Internal Magnesium, 2,3-Diphosphoglycerate, and the Regulation of the Steady-State Volume of Human Red Blood Cells by the Na/K/2Cl Cotransport System

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ABSTRACT This study is concerned with the relationship between the Na/K/Cl cotransport system and the steady-state volume (MCV) of red blood cells. Cotransport rate was determined in unfractionated and density-separated red cells of different MCV from different donors to see whether cotransport differences contribute to the difference in the distribution of MCVs. Cotransport, studied in cells at their original MCVs, was determined as the bumetanide (10 μM)-sensitive ^{22}Na efflux in the presence of ouabain (50 μM) after adjusting cellular Na (Na$_i$) and K$_i$ to achieve near maximal transport rates. This condition was chosen to rule out MCV-related differences in Na$_i$ and K$_i$ that might contribute to differences in the net chemical driving force for cotransport. We found that in both unfractionated and density-separated red cells the cotransport rate was inversely correlated with MCV. MCV was correlated directly with red cell 2,3-diphosphoglycerate (DPG), whereas total red cell Mg was only slightly elevated in cells with high MCV. Thus intracellular free Mg (Mg$_i^{free}$) is evidently lower in red cells with high 2,3-DPG (i.e., high MCV) and vice versa. Results from flux measurements at their original MCVs, after altering Mg$_i^{free}$ with the ionophore A23187, indicated a high Mg$_i$ sensitivity of cotransport: depletion of Mg$_i^{free}$ inhibited and an elevation of Mg$_i^{free}$ increased the cotransport rate. The apparent $K_{0.5}$ for Mg$_i^{free}$ was ~0.4 mM. Maximizing Mg$_i^{free}$ at optimum Na$_i$ and K$_i$ minimized the differences in cotransport rates among the different donors. It is concluded that the relative cotransport rate is regulated for cells in the steady state at their original cell volume, not by the number of copies of the cotransporter but by differences in Mg$_i^{free}$. The interindividual differences in Mg$_i^{free}$, determined primarily by differences in the 2,3-DPG content, are responsible for the differences in the relative cotransport activity that results in an inverse

relationship with in vivo differences in MCV. Indirect evidence indicates that the relative cotransport rate, as indexed by Mg_i^{free}, is determined by the phosphorylated level of the cotransport system.

INTRODUCTION

The primary aim of the work described in this paper was to investigate the extent to which the Na/K/2Cl cotransport system (hereafter referred to as the cotransport system) is involved as a determinant in the in vivo steady-state volume of oxygenated red blood cells. This is in light of the fact that the cotransport system is known to promote coupled, bidirectional net movements of Na, K, and Cl in response to perturbations in cell volume (Kregenow, 1981; Siebens, 1985; Hoffmann and Simonsen, 1989) and/or changes in the net chemical potential driving forces (Schmidt and McManus, 1977; Duhm and Göbel, 1984). It has been reported by others (Adragna et al., 1982; Duhm and Göbel, 1982; Stewart, 1988) that there was a significant interindividual variability in the size of the cotransport flux, and that this flux also varied inversely with both the mean red cell volume (MCV) and K content (Duhm and Göbel, 1984). Because the relationship between the MCVs the cells were studied at and their original (in vivo) MCVs was not clear in these studies, we felt that a reexamination was warranted, particularly since the available evidence (see Kregenow, 1981; Siebens, 1985; Hoffmann and Simonsen, 1989) indicated that, for instance, in an avian red cell or in an Ehrlich ascites tumor cell, the cotransport system's activity was higher in hypertonic than in isotonic solutions, i.e., during cell volume readjustment. It would therefore seem that a more critical evaluation of whether or not the cotransport system was a contributor to steady-state, in vivo cell volume would best be studied in the bloods of individuals that expressed high and low cotransport activities at their in vivo MCVs, where the magnitude of their Na/K pumps and electrodiffusional leaks was comparable. The idea of having comparable pumps and leaks would act to normalize their input as primary determinants of cell volume (Tosteson and Hoffman, 1960; Milanick and Hoffman, 1986) and perhaps thereby amplify the relative effects of cotransport. These considerations thus represent the basis for the approach taken and the selection of the individuals whose red cell populations were analyzed in this study. It should also be noted that since the activity of the cotransport system is affected by the concentrations of K_i and Na_i (Canessa et al., 1986) at any volume measured, we only measured cotransport activity under conditions where the internal concentration of these ions was changed to optimize the cotransport rates. This was done to relate the maximum cotransport capacity of the cells to their original in vivo MCV and avoid variations in cotransport activity that might be arbitrarily dependent on variations in Na_i.

