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STUDY ON THE DEHYDRATING EFFECT OF THE RED CELL Na^+/K^+-PUMP IN NYSTATIN-TREATED CELLS WITH VARYING Na^+ AND WATER CONTENTS *

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Using the antibiotic Nystatin, we have developed a systematic method for the preparation of red blood cells with independently selected levels of intracellular Na^+ concentrations and water content. Such cells provided an experimental model to study the effect of Na^+/K^+ pump stimulation on red cell water content. Even in initially dehydrated cells, stimulation of the Na^+/K^+ pump by elevated intracellular Na^+ caused subsequent further loss of cell water. Cell water loss was reflected in decreased monovalent cation content per unit mass of hemoglobin and by a shift in the density distribution of the cell populations to higher densities on discontinuous Stractan gradients. We conclude that the 3 Na^+_{out} : 2 K^+_{in} stoichiometry of the Na^+/K^+ pump results in a net desalting effect with increased pump activity. Under the conditions of these experiments, the cell appears to have no effective mechanism to compensate for a net loss of ions and water.

Introduction

The remarkable deformability of the human red cell is a critical property in the cell's oxygen transport function. Loss of deformability compromises the ability of red cells to circulate freely through the small vessels of the microvasculature and is associated with shortened cell survival [1–3]. One important aspect in the maintenance of normal deformability is the precise regulation of the red cell water content. A cell with too little water will have reduced deformability because of the high viscosity of the concentrated hemoglobin solution inside [4,5]. Conversely, a cell with too much water will have reduced deformability because of decreased surface area-to-volume ratio and the resulting lack of reserve membrane to accomodate cellular distortion [4,5]. Naturally occurring aberrations in the regulation of red cell water content encompass both of these extremes, in the hemolytic disorders 'desiccytosis' and hereditary stomatocytosis [6–10]. A major role for reduced deformability in the premature destruction of cells in these disorders is suggested by the ameliorative effects of splenectomy [7,9].

Because the red cell membrane has a very low permeability to various cations, its water content is governed primarily by its sodium and potassium content. Maintenance of a constant red cell volume has been modeled mathematically as a dynamic balance between the energy-requiring Na^+/K^+ pump in the membrane and the passive permeation of Na^+ and K^+ along the concentration gradients established by the pump [11]. The pump/leak model suggests that abnormalities in any component of the pump/leak network could lead to failure of cell volume regulation and potentially to reduced deformability and premature cell destruction. Recent studies have suggested the existence of an additional path for cation

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Abbreviation: MCHC, mean cell hemoglobin concentration; MCH, mean cell hemoglobin content.

transport, namely an Na^+/K^+-co-transport route [12]. In this process, the downhill gradient for one ion drives an uphill flux of the other ion, resulting in coupled movement of both ions in the same direction. This could then produce an efflux or influx component, depending on the direction and magnitude of transmembrane ion gradients.

The inherent characteristics of the active Na^+/K^+ pump provide it with the capacity to compensate for some defects in normal membrane permeability properties. The Na^+/K^+ pump normally maintains low intracellular Na^+ concentrations and is stimulated by increases in intracellular Na^+ [13]. Thus leakage of Na^+ into the cell causes pump activation and an increased rate of Na^+ extrusion, which tends to compensate for increased Na^+ influx. As the pump expells Na^+ from the cell interior, it brings K^+ into the cell, at an apparently fixed stoichiometric ratio of $3\ Na^+_{out} : 2\ K^+_{in}$ [14]. This results in a net pumping of ions out of the cell [15], which under normal circumstances provides a balanced counterflow of Na^+ and K^+ against the fluxes driven by the normal ion gradients. Furthermore, in the event of a non-selective increase in the permeability of the membrane to monovalent cations, there is a tendency towards water and Na^+ accumulation because of the large plasma reservoir of Na^+. Thus, acceleration of pump activity, with its capacity for net cation extrusion, would provide compensation for the threatened increase in cell volume. However, in some instances, it appears that changes in membrane permeability are restricted to a selective leak for K^+, with continued low permeability to Na^+. The Gardos effect, a selective increase in K^+ permeability that results from intracellular accumulation of Ca^{2+}, is one such example [16]. Glader and Nathan [17] have suggested that cells with reduced K^+ content and only slightly elevated Na^+ concentrations are inherently unable to restore their lost K^+ content because of the selective pump activation by elevation of internal Na^+. We have further proposed that pump stimulation in a low K^+, dehydrated cell, such as an irreversibly sickled cell, would in fact worsen the deficiency in total ion and water content, rather than correct it [18]. This argument is based on the $3\ Na^+_{out} : 2\ K^+_{in}$ stoichiometry of the pump, leading to the conclusion that stimulation of pump activity would inevitably result in a further net loss of intracellular ions and water. Underlying this view is the assumption that the permeability characteristics of the membrane do not change with variations in cell water content. However, Poznansky and Solomon [19] have reported that changes in the volume of human red cells are accompanied by alterations in Na^+ and K^+ permeability in a direction that tends to compensate for the volume change.

