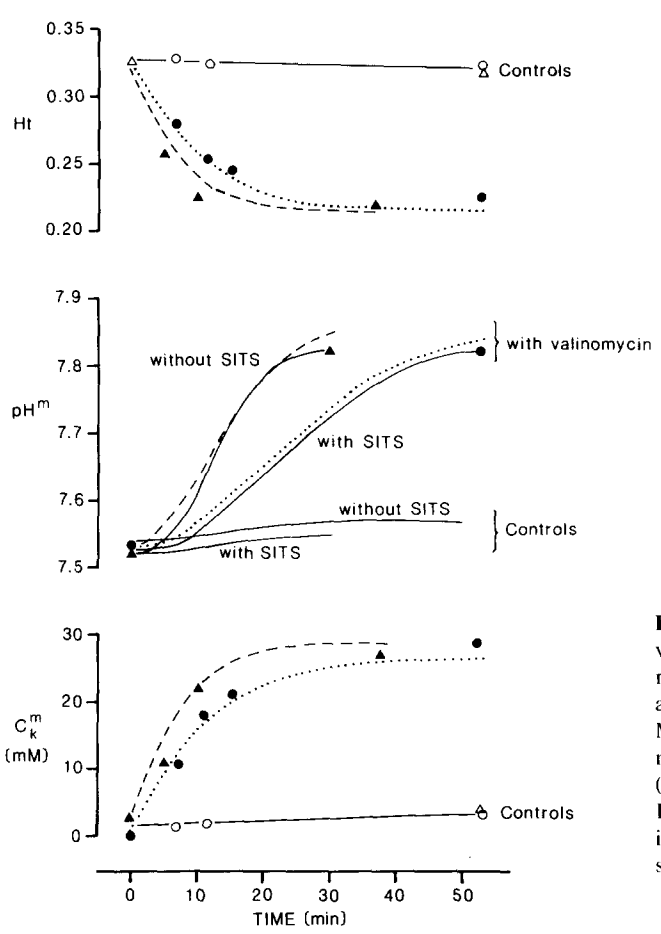
$$\Sigma\bar{C}_i^E = \bar{C}_A^E + \bar{C}_K^E \quad (4)$$

Results

Following valinomycin addition, the pH of a suspension always increased, as shown in Figs. 1 and 2. There were differences in the rate and extent of the pH changes in different experiments; with K-permeabilized bank cells (experiment I) medium pH increased faster but to a lesser extent than with fresh cells (experiments II and III).

In experiment III (Fig. 2) the time course of the changes in hematocrit and in external K concentration were compared with that of the pH change. It can be seen that valinomycin triggered rapid cell K loss and dehydration and delayed medium alkalinization. Cell dehydration and K depletion preceded medium alkalinization both in the presence and absence of SITS, which slowed down all the measured valinomycin-induced changes.

The predicted and observed behavior of the red cell suspensions following valinomycin addition is compared for quasi-steady states in Table 1 and for time-dependent changes in Fig. 2. The measured changes in quasi-steady state were: (i) cell dehydration to about 70% of the original cell volume; (ii) alkalinization of the medium by about 0.2–0.4 pH units; (iii) increase in the external K concentration by 23–26 mM; (iv) dilution of the external Na concentration by 15–30 mM; (v) increased medium osmolarity by between 9 and 15 mosmol/liter; (vi) reduction in cell K contents by about 50–70 mM, and a small cell Na gain, of 1–4 mM. It can be seen that the experimental values are quite similar to those predicted by the model. For the present purposes of a semi-quantitative level of comparison, the minor differences between measured and predicted values do not justify analysis. Since precise quantitative comparisons were outside the scope of these studies, no attempt was made to correct for nonideal behavior or to incorporate the measured Na/K content of the cells into the simulations.