We found not only that the interindividual variation in the red cell MCVs was inversely correlated with the extent of cotransport, but that the variation in MCV was directly related to the cellular concentration of 2,3-diphosphoglycerate (DPG). This latter relationship was shown to be the result of variations in the cellular concentration of free Mg_i (Mg_i^{free}), indicating that the cotransport was directly related to Mg_i^{free}. Thus cells with small MCVs have low concentrations of 2,3-DPG but high cotransport rates due to high Mg_i^{free}, the converse applying to cells with large MCVs. Maximization of Mg_i^{free} (by use of the ionophore A23187 and a Mg_o buffering system)

was shown to essentially eliminate the interindividual variability in cotransport rates. It thus appears that the activity of the cotransport system is not only highly regulated, but that for normal cells the cotransport system contributes to their steady-state MCVs.

Preliminary accounts of this work have been previously presented (Mairbäurl and Hoffman, 1989, 1990).

METHODS

Definitions

In this paper the term cotransport is used to denote Na/K/2Cl cotransport. This flux is taken as the bumetanide-sensitive Na efflux ($^oM_{Na}^{bumet}$) that is measured in the presence of ouabain. Thus it is assumed that other anion-dependent cotransport systems (e.g., K/Cl) are not involved in these measurements.

Na/K pump activity is taken as the ouabain-sensitive Na efflux ($^oM_{Na}^{ouab}$) or Rb influx ($^iM_{Rb}^{ouab}$). Na/K pump fluxes are not affected by the presence of bumetanide. The residual efflux of Na ($^oM_{Na}^{leak}$) that occurs in the presence of ouabain and bumetanide is taken to represent the passive diffusion component. These operational definitions serve to separate the three types of fluxes studied in this paper. It should be noted that ^{86}Rb is used as a surrogate tracer for the unidirectional movement of K. Separate comparisons of ^{86}Rb with ^{42}K fluxes have shown the measurements to be completely comparable.

Subscripts i and o are used to refer to intracellular and extracellular concentrations, respectively, of ions such as Na, K, Mg, and Cl. Hb refers to hemoglobin concentration; Hct to hematocrit; MCHC to mean corpuscular Hb; MCH to mean corpuscular hemoglobin content; MCV to mean cell volume.

The term unfractionated refers to the whole cell population in contrast to fractionated cells where the population has been separated by buoyant density centrifugation. Unmodified refers to normal cells fractionated or unfractionated. Modified refers to cells whose Na_i, K_i, and/or Mg_i has been altered by preincubation procedures as described below.

Materials and Analytical Methods

All solutions were prepared from deionized water using analytical grade reagents. The osmolarity of all solutions was measured with an osmometer (model 3MO; Advanced Instruments, Inc., Needham Heights, MA). Tris and HEPES were from ICN Biomedicals, Inc., Costa Mesa, CA. A23187 was from Boehringer Mannheim Corp., Indianapolis, IN. EDTA, EGTA, bovine serum albumin (BSA; Fraction V), choline chloride, ouabain, and nystatin were from the Sigma Chemical Co., St. Louis, MO. Choline chloride was recrystallized from hot ethanol before use. Bumetanide was a gift from Hoffman La Roche, Nutley, NJ. All isotopes were from New England Nuclear (DuPont Company, Boston, MA).