In an effort to define the influence of (Na^+/K^+)-pump stoichiometry on the capacity of the pump to correct abnormalities in cell water content, we have studied pump-dependent volume changes in red cells with various intracellular cation contents and compositions. Using the antibiotic Nystatin, which introduces a reversible leak for Na^+ and K^+ into the red cell membrane, we prepared red cells with high and low Na^+ concentrations, combined with increased or decreased total cation and water content. We found that stimulation of Na^+/K^+ transport rates by elevation of intracellular Na^+ concentration was accompanied by a net loss of intracellular ions and water. For cells with excess water, this process tended to return the cell to normal volume and water content. For dehydrated cells, however, it resulted in further dehydration or 'desalting', with consequent increase in intracellular hemoglobin concentration. These studies thus provide experimental support for the idea that red cells are ill-equipped to compensate for defects that tend to produce cellular dehydration.

Materials and Methods

Adjustment of Intracellular Na^+ and water contents. Using the antibiotic, Nystatin [20], we developed a systematic method for the preparation of red cells with specified Na^+, K^+ and water contents. The basis of the method was to add either dilute or concentrated salt solutions of Na^+ and K^+ to red cells in the presence of Nystatin, which permitted the equilibration of Na^+ and K^+ across the membrane. This dilution was carried out gradually to minimize the concentration gradients and thereby prevent changes in the cell volume at this stage. The desired final value for the mean cell hemoglobin concentration(MCHC) determined the choice of total salt concentration during the equilibration stage. Cells equilibrated in hypotonic salt solutions lost total ion content. Then, after removal of Nystatin and return to isotonic medium, they lost water and increased their MCHC. In

contrast, cells equilibrated in hypertonic salt solutions increased their salt concent. After Nystatin removal and return to isotonic medium, they took up water and decreased their MCHC.

For each experiment, the values for final MCHC and Na^+ concentration were first chosen. The choice of MCHC dictated the osmotic strength to be attained during Nystatin equilibration (see Fig. 1 in Results). The Na^+ concentration of the complete equilibration suspension was then determined from the desired final Na^+ concentration according to the relationship:

$$Na^+_{equil}\ (\text{mequiv./l } H_2O) = \\ = (Na^+_{final}\ (\text{mequiv./l } H_2O)/r_{Na^+}) \\ \times (1/0.717)\ (\text{l cells/l } H_2O) \\ \times ((35/MCHC_{final}) - 0.283)\ (\text{l } H_2O\text{/l cells})$$

For this calculation, the following were assumed:
MCHC (initial) = 35 g/100 ml
Volume cell water (initial) = 0.717 ml/ml cells (Ref. 21)
Volume cell solids = 0.283 × initial cell volume
Na^+ distribution ratio $r_{Na^+} = Na^+_{in}/Na^+_{out} = 1.36$

We determined experimentally that the distribution ratio $r_{Na^+} = Na^+_{in}/Na^+_{out}$ in the presence of Nystatin was constant, and that the subsequent change in cell water content either concentrated or diluted the trapped Na proportionally (see Results, Fig. 2).

Swollen cells. To prepare low MCHC cells, we added a small volume of concentrated salt solution to dilute cell suspensions, prepared from freshly drawn blood as follows: After removal of plasma and one wash in buffered saline with potassium and glucose *, the cells were washed twice in 'internal cation buffer' **, prepared with the same concentrations of Na^+ and K^+ as those found inside normal red cells. The cells were then resuspended to 5% hematocrit (usually 0.7 ml packed cells plus 13.3 ml internal cation buffer). Sufficient Nystatin stock solution (5 mg Squibb Nystatin in 1 ml anhydrous methanol) was added to give a concentration of 30 μg Nystatin/ml cell suspension. The Nystatin-treated cells were kept in an ice water bath for 10 min, and then the concentrated ice-cold NaCl/KCl solution was slowly added in seven equal portions, with a 5 min interval between each addition. The salt solution was composed of a mixture of 2 M NaCl and 2 M KCl in the required proportions. The volume added was chosen to give the required elevation in osmolality of the cell suspension. The proportion of NaCl was then determined to give the desired concentration of Na^+ in the cell suspension at the final stage of equilibration. The cell suspension was stirred in the ice bath during salt addition and was permitted to equilibrate for 5 min. After all the salt solution was added, the cells were then centrifuged at 2 000 rev./min for 3–5 min, and the supernatant removed and retained for subsequent measurement of Na^+ and K^+ concentrations, and osmolality. The cells were then washed four times in a medium identical in ion composition to the equilibrium medium but free of Nystatin. In addition, 0.3–0.5% (w/v) of bovine serum albumin (Fraction V powder, Sigma Chemical Co., St. Louis, MO) was added to the wash medium, and the solution was warmed to 37°C before using to insure the removal of Nystatin and restoration of the normal membrane permeability barrier. Measurement of the ouabain-insensitive rate of K^+ efflux from Nystatin-treated cells with normal Na^+ and K^+ content confirmed that the Nystatin treatment had no residual effect on cell permeability under the conditions of the pumping experiments.