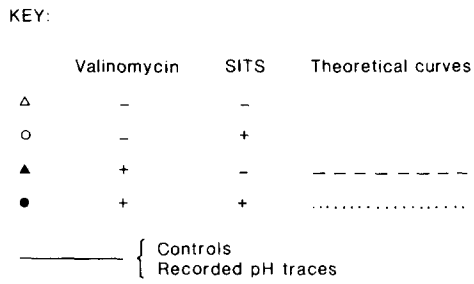


Fig. 2. Time course of changes in hematocrit (expressed as cell volume fraction), external K concentration and pH in experiment III, in the presence and absence of SITS. Valinomycin was added at $t = 0$. The detailed protocol is given in Materials and Methods. The theoretical curves were computed from the parameters found to provide the best fit to the experimental points (*see* Results). The three parameters adjusted were the diffusional K and A permeabilities and the turnover of the rate-limiting step in the Jacob-Stewart cycle. Note that all individual points represent experimental values

The predicted and observed mean compositions of the cell effluents were estimated from the quasi-steady state values in Table 1 (*see* Materials and Methods, "Analysis of Results") and are reported in Table 2. It can be seen that the mean composition of predicted and measured effluents approximates that of a hypertonic solution containing over 350 ideal mOsmol/liter of solutes, about 180 mM of KCl (SCN) and 8–11 mM KOH.

The three parameters used to compute the theoretical curves of Fig. 2 were P_K^G, P_A^G, and k_{HA}. The rate of increase in the medium K concentration in the absence of SITS uniquely determined the value of P_K^G. The best fit was found with $P_K^G = 9.8\ h^{-1}$, which represents a 10^4-fold increase in the ground K permeability of the red cell membrane [23] and is within the observed range of valinomycin-induced effects. This value of P_K^G also accounts for the rate of fall in hematocrit (Ht) as long as P_A^G is equal to or above a limiting value of $50\ h^{-1}$. In the controls without SITS, therefore, P_A^G has an indeterminate value above $50\ h^{-1}$. This means that the diffusional SCN^- permeability is likely to be at least 100 times higher than that estimated for Cl ions [17, 18].

The time course of medium alkalinization is mainly determined by k_{HA} and is only marginally affected by P_A^G. Values of 5×10^7 for k_{HA} and of $50\ h^{-1}$ for P_A^G provide the best fit of the results in the absence of SITS. In the condition with SITS present the best fit is with P_A^G and k_{HA} values of 8 and 1.5×10^7, respectively; P_K^G remains unchanged. The effects of SITS, at the concentration used, can therefore be attributed to similar inhibition, by about 70–80%, of the diffusional and exchange components of anion transport. This interpretation assumes that inhibition of k_{HA} by SITS represents inhibition of the anion exchange step in the Jacobs-Stewart mechanism, which is considered to be the rate-limiting step in the cycle [24]. The results in Fig. 2 demonstrate that the parameter values required to fit the experimental results are uniquely determined (except for P_A^G in the absence of SITS) and that the values are consistent with the well-characterized effects of SCN^-, valinomycin and SITS [3, 17, 18, 25].

Table 1. Comparison of the measured and predicted values in quasi-steady state following valinomycin-induced K permeabilization of human red cells in low-K media[a]

Experimental condition of simulation or experiment			Ht	pH^m	C_K^m	C_{Na}^m	ΣC_i^m	Q_K	Q_{Na}
					(mM)			(mmol/loc)	
Simulations	P_A^G (h^{-1})	k_{HA}							
Reference state	50	10^7	0.325	7.53	3.0 (0.1)	147	305	105	7.5
Increased K permeability without SITS	50	10^7	0.217	7.86	28.8	125	311	46	7.6
Difference			−0.108	0.33	25.8	−22	6	−59	0.1
Increased K permeability with SITS	8	1.5×10^7	0.215	7.86	26.7	127	311	45	7.7
Difference			−0.110	0.33	26.6	−20	6	−60	0.2
Experiment I									
Before			0.332	7.39	0.1	143	277	93	21
valinomycin			(0.002)		(0.1)	(2)	(1)	(2)	(1)
After			0.234	7.59	26.5	116	292	27	23
valinomycin			(0.001)		(1.2)	(1)	(2)	(1)	(1)
Difference			−0.098	0.20	26.4	−27	15	−66	3
			(0.002)		(1.2)	(2)	(2)	(2)	(1)
Experiment II									
DMSO controls			0.347	7.56	3.5	143	281	87	12
			(0.002)		(0.3)	(1)	(0.4)	(4)	(1)
With valinomycin			0.251	7.86	26.8	122	290	36	16
			(0.002)		(0.9)	(2)	(1)	(2)	(1)
Difference			−0.096	0.30	23.3	−21	9	−52	4
			(0.003)		(1)	(2)	(1)	(5)	(1)
Experiment III SITS absent									
DMSO controls			0.321	7.55	4.0	140	284	95	14
With valinomycin			0.220	7.82	27.0	127	297	41	16
Difference			−0.101	0.27	23.0	−13	13	−54	2
Experiment III SITS present									
DMSO controls			0.323	7.57	3.0	141	282	93	14
With valinomycin			0.226	7.82	29.0	127	297	40	17
Difference			−0.097	0.25	26.0	−14	15	−53	3