Hb was measured by the cyanmethemoglobin method with Drabkin's reagent (Sigma Chemical Co.). Hct was determined by microcentrifugation. Red cell counts were determined with a Coulter Counter, model Z_{BI}, after suitable dilution of the cells with Isotone II (Coulter Corp., Hialeah, FL). From these data MCV, MCHC, and MCH were calculated. MCV determinations were carried out in duplicate. MCV ± SEM values (in cubic micrometers) for the bloods numbered 1–6 in the figures (see below) were, respectively, 88.7 ± 0.4(7), 90.9 ± 0.5(6), 90.3 ± 0.3(7), 91.5 ± 0.8(3), 94.2 ± 0.3(3), and 95.3 ± 0.6(5), where the value in the parentheses gives the number of separate measurements. Red cell 2,3-DPG and ATP were measured from trichloroacetic acid extracts by a colorimetric method (procedure 665; Sigma

Chemical Co.) and a bioluminescence assay kit (FL-AA; Sigma Chemical Co.) using a luminometer (model TD-20e; Turner Designs, Sunnyvale, CA), respectively.

Na_i and K_i were determined in red cells washed three times with a solution containing 109 mM $MgCl_2$ or 155 mM choline Cl (2 min, 15,000 g, 4°C, Sorvall SS-34 rotor) before being packed by centrifugation (10 min, 12,000 g, 4°C, Sorvall HB-4 rotor) into a narrow-bore column in special lucite tubes (Dunham and Hoffman, 1971). After packing, the supernatant was carefully removed and aliquots of the packed red cells were removed for Na_i and K_i analysis by flame photometry (Corning 400; Corning Medical, Ltd., Halstead, UK) in appropriately diluted lysates of the packed cells. Cell water (wt/wt) was determined by the wet weight/dry weight difference after drying overnight at 80°C.

Cl_i (millimoles per liter cell water) was determined from the equilibrium distribution of ^{36}Cl after incubation of red cells in a medium containing $^{36}Cl_o$ and a known Cl_o as previously described (Hoffman and Laris, 1974). The chloride ratio (r_{Cl}) was calculated as Cl_i/Cl_o. Direct measurements of pH_i were carried out with the method used by Funder and Wieth (1966a).

Measurements of total cellular Mg (Mg_i^{tot}; accuracy of ±5%) were carried out on red cells washed three times with a solution containing 155 mM choline Cl and then packed as described above. The packed cells were lysed and suitably diluted with a medium containing 0.1% (wt/vol) $LaCl_3$ prior to Mg measurements by atomic absorption (model 560; The Perkin-Elmer Corp., Norwalk, CT). Estimates of intracellular free Mg (Mg_i^{free}) were calculated from Mg_0^{A23} after permeabilizing the cells for Mg with the ionophore A23187 (Reed, 1976; Flatman and Lew, 1977; Yingst and Hoffman, 1978). Therefore cells (Hct ~6%) were loaded with Mg and ^{36}Cl by incubation for 15 min at 37°C in media containing varying concentrations of Mg_0 in the presence of 5 μM A23187. Depending on the Mg content of the intact cells, Mg_0 changed in the presence of A23187 (i.e., Mg_0^{A23}) such that after equilibration Mg_i^{free} was estimated from the measured values of Mg_0^{A23} and r_{Cl} using the formula (Flatman and Lew, 1980):

$$Mg_i^{free} = Mg_0^{A23} \cdot (r_{Cl})^{-2}$$

Mg_i^{free} was also estimated from the total cellular concentration of Mg, ATP, 2,3-DPG, Hb, and Cl (in millimoles per liter cell water) and the binding constants reported by Gerber et al. (1973), Gupta et al. (1978), and Chiancone et al. (1972) by using the methods described by Gerber et al. (1973) and Gupta et al. (1978). The binding constants used were (mM): Mg[ATP], 0.038; Hb[ATP], 3.43; Hb[MgATP], 5.4; Mg[DPG], 1.50; Hb[DPG], 4.75; and Hb[Cl], 1,000. An oxygen saturation of 100% was assumed.

Preparation of Red Cells

Heparinized (10 IU/ml) blood was collected after informed consent by puncture of antecubital veins from randomly selected male and female Caucasian donors with no known blood dyscrasias. Plasma and buffy coat were carefully removed during three washes (2 min, 15,000 g, 4°C, Sorvall SS-34 rotor) of the red cells with ~8 vol of washing medium (mM): 150 NaCl and 3 Tris-HEPES (pH 7.4 at 4°C). It is important to note that <2% of the red cells were lost during aspiration of the buffy coat.