Shrunken cells. We prepared high MCHC cells by adding a large volume of hypotonic buffered medium to a small volume of packed cells. The procedure was similar to that used for preparation of swollen cells, except that: Hypotonic cation exchange solutions included (1) 10 mM phosphate buffer, pH 7.4, as either the Na^+ or K^+ salt, depending on the desired Na^+ concentration; (2) 27 mM sucrose, to balance the osmotic strength of impermeant intracellular species; (3) NaCl at a concentration sufficient to produce the desired Na^+ concentration at the final stage of dilution (taking into account the Na^+ contributions of the phosphate buffer, and the total Na in the packed cell suspension); and (4) sufficient KCl to adjust the

* Buffered saline with potassium and glucose: 135 mM NaCl, 5 mM KCl, 8.6 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM $NaH_2PO_4 \cdot H_2O$, 11 mM glucose, made up to 1 liter. Osmolality and pH adjusted to 290–295 mosmol/kg and 7.4 as necessary.

** Internal cation buffer: 12 mM NaCl, 135 mM KCl, 8 mM KCl, 8 mM K_2HPO_4, 2 mM KH_2PO_4, 27 mM sucrose, made up to 1 liter. Osmolality and pH adjusted to 290–295 mosmol/kg and 7.4 as necessary.

osmolality to the value required at the final stage of dilution (again taking into account the contribution from the initial cell suspension). After addition of Nystatin to the 5% suspension of cells in Internal Cation Buffer, the cells were allowed to equilibrate on ice for 5 min, and were then sedimented by centrifugation (2 000 rev./min for 3 min) in a graduated centrifuge tube. The supernatant was removed, the volume of packed cells noted, and ice cold hypotonic salt solution containing 30 μg Nystatin per ml was added in seven portion at 5-min intervals. Instead of adding portions of equal volume, however, we added portions divided to give approximately equal decrements in the total osmolality. After the final stage of equilibration, these cells were also washed in 37°C medium identical to the final equilibration medium except for the absence of Nystatin and the presence of bovine serum albumin.

Characterization of Nystatin-treated cells. The degree of dehydration or hydration in the Nystatin treated cells was determined from the measured MCHC, using spun hematocrits and spectrophotometric measurements of hemoglobin as the cyanomethemoglobin complex. To completely lyse the most dehydrated cells, 0.1% Triton X-100 was added to the cyanomethemoglobin reagent. Intracellular and buffer concentrations of Na^+ and K^+ were measured with a flame photometer (Instrumentation Laboratories, Lexington, MA), using cell samples washed three times in isotonic Tris-HCl buffered (10 mM, pH 7.4) magnesium chloride (Tris-HCl $MgCl_2$). Ion concentrations, based on hematocrit measurements of the washed cells, were obtained in units of mequiv./l cells and were converted to units of mequiv./l of cell water using the measured MCHC and the assumption that the volume of cell solids was 0.283 ml/ml of original cells at the normal MCHC of 35 g/100 ml [21]. A measure of total cellular content of monovalent cations was obtained by dividing the concentration of $Na^+ + K^+$ in mequiv./l cells by the MCHC, thus giving $Na^+ + K^+$ per 10 g hemoglobin. Osmolalities were measured using a Wescor vapor pressure osmometer (Wescor, Inc., Logan, UT).

Pumping experiments. To study the volume regulation in the Nystatin-treated cells, we incubated them for periods of 4–24 h at 37°C in buffered saline with potassium and glucose, with added penicillin (100 U/ml) and streptomycin (100 μg/ml) (Penicillin-Streptomycin, Grand Island Biological Co., Grand Island, NY) to inhibit bacterial growth. Paired samples, with and without 10^{-4} M ouabain to inhibit (Na^+/K^+)-pump activity, were incubated at a hematocrit of ≃1%. Before and after the incubation, the cells were analyzed for Na^+, K^+ and water content. Changes in cell water were assessed by centrifugation of cell samples on discontinuous Stractan (St. Regis Paper Co., Tacoma, WA) density gradients [22,23]. The Stractan solutions contained from 0.258 to 0.333 g Stractan/ml solution, corresponding to a density range of 1.098 to 1.127 g solution/ml solution. In addition, the gradient solutions contained 15 mM potassium phosphate, pH 7.4, 5 mM $MgCl_2$, 10 mM glucose, 3 g/100 ml bovine serum albumin and enough NaCl to bring the total osmolality to 290 ± 5 mosmol/kg. Density analysis on the gradients provided a more sensitive measure of changes in cell water than did MCHC, and it also provided information concerning the heterogeneity of each cell population. Loss of total ions and water during incubation caused the cells to equilibrate at higher density positions along the gradient. Conversely, a gain in ion and water content caused the cells to equilibrate at lower density positions. To obtain quantitative information about the cell density distribution, we removed the cells from the gradient interfaces and measured the amount of hemoglobin in each subpopulation. Density profiles were then plotted, showing the percentage of total sample hemoglobin at each interface as a function of the density of the Stractan layer on which the cells rested.