[a] For explanation of the symbols, *see* "Analysis of Results." The values in brackets represent SEM of 4 to 10 duplicate samples. The simulations were performed using the model of Lew and Bookchin [24]. The simulated increase in potassium permeability was from a reference state value of 0.016 h^{-1} to the value of 9.8 h^{-1} which was found to fit the K loss curves in the experiment of Fig. 2. The initial medium K concentration used for the computations was as measured in experiment III. This was 3 mM in the condition without SITS and 0.1 mM in the condition with SITS. The values of P_A^G and k_{HA} given in the Table are also those used for the fits of the experiment in Fig. 2. This is not critical because, unlike the time-dependent processes analyzed in Fig. 2, predicted quasi-steady state quantities are little affected by the value of those parameters as long as they are large enough to allow quasi-steady state to be attained within about 20–30 min.

Discussion

The results presented here confirm predictions of the integrated red cell model of Lew and Bookchin [24], that K permeabilization of human red cells causes the loss of a hypertonic, alkaline fluid containing K salts and KOH. The mechanism of these effects, as explained by the model, is outlined in Fig. 3. The increase in K permeability hyperpolarizes the cells [22] and generates a driving force for net anion efflux through diffusional pathways. This results, initially, in the loss of an isotonic fluid con-

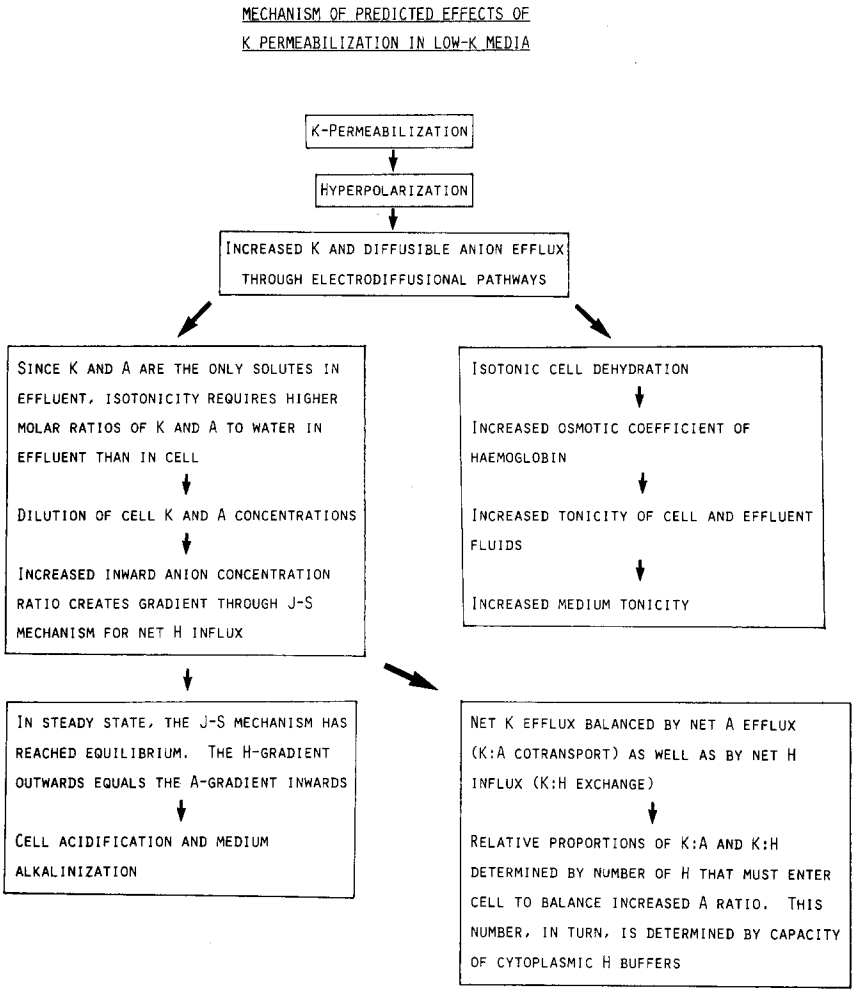


Fig. 3. Diagram outlining the mechanism of the effects predicted by the integrated red cell model for K-permeabilized cells suspended in low-K media

Table 2. Mean composition and osmolarity of the valinomycin-induced cell effluent[a]

Variables (mM)	Mean values in valinomycin-induced effluent				
	Predicted	I	II	III	III(SITS)
$\bar{C}_K^E$	190	183	185	182	210
$\bar{C}_A^E$	179	176	174	172	200
$\bar{C}_{OH}^E$	12	8	10	10	10
ΣC_i^E	368	359	359	354	410

[a] The composition of the cell effluent was computed from the values in Table 1 as detailed in "Analysis of Results." There were no differences in the predicted values for the simulations with or without SITS.

taining only K and diffusible anions. As the cells become dehydrated, the rising cell concentration of impermeant solutes leads to intracellular dilution of K and diffusible anion. The consequent increase in inward anion concentration ratio induces an inward proton flux through the Jacob-Stewart mechanism [15, 19, 20]. This, in turn, acidifies the cells and alkalinizes the medium. The extent of the proton shift into K-permeabilized cells is determined by the titration properties of the