Fractionation of Red Cells

Red cells were fractionated by centrifugation on self-forming Percoll (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) density gradients. Pure Percoll of a density of 1.130 g/ml was adjusted to an osmolality of ~285 mosmol/liter and brought to a density of 1.095 g/ml by mixing with a medium containing (mM): 110 NaCl, 50 KCl, 50 glucose, and 150 Tris-HEPES (pH 7.4 at 4°C). 6 ml of freshly drawn heparinized whole blood was layered on 30 ml of gradient medium in 50 ml polycarbonate tubes and then centrifuged (30 min, 20,000 g, 4°C,

Sorvall SS-34 rotor). After centrifugation, supernatant plasma and leukocytes that did not penetrate the density gradient were removed by suction and the isopycnically banded red cells were fractionated into several preselected portions by aspiration from the top to the bottom of the gradient using a peristaltic pump. Eight centrifuge tubes of the same subject's blood were processed at the same time in this way and cell fractions of equal density from the eight tubes were pooled. The percentage of cells in each fraction was determined by measurements of Hb on aliquots after pooling. Several contiguous fractions were combined to obtain sufficient cells in each of three to five fractions of the gradient, although most of the middle fractions, containing the bulk of the cell population, were usually discarded. The cells from each pooled fraction were washed free from Percoll with washing medium before use in experiments. Thus it should be clear that the different fractions separated for study, while varying in density, were not proportional to their concentrations in the total cell population.

Modifications of Na$_i$ and K$_i$

Na$_i$ and K$_i$ of unfractionated or fractionated red cells were each adjusted to ~50 mmol/liter cells by using a modification of the nystatin procedure (Cass and Dalmark, 1973) to be able to evaluate all transport parameters at their respective maximal values. At these concentrations of Na$_i$ and K$_i$, the Na/K pump is saturated (Post and Jolly, 1957) and the cotransport activity is also essentially maximal under these circumstances (Garay et al., 1981; Canessa et al., 1986; Kracke et al., 1988). These latter estimates were carried out where Na$_i$ and K$_i$ were varied reciprocally. On the other hand, we have carried out, with the nystatin technique, separate experiments where $^oM_{Na}^{bumet}$ was measured at increasing concentrations of Na$_i$ (5–60 mmol/liter cell water) at constant K$_i$ (20–30 mmol/liter cell water) with choline being used to maintain the osmolarity constant. We found that regardless of the resultant MCV the cotransport activity plateaued between 40 and 50 mmol Na$_i$/liter cell water (data not shown). We also found in other experiments that Cl$_i$ was saturating under our conditions (Mairbäurl and Hoffman, 1991).

In the nystatin protocol, the loading medium contained (mM): 70 NaCl, 70 KCl, 1 NaH$_2$PO$_4$, 1 MgCl$_2$, 5 glucose, 25 sucrose, and 20 Tris-HEPES (pH 7.2 at 0°C). Three loading steps, each lasting 25 min, were performed on ice and in the dark, with fresh loading medium used in each step. In the first step, nystatin (dissolved in methanol) was added to a final concentration of 40 μg/ml to a 20% Hct cell suspension. In the second step, after packing the cells by centrifugation and removing the supernatant, the cells were suspended in fresh nystatin-free loading medium (5% Hct). In the third step, after removal of the previous medium the cells were suspended in fresh medium (30% Hct) which, when desired, also contained 20 μCi/ml of ^{22}Na in order to label cells for Na efflux measurements. To remove all cell-associated nystatin, the cells were then washed seven times with warm loading medium (pH 7.4 at 30°C) containing 0.2% BSA, followed by two washes with ice-cold flux medium. MCV, MCHC, and percentage of cell water remained unchanged throughout this procedure without significant hemolysis occurring. The modified cells were then used directly for flux measurements.