ATP was measured in Tris borate extracts of washed red cell suspensions using the luciferin-luciferase assay system [24].

Results

Preparation of cells with specified Na^+ and water contents

Using the method described, we found that the final MCHC of the Nystatin-treated cells was determined by the total osmolality of the cell suspension at the time that Nystatin was removed. The relationship between final MCHC and this equilibration osmolality, using normal cells with an initial MCHC of approx. 35 g/100 ml cells, is shown in Fig. 1. The curved line in Fig. 1 was obtained from a simple

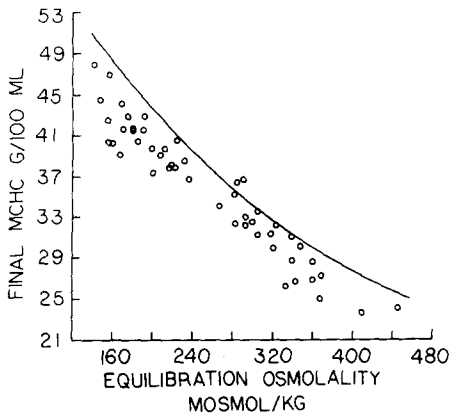

Fig. 1. Dependence of final MCHC on equilibration osmolality. Experimental points show the measured final MCHC in relationship to the measured osmolality at the stage of Nystatin washout. The solid curve represents the relationship that was predicted on the basis of a simple model (Appendix).

mathematical model that assumed that the intracellular osmotic strength comprised contributions from monovalent ionic species and impermeant anions, whose osmotic contribution was proportional to their concentration and from hemoglobin, whose osmotic contribution varied in a non-linear, but predictable way with its concentration (See Appendix). At present, we have no explanation for the consistent overestimation of MCHC by this simple model.

At any particular medium osmolality, the osmotic contribution of the small ionic species inside the cell (Na^+, K^+ and Cl^-) will be linearly related to cell water content. The reciprocal of MCHC is also a linear function of cell water, since $1/MCHC = (V_{H_2O} + V_{cell\ solids})/MCH$ where the Vs denote the volume occupied by intracellular water and cell solids, and MCH is the mean cell hemoglobin content. In the modified cells we verified a common dependence on cell water of MCHC and cation content by plotting $(Na^+ + K^+)/(290 - O_{Hb})$ versus 1/MCHC where O_{Hb} was the calculated osmolality of the hemoglobin at the final MCHC (See Appendix), and $Na^+ + K^+$ was the measured final amount of cations in mequiv./10 g of hemoglobin. The relationship was linear, with a correlation coefficient of 0.979. For large variations in water content, as represented by the starting cells for the pumping experiments, either MCHC or Na + K (mequiv./10 g Hb) or cation concentrations (mequiv./l cells) provided a useful measure of cell water content. But for the small changes in water content that occurred in the pumping experiments, only the cation content provided a sufficiently sensitive indicator.

Several aspects of the Nystatin loading procedure were important in ensuring reproducible results. First, it was necessary to include sucrose (27 mM) and Nystatin (30 μg/ml) in the hypotonic diluent solutions used to prepare dehydrated cells. Omission of either substance maintained the MCHC near the normal value. It was not necessary to add sucrose and Nystatin to the small volumes of concentrated salt solutions used to prepare swollen cells. A second im-

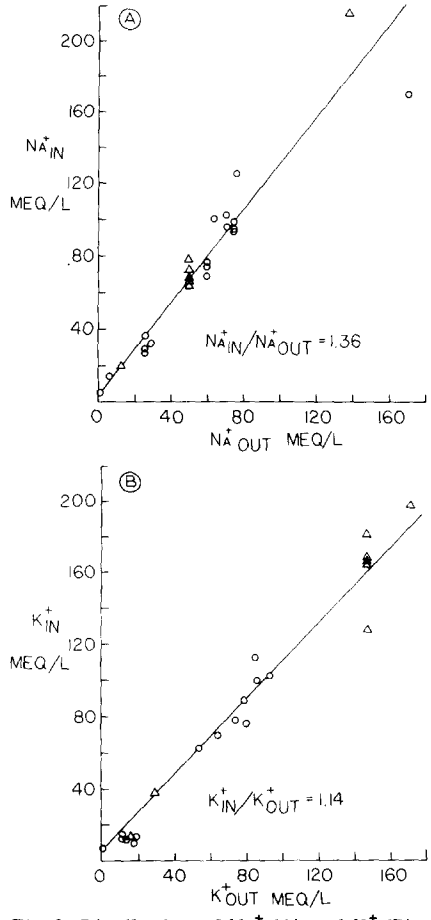

Fig. 2. Distribution of Na^+ (A) and K^+ (B) across the red cell membrane in the presence of Nystatin. Na^+_o and K^+_o were the measured supernatant concentrations of Na^+ and K^+ just before Nystatin washout. Na^+_i and K^+_i were the intracellular concentrations in mequiv./l cell H_2O, calculated from the measured final cation and hemoglobin concentrations (see text).