cell buffers [7, 9]. Part of the K lost from the cells would then appear as K : H exchange or net KOH loss. Dilution of the internal K and diffusible anion concentrations increases with time, as does the anion gradient. Although the loss of cell water directly follows the anion loss, the C_A^m/C_A^c ratio, which determines the C_H^c/C_H^m ratio, changes more slowly initially and increases more rapidly with time as C_A^c falls. This explains the delay in pH change relative to cell dehydration documented in Fig. 2. As the cells dehydrate, the increase in osmotic coefficient of hemoglobin [1, 10, 11, 12, 16, 26, 27] "creates" osmotic particles which equilibrate between cells and medium in the form of a progressively more hypertonic KCl (SCN) and KOH effluent.

The composition and tonicity of the cell effluent varies continuously as the cells dehydrate, with a time course determined by the magnitude of the permeability increase and by the kinetics of the transport pathways involved. The extent of cell

acidification and dehydration in the quasi-steady state, on the other hand, are almost entirely determined by the charge and osmotic properties of the nondiffusible cell ion, mainly hemoglobin [7, 9, 24].

Previous studies have reported, without explanation, a decrease in the total monovalent cation concentrations (as expressed per volume of cell water) in dehydrated red cells in sickle cell anemia (SS), homozygous hemoglobin C disease and in hereditary xerocytosis. These cells have undergone a large loss of potassium [2, 8, 13, 14, 21]. The relative acidification of the denser cell fractions of normal and SS cells has also been described [21]. The present studies show that both dilution of cell cation and cell acidification are features of red cells that have sustained a net cation loss. These features can be attributed to the nonideal osmotic properties and proton buffering behavior of hemoglobin. The model provides a clear explanation of the mechanism of the observed osmotic and proton shifts, both of which are verified experimentally.

The experiments reported here critically test interactions among transport, pH and volume regulatory functions of human red cells, as represented by the model. The substantial agreement found between predicted and observed behavior validates the basic assumptions and structure of the model. The prediction and confirmation of unexpected side effects of potassium permeabilization, such as cell acidification and the increase in medium osmolarity, points out the potential shortcomings of studying isolated functions of intact cells and thus the need for an integrated model.

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