Na Efflux

Na efflux was measured after washing the cells once with flux medium that was usually (unless otherwise stated) composed of (mM): 135 NaCl, 5 KCl, 1 NaH$_2$PO$_4$, 1 MgCl$_2$, 5 glucose, and 20 HEPES-Tris (pH 7.4 at 37°C). When present, the final concentration of ouabain was 50 μM and that of bumetanide (dissolved in DMSO) was 10 μM. When bumetanide was added to experimental flasks, DMSO (final concentration, 0.1%) was added to all control flasks. The flux measurement was begun by addition of red cells to a final Hct of ~1%. Triplicate samples were removed at 3 min (to allow for temperature equilibration) and at 63 min. Fluxes were stopped

by cooling the samples for 3 min in an ice-water bath before centrifugation in a microfuge. Radioactivity was determined on samples of the supernatants taken at different time points as well as from the whole suspension mixture.

Where applicable, Mg_i^{tot}, and therefore Mg_i^{free}, was altered by varying the concentration of Mg_o in the flux medium and by adding 5 μM A23187. The cells were then incubated for 15 min at 37°C in this flux medium to allow Mg to equilibrate between cell and medium before the flux measurements. No difference in $^oM_{Na}^{bumet}$ was found whether A23187 was absent or present during the flux measurement.

Rb Influx

Influx of ^{86}Rb was determined under the same conditions (1% Hct, 37°C) and in the same medium (± 50 μM ouabain) as used for Na efflux after addition of 20 μCi of ^{86}Rb per 10 ml of medium. The flux was begun by addition of cells (final Hct $\sim 1\%$). Triplicate samples were removed at 5 and 35 min and immediately centrifuged. The cells were then washed four times with chilled flux medium (4°C) to remove $^{86}Rb_o$. The packed red cells were lysed with deionized water for radioactivity determinations. The red cell content of the lysate was calculated by using the Hct and Hb of the initial suspension and the Hb in the lysate. Total counts were also determined for samples of the whole cell suspension.

Calculations

All fluxes were calculated from determinations of the rate constants (h^{-1}) and either Na_i (millimoles per liter cells) or the medium K_o (Rb_o) for Na efflux and K influx, respectively. It was confirmed in separate measurements that the time course of isotope movement during efflux and influx followed a single exponential over the time periods studied.

Results from single experiments are presented as mean values of triplicate measurements. The average coefficient of variation of the fluxes calculated from triplicate measurements was 1.5 $\pm$ 1.2% (mean $\pm$ SD). Correlations between parameters were calculated by linear regression analysis, with regression lines being estimated by least-squares fits. Regression lines are only shown where the level of significance (P) was <0.05. Testing for differences of parameters among density-separated red cells was performed by one-way analysis of variance. Statistics and graphics were performed using the STATGRAPHICS® (STSC Inc., Rockville, MD) and SIGMAPLOT® (Jandel Scientific, Corte Madera, CA) software.

RESULTS

The original aim of this work was first to identify individuals whose normal, red cell steady-state MCVs were different from each other, and second, to survey various membrane transport parameters to see whether there were any consistent differences in the ion flux patterns that might indicate a basis for the differences in MCV. It must be emphasized that all measurements of MCV were carried out on fresh, unmodified cells, whereas all transport parameters were evaluated at their respective maximal values by modification first of Na_i and K_i, and subsequently of Mg_i. The resultant flux values were then used to evaluate a possible correlation with MCV.

Properties of Unfractionated Red Cells

Fig. 1 presents some characteristics of the red cells from the various individuals used in this study. It shows the relationship between the values of MCV and the cellular contents of $Na_i + K_i$ (Fig. 1 A), K_i (Fig. 1 B), and Na_i (Fig. 1 C) for the whole