portant aspect of the procedure was the initial equilibration of the cells with Nystatin in a medium that mimicked the intracellular cation concentrations. Abrupt changes in medium cation concentrations in the presence of Nystatin resulted in variable volume changes, depending on the ion gradients across the cell membrane. A third important consideration was the temperature at which the various steps of the procedure were conducted. Because the rate of ion flux mediated by Nystatin is highest at low temperature [20], it was important to maintain the cell suspension at ice temperature during the ion exchange process. However, higher temperatures were required to remove the Nystatin from the cells after ion equilibration. We found that warming the wash solutions to 37°C before adding them to the cold, sedimented cells was adequate for restoration of normal Na^+ and K^+ permeability as measured at 37°C. Cells washed in cold solutions remained leaky to Na^+ and K^+ and continued to exchange cations with the extracellular medium. For example, when low Na^+ cells resuspended in solutions containing high Na^+ concentrations after four to five cold washes, they increased their Na^+ content substantially.

In order to control the final Na^+ concentration in Nystatin-treated cells, it was necessary to know how the Na^+ and K^+ were distributed across the membrane in the presence of Nystatin. It was not possible to measure the intracellular cations directly during the equilibration stage, so we measured the final concentrations after washing out the Nystatin and restoring isotonicity. We then calculated the concentrations of Na^+ and K^+ that would have been present at the equilibration MCHC of 35 g/100 ml cells. Assumptions for this calculation were that the relative volume of cell solids at 35 g/100 ml MCHC was 0.283, and that the Na^+ and K^+ content of the cells did not change during Nystatin washout and subsequent osmotic shrinkage or swelling. Thus the cation concentration changes were assumed to be inversely proportional to the changes in cell water volume. We then plotted the derived values for intracellular Na^+ and K^+ concentrations at the final equilibration stage against the measured concentrations in the extracellular medium. Fig. 2 shows the graphs for Na^+ and K^+. A linear relationship was found for both cations, from which we obtained distribution ratios of $Na^+_{in}/Na^+_{out} = 1.36$ ($r = 0.939$) and $K^+_{in}/K^+_{out} = 1.14$ ($r = 0.982$). Brumen and Glaser [25] recently proposed a model for cation permeable cells. Applying this treatment for a constant medium sucrose concentration of 27 mM, one would predict that as the medium electrolyte concentration was increased from 40 to 200 mM the membrane potential would have gone from approximately −3 mV down towards −1 mV. This would predict a change in the cation distribution ratio from 1.12 down to 1.04. In our experiments we observed no dependence of cation distribution ratios on the electrolyte concentration during ion equilibration. However, our experimental uncertainty was too large to reveal an effect of the predicted magnitude. Freedman and Hoffman [21] measured the distribution coefficients of Na^+ and K^+ in experiments in which cells were treated with Nystatin in two different media containing Na^+ and K^+ at concentrations different from the initial cell concentrations. At pH 7.3–7.4, they obtained values of $r_{Na^+} = 1.17–1.27$ and values of $r_{K^+} = 1.14–1.42$, depending on the Na^+ and K^+ concentrations.

TABLE I

OUABAIN-SENSITIVE CHANGES IN TOTAL CATION CONTENT OF Na^+-LOADED CELLS

Cells with initial Na^+ concentrations ranging from 31 to 116 mequiv./l cells and MCHC from 26 to 47 g/100 ml cells were incubated for 20–24 h at 37°C with and without 10^{-4} M ouabain to inhibit (Na^+/K^+)-pump activity. As shown here, the total monovalent cation content, expressed as mequiv./10 g hemoglobin or mequiv./l cells showed a net decrease when (Na^+/K^+)-pump activity was permitted. Figures are presented as mean ± S.D.

	n	$\Delta(Na^+ + K^+)$ mequivalents	
		per 10 g hemoglobin	per l cells
Swollen cells MCHC < 35			
+ ouabain	7	−0.12 ± 0.22	−1.8 ± 6.6
− ouabain	7	−0.72 ± 0.30	−11.9 ± 7.5
Shrunken cells MCHC > 35			
+ ouabain	14	+0.10 ± 0.19	−2.2 ± 5.4
− ouabain	14	−0.30 ± 0.19	−11.1 ± 5.9