unfractionated and unmodified populations of red cells studied. It is apparent that $Na_i + K_i$ and K_i are directly correlated with MCV, whereas Na_i decreases with increased MCV. The correlation of MCV with $Na_i + K_i$ is not only consistent with the results of Funder and Wieth (1966b) and Lee et al. (1984) but also emphasizes that the prime determinant of cell water (volume) is the cell's content of Na + K, thus focusing attention on the transport parameters that underlie the cell's sum of Na + K. Statistics for the regression lines shown in Fig. 1 are presented in Table I. It should be noted that while the correlation of MCV with Na_i is statistically significant
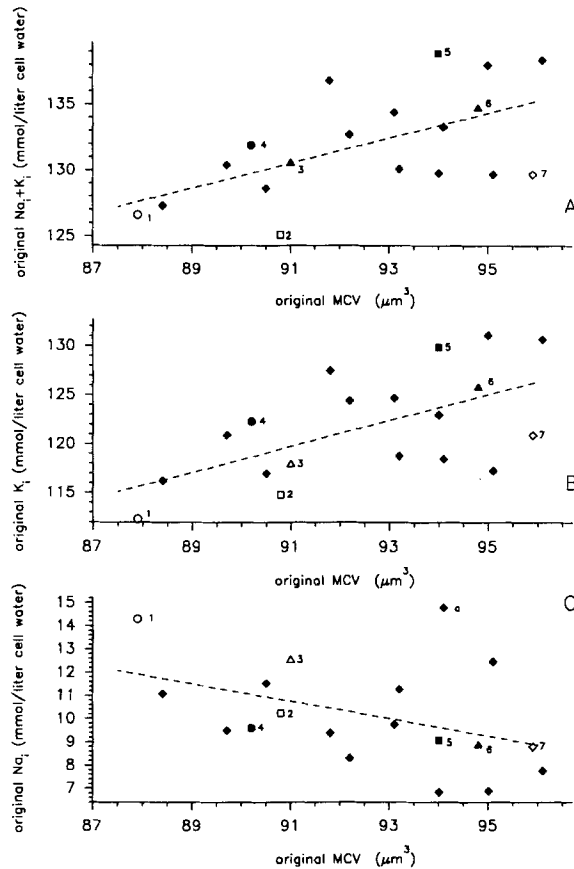

FIGURE 1. The relationship between original red cell volume (MCV) and (*A*) ($Na_i + K_i$), (*B*) K_i, and (*C*) Na_i from unmodified red cells from 19 different donors. The same values of MCV on the abscissa refer to the same individuals in all three panels and are also the same as shown in Fig. 2, *A–C*. Blood samples from donors numbered 1–6 (symbols other than filled diamonds, except for donor 7) were used in studies on density-separated red cells (see below). Regression statistics are presented in Table I.

only at the level of $P \leq 0.076$, it is not clear whether inclusion of more individuals would have changed this relation. It may be of interest that elimination of the data point *a* in Fig. 1 *C* increases the level of significance to $P \leq 0.010$. Mention should be made that an indirect relationship was also found (results not shown) between MCV and MCHC ($r = -0.708$; $P \leq 0.0007$; MCHC (grams per liter) $= 581.7 - 2.730 \times$ MCV).

Fig. 2 shows the results of measurements of the various transport parameters for the same individuals represented in Fig. 1. It is apparent that there is an inverse

TABLE I

Regression Statistics for the Results Presented in Fig. 1, A–C, and Fig. 3, A–C

MCV vs.	Intercept	Slope	r	P values
Unfractionated red cells (Fig. 1)				
$(Na_i + K_i)$	44.19	0.949	0.581	0.009
K_i	−1.557	1.333	0.602	0.006
Na_i	44.98	−0.376	−0.417	0.076
Fractionated red cells (Fig. 3)				
K_i	−3.934	0.998	0.774	0.0001
Na_i	31.36	−0.228	−0.551	0.0002
MCHC	576.9	−2.644	−0.876	0.00001

r and P values are the correlation coefficient and the level of significance of the regression coefficients, respectively.
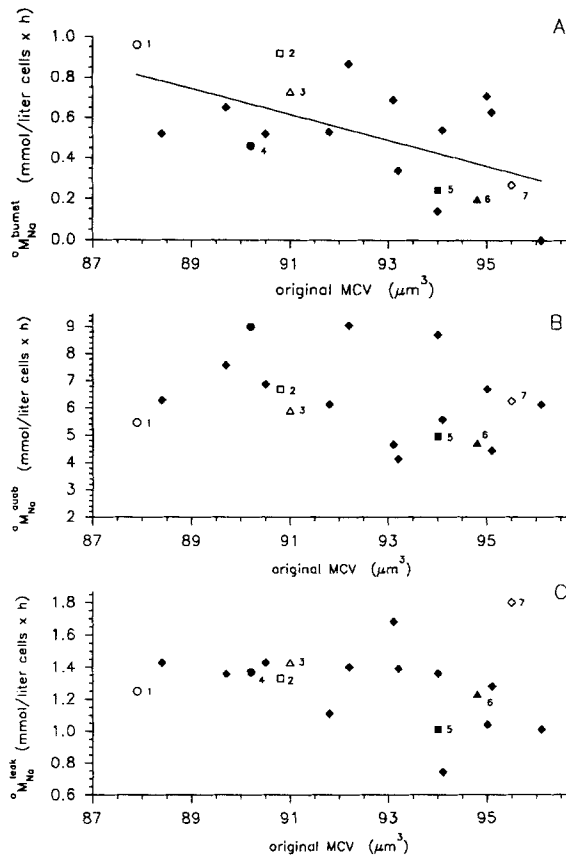