Effects of Na^+/K^+ pump on cell water content

Cells loaded with Na^+ were studied for indications of ouabain-sensitive changes in cell water content. Criteria used to detect such changes were alterations in total intracellular monovalent cation content or concentration and in the distribution of cell populations along Stractan density gradients. When cells were incubated overnight at 37°C in the absence of ouabain, a reduction in intracellular Na^+ content and an increase in intracellular K^+ content provided evidence of (Na^+/K^+)-pump activation. In all 21 experiments where initial Na^+ concentrations were greater than 40 mequiv./l cell H_2O, this active transport of Na^+ and K^+ resulted in a net loss of total $Na^+ + K^+$. Mean values for the net changes in Na^+ and K^+ in those experiments are summarized separately for initially swollen and shrunken cells in Table I. Although the average loss in cation content per 10 g Hb from swollen cells was larger, all the shrunken cells, even severely dehydrated ones, lost significant amounts of $Na^+ + K^+$ during the long incubation (p = 0.05 by two-tailed t-test). The loss of total cations in these experiments was modest, and it was not consistently reflected by increases in MCHC. However, analysis of the density distributions of the various cell populations confirmed a ouabain-sensitive loss of cell water. Fig. 3 contains photographs of the density gradients from a representative experiment involving dehydrated cells. Fig. 4 contains density profiles for several experiments covering a range of initial cell water contents, and Table II summarizes the properties of the cells in these experiments. The percentage of hemoglobin at each gradient interface was used to provide a measure of the percentage of cells with densities bracketed by those of the Stractan layers between which they were trapped. From these density profiles it is evident that cell incubated in the absence of ouabain showed a shift to higher densities, reflecting an increase in the proportion of higher density, low water cells. Initially dehydrated cells incubated in the presence of ouabain showed a shift to

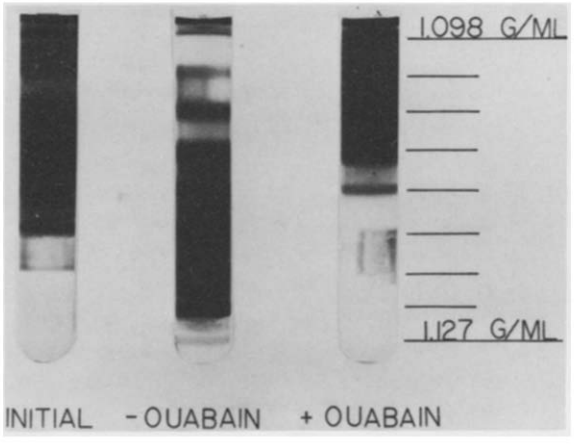

Fig. 3. Photograph of the distribution of cells on Stractan density gradients from a representative experiment. The tube on the left contained cells with MCHC of approx. 46 g/100 ml of cells, centrifuged on the gradient immediately after preparation. The initial Na_i^+ was 106 mequiv./l cells. The tube in the center contained cells incubated overnight in the absence of ouabain, and the one on the right contained cells incubated with ouabain. Note the increased proportion of high density cells in the sample incubated without ouabain.

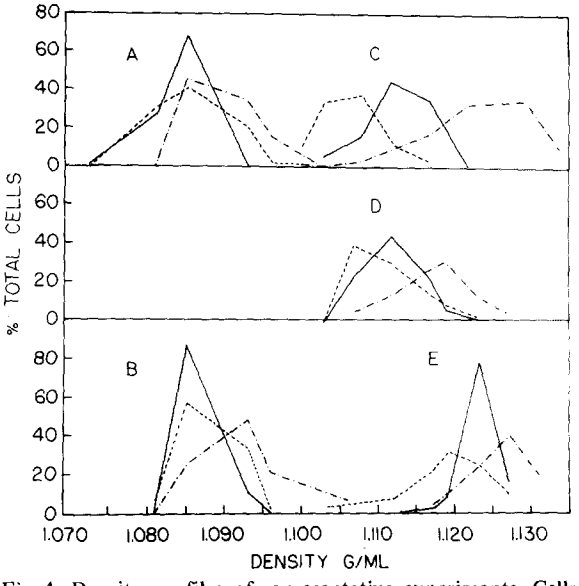

Fig. 4. Density profiles of representative experiments. Cells were prepared with various MCHC and Na^+ concentrations as summarized in Table II and were analyzed on Stractan density gradients before and after overnight incubation with and without 10^{-4} M ouabain. The percentage of total hemoglobin collected from each gradient layer was then plotted versus the density of the Stractan layer on which that subpopulation of cells rested. Experiments A and B involved initially low density, swollen cells. Experiments C, D and E involved initially high density, shrunken cells. ———, pre-incubation samples; – · – · –, samples incubated without ouabain; - - - - -, samples incubated with ouabain. Note the increased proportion of high density cell populations in all samples incubated without ouabain, including initially dehydrated cells.

TABLE II

CHARACTERISTICS OF CELLS USED IN EXPERIMENTS FOR FIG. 4

Na^+-loaded cells of various initial MCHC were incubated for 20–24 h with and without ouabain. The experiment letters in the table correspond to the same letters in Fig. 4, which shows the density gradient distributions for these illustrative examples.

Expt.	Initial MCHC (g/100 ml cells)	Initial Na (mequiv./l cells)	Initial Na + K (mequiv./l cells)	Final $Na^+ + K^+$ (mequiv./l cells)	
				+Ouabain	−Ouabain
A	29.9	68.9	128.2	117.1	113.1
B	31.4	64.5	124.8	121.2	112.4
C	41.6	99.6	109.9	109.1	91.6
D	40.4	70.6	112.2	103.8	94.2
E	44.6	48.0	88.1	89.8	77.4

lower densities, reflecting an increase in water. In general, cell density changes were not uniform throughout the cell population, and some cells showed more shrinkage or swelling than others, resulting in some broadening as well as movement of the original density distribution. Measurement of Na^+ and K^+ concentrations in individual subpopulations from samples incubated without ouabain showed that the denser cells contained relatively higher K^+ concentrations and lower Na^+ concentrations. This observation indicates that the heterogeneity in cell densities was associated with differences in the degree of pump activation in various subpopulations. In addition, the heterogeneity in density provided an explanation for the fact that changes in MCHC in Na^+-loaded cells during prolonged incubation were minor and inconsistent, since the shrinkage of cells that were pumping was partly obscured by swelling of cells that were not.