FIGURE 2. The relationship between MCV and (A) $^oM_{Na}^{bumet}$, (B) $^oM_{Na}^{ouab}$, and (C) $^oM_{Na}^{leak}$. Na_i and K_i were modified in order to bring these concentrations to near maximal values for the cotransport system (Canessa et al., 1986) and Na/K pump (Post and Jolly, 1957) using the nystatin technique described in Methods. The modification resulted in values of Na_i of ~48 mmol/liter cells and of K_i of ~52 mmol/liter cells, respectively. The effluxes were measured at 37°C in a medium composed of (mM): 135 NaCl, 5 KCl, 1 MgCl$_2$, 1 NaH$_2$PO$_4$, 5 glucose, and 20 HEPES-Tris, pH 7.4 at 37°C, osmolarity ~290 mosmol/liter. When present, the media also contained 10 μM bumetanide ±50 μM ouabain in order to differentiate the flux components into a bumetanide-sensitive ($^oM_{Na}^{bumet}$) cotransport, a ouabain-sensitive ($^oM_{Na}^{ouab}$) Na/K pump exchange, and a leakage component ($^oM_{Na}^{leak}$) that is insensitive to both ouabain and bumetanide. Each point represents the mean value of triplicate measurements. The same values of MCV on each of the abscissa refer to the same individuals in all panels and are the same as in Fig. 1. Blood from donors numbered 1–6 was used in the studies on fractionated red cells (see below).

relationship between $^oM_{Na}^{bumet}$ and MCV (Fig. 2 *A*) but not between either the Na/K pump (Fig. 2 *B*) or the leak (Fig. 2 *C*). The correlation between $^oM_{Na}^{bumet}$ and MCV was statistically significant ($r = -0.586$; $P \le 0.008$; flux (millimoles per liter cells per hour) $= 6.447 - 0.064 \times$ MCV) but not between $^oM_{Na}^{ouab}$ or $^oM_{Na}^{leak}$ and MCV (MCV vs. Na/K pump: $r = -0.273$, $P \le 0.257$; MCV vs. leak: $r = -0.216$, $P \le 0.375$ (fluxes: millimoles per liter cells per hour). Repeated measurements of cotransport on red cells from the same donors showed a high degree of reproducibility ($\pm 6\%$). These findings are consistent with Duhm and Göbel (1984) and Stewart (1988), who reported an increase in the Na/K/2Cl cotransport flux with decreasing MCV. It should be noted that the results presented in Fig. 2 were obtained on red cells after modification of Na_i and K_i to achieve maximal levels of cotransport, whereas Duhm and Göbel (1984) and Stewart (1988) measured fluxes on unmodified human red cells. Duhm and Göbel also used furosemide as an inhibitor for Na/K/2Cl cotransport, while bumetanide was used in the present study as well as by Stewart (1988).

Note that in Fig. 2 $^oM_{Na}^{bumet}$ is expressed in millimoles per liter of cells. Since the number of cells per liter increases as MCV decreases, it would be more accurate to relate the fluxes to a constant surface area. Because it was not possible to assess the latter parameter, it is an arbitrary choice whether to relate the fluxes to a liter or constant number of cells. It is therefore of interest that when the fluxes were calculated on the basis of cell number (the most stringent basis) the inverse relationship between MCV and $^oM_{Na}^{bumet}$ was still statistically significant although the slope of the regression line was reduced ($r = -0.546$; $P \le 0.016$; flux (millimoles per 10^{13} cells per hour) $= 5.466 - 0.054 \times$ MCV).