In the presence of ouabain there was a tendency for swollen cells to shrink and for shrunken cells to swell, but this effect was not consistent. Linear regression analysis of the change in ion content (in mequiv./10 g Hb) versus initial ion content gave an r value of −0.57.

When cells were adjusted to initial Na^+ concentrations below about 40 mequiv./l cell H_2O consistent pump-associated changes in cell density distribution were not detected, presumably, because restoration of normal Na^+ concentrations was accomplished with small, undetectable changes in total ion and water content. This is exemplified by the experiment with initially swollen cells summarized in Table III. Even with higher Na^+ concentrations, prolonged incubation was required to detect the cell shrinkage; cells incubated for only 6 h had density profiles indistinguishable from those of the initial cells. A modest correlation was found between ouabain-sensitive cation changes (per 10 mg Hb) and initial Na^+ content (mequiv./10 g Hb), with an r value of −0.64. This was consistent with our anticipation that the pump effect should be more evident in cells with large quantities of Na^+ to be extruded. The ratio of the ouabain-sensitive change in Na^+ content to that in K^+ content was 1.60 (S.D. = 0.25). Such a simple ratio does not accurately measure pump stoichiometry because it neglects differences in cation gradients in cells with and without ouabain. However, this observed ratio is in general agreement with a 3 : 2 stoichiometry for Na^+/K^+ transport.

TABLE III

ABSENCE OF CELL SHRINKAGE FOR LOW INITIAL Na^+

In preparation for this experiment, cells were treated with Nystatin in high K^+, hypertonic medium so that low Na^+, swollen cells would be obtained. Subsequent incubation in the absence of ouabain caused negligible change in Na^+ or K^+ content, whereas incubation with ouabain resulted in net ion uptake and further swelling.

	Na^+ (mequiv./ l cells)	K^+ (mequiv./ l cells)	$Na^+ + K^+$ (mequiv./ l cells)
Initial	13.2	111.2	124.4
24 h + ouabain	33.5	100.6	134.1
24 h − ouabain	11.8	111.0	122.8

TABLE IV

ATP CONCENTRATIONS BEFORE AND AFTER OVERNIGHT INCUBATION

ATP was measured before and after 24 h incubation to determine whether metabolic depletion occurred during the (Na^+/K^+)-pump experiments.

	Na^+ (mequiv./l cells)	K^+ (mequiv./l cells)	ATP (mmol/l cells)
Expt. a			
Initial	89.1	12.1	1.25
24 h + ouabain	94.1	7.9	1.27
24 h – ouabain	34.9	53.6	1.41
Expt. b			
Initial	106.4	14.1	1.54
24 h + ouabain	107.5	9.5	1.23
24 h – ouabain	64.0	42.1	1.33

The pH of the cell suspensions did not decrease more than 0.1 pH unit during overnight incubation. Moreover, normal intracellular ATP concentrations were maintained, regardless of pumping activity (Table IV).

Discussion

These experiments show that substantial elevation of red cell Na^+ concentration in the absence of appreciable changes in Na^+ permeability results in a subsequent decrease in total monovalent cation and water content, even in initially dehydrated cells. The inhibition of this dehydration process by ouabain suggests that it is associated with the known stimulatory effect of Na^+ on active transport of Na^+ and K^+. We further conclude that the observed ouabain-sensitive water loss is a reflection of the 3 Na^+_{in} : 2 K^+_{out} stoichiometric ratio of the Na^+/K^+ pump.

The observations imply, as suggested by Glader and Nathan [17], that the red cell is ill-equipped to compensate for processes that tend to cause an unbalanced efflux of K^+ (and water), such as the specific K^+ leak associated with Ca^{2+} accumulation [16] or that associated with irreversible sickling [17]. Knight and Welt [26] found that if intracellular Na^+ were low enough, reduction of intracellular K^+ itself caused pump stimulation. Thus the low K^+ dehydrated cell would appear to be in a vulnerable state with either high or low internal Na^+. On the other hand, Na^+ stimulation of the Na^+/K^+ pump does provide an appropriate compensatory response to increased influx of Na^+ (and water). Pump stimulation by elevation of internal Na^+ results in accelerated extrusion of Na^+ in greater quantities than the K^+ for which it is exchanged, causing a net extrusion of total ions and water. Poznansky and Solomon [19] have observed changes in the flux of isotopically labeled cations in osmotically swollen and shrunken red cells. They concluded from these observations that human cells undergo volume-dependent permeability changes that tend to restore normal cell volume. Since we did not measure isotopic ion flux, we cannot clearly determine whether such permeability changes occurred in our Nystatin treated cells in isotonic medium. If such compensatory changes in membrane permeability did occur, they were overwhelmed by the larger effects of active transport. It is possible that the small negative correlation between ouabain-insensitive changes in total ion content versus initial ion content that we observed was a reflection of such a pump-independent contribution to volume regulation.

It is difficult to define the possible contribution of Na^+/K^+ co-transport to the cell volume changes in these experiments. If the normal cell has a co-transport process in which Na^+ extrusion is driven by the outward K^+ gradient, then our Na^+-loaded cells with reduced internal K^+ concentrations should have had a reduced rate of co-transport. This would presumably confer a decreased tendency to lose total ions and water. As the pump restored the potassium gradient towards its normal level, outward co-transport could have become reactivated, augmenting the pump's effect on ion extrusion. At present however, there are no experimental data to delineate the role of co-transport in regulation of red cell water content.

The Nystatin loading method developed for these studies provides a convenient means of preparing red cells with preselected and independently varying Na^+ and water contents. Such preparations should be generally useful in studying regulation of cell volume and other functions over a wide range of water content. In this study, the Nystatin-treated cells provide a model for the situation in which intracellular ion or water contents have been altered without substantial modification of passive permeability. This model

could be appropriate for sickle cells, whose Na^+ concentrations become elevated during a transient period of deoxygenation [17]. After reoxygenation, transiently elevated cation permeabilities return to normal, and a Na^+-stimulated pump reduces intracellular Na^+ and increases K^+. The model suggests that during the process of restoring the Na^+ and K^+ gradients across the membrane, the cells could lose some water. The effect would be expected to be small, since the pump would operate at an elevated rate only as long as the Na^+ was elevated. If Na^+ permeability were not enhanced, cell shrinkage would cease when cation flux through the pump decreased to a level that equalled the net passive ion flow. This steady state volume would depend on the extent to which the normal volume and Na^+ had been perturbed initially. Even with the large increases in intracellular Na^+ used in these experiments, the ouabain-sensitive reductions in cation and water content were modest. Further study will be required to determine whether elevation of intracellular Na^+ can augment cell water loss in physiologic situations.

Appendix

A model to predict the relationship between final MCHC and osmolality during Nystatin-mediated ion exchange.

Let the final equilibration stage, just before Nystatin removal, be designated stage 1, and the final stage, after removal of Nystatin and restoration of isotonicity, be designated stage 2. Assume $MCHC^1$ = 35 g/100 ml cells, $V^1_{H_2O}/V^1_{cell} = 0.717$, and $V^1_{cell\ solids}/V^1_{cell} = 0.283$, where V_{H_2O} is the volume of the cell that is occupied by water. At the final stage, the medium osmolality, O^2 = 290 mosmol/kg.

$$O^1_{medium} = O^1_{cells};\ O^2_{medium} = O^2_{cells}$$
$$O_{cells} = ((\Sigma_i n_i \alpha_i + P\alpha_p)/V_{H_2O} + O_{Hb}$$

where n_i is the number of each osmotically active species i in the cell, α_i is the osmotic coefficient of species i, P is the number of impermeant molecules other than monovalent cations and hemoglobin, with osmotic coefficient α_p, and O_{Hb} is the osmotic contribution of hemoglobin. This equation simply states that the osmotic strength inside the cells is the sum of the contributions from the monovalent ionic species, and the hemoglobin and impermeant anions. It applies to stage 1 and stage 2, and $\Sigma_i n^1_i \alpha_i = \Sigma_i n^2_i \alpha_i$ since the permeability barrier is restored by removal of Nystatin, and the ions present at stage 1 are trapped inside the cell.

When the extracellular osmolality is changed, the number of ions and hemoglobin molecules remains constant, but water enters or leaves the cell to balance the extracellular osmotic strength. The osmotic contribution of the small ions is taken to be inversely proportional to the water volumes, but the hemoglobin contribution varies non-linearly with hemoglobin concentration.

$O_{Hb} = \phi_{Hb} \times (Hb)$, and $\phi_{Hb} = 1 + 0.0645(Hb) + 0.0258(Hb)^2$ where (Hb) is the molal concentration of hemoglobin [21]. So $O_{Hb} = (Hb) + 0.0645(Hb)^2 + 0.0258(Hb)^3$. Combining and rearranging the equations above, we have:

$$\Sigma_i n_i \alpha_i + P\alpha_p = (O_{medium} - O_{Hb}) \times V_{H_2O}$$

Therefore

$$(O^1_{medium} - O^1_{Hb}) \times V^1_{H_2O} = (O^2_{medium} - O^2_{Hb}) \times V^2_{H_2O}$$

and

$$O^1_{medium} = (V^2_{H_2O}/0.717) \times (290 - O^2_{Hb}) + 20.05$$

and for any final MCHC

$$V^2_{H_2O} = 35/MCHC^2 - 0.283$$

Calculation of the osmolalities required at the equilibration stage to produce specific MCHC from 25 to 51 g/100 ml of cells resulted in the line included with the actual data in Fig. 2.

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