To study whether modifying Na_i and K_i with the nystatin technique changes the pump's stoichiometry and to what extent Na/Na or K/K exchange might be involved in these measurements we determined $^oM_{Na}^{ouab}$ in the presence and absence of Na_o and $^iM_{Rb}^{ouab}$ in the presence and absence of K_i (Table II). The ratio of $^oM_{Na}^{ouab}$ to $^iM_{Rb}^{ouab}$ under normal conditions (see legend to Fig. 2) was 1.55 ± 0.11 (mean $\pm$ SD, $n = 11$), which is essentially the same as the coupling ratio found by Post and Jolly (1957). It is also clear from the results shown in Table II that the pump's stoichiometry was not significantly changed by use of the nystatin procedure, nor was there any evidence that Na_i/Na_o or K_i/Rb_o exchange diffusion components were involved in the measurements since the coupling ratios were the same in the presence and absence, respectively, of Na_o and K_i. These results, in representing appropriate controls, underscore the validity of the measurements in Fig. 2 *B* of $^oM_{Na}^{ouab}$ as an indicator of the relative Na/K pump activity of the different bloods studied in this paper.

Characteristics of Fractionated Red Cells

Since differences in MCV among individuals must be based on differences in the population distribution of red cell volumes (shifts in the mean and/or shape of the distribution curve for MCVs), a possible cause for MCV-related individual differences in $^oM_{Na}^{bumet}$ could be the contribution of subpopulations of red cells with markedly different values of $^oM_{Na}^{bumet}$.

To obtain subpopulations of cells with different MCVs, red cells from individuals with high $^oM_{Na}^{bumet}$ and low MCV or low $^oM_{Na}^{bumet}$ and high MCV were separated according to their buoyant density. This provides a way to see what the relationship is

between $^oM_{Na}^{bumet}$ and MCV within the cell population distribution of the different individual bloods, and whether a subpopulation of cells with very high or low values of $^oM_{Na}^{bumet}$ exists. The fractionation of red cells according to density is based on changes in the MCHC at almost constant MCH (Borun et al., 1957; Hoffman, 1958; Murphy et al., 1974; Lee et al., 1984). The increase in MCHC with cell density presumably reflects a loss of solutes (e.g., K_i) and cell water, causing a decrease in

TABLE II

Stoichiometry of the Na/K Pump at V_{max} in Unfractionated Red Cells

	Na/K cells	Na/Chol cells
Na_i, mmol/liter cells	57.9	38.6
K_i, mmol/liter cells	51.6	<0.1
MCHC, g/liter cells	330.0	345.4

	Flux media			
	Na/Rb	Chol/Rb	Na/Rb	Chol/Rb
Na efflux				
Total	5.85	5.79	4.63	4.05
Ouabain	1.68	1.27	1.54	1.42
Ouabain-sensitive	4.17	4.52	3.09	2.63
Rb influx				
Total	3.40	3.57	2.46	2.04
Ouabain	0.59	0.35	0.38	0.23
Ouabain-sensitive	2.81	3.22	2.08	1.81
Na/K stoichiometry	1.48	1.41	1.49	1.45

Ouabain-sensitive Na/K fluxes were measured at V_{max} in cells after modification of Na_i and K_i with the nystatin technique as described in Methods. One set of red cells (Na/K cells) was prepared to contain the indicated concentrations of Na_i and K_i. In a second set internal K was replaced by choline (Na/Chol cells). To prepare Na/Chol cells, the loading step was extended to 6 h and the osmolarity of the loading medium was raised to 380 mosmol/liter with choline-Cl to promote better loading with choline and to obtain cells with normal MCV after washing with flux media. The flux media contained (mM): 135 NaCl (Na/Rb medium) or 135 choline-Cl (Chol/Rb medium), 5 RbCl, 1 NaH_2PH_4, 1 $MgCl_2$, 5 glucose, and 20 HEPES-Tris, pH 7.4 at 37°C. The concentration of ouabain, when present, was 50 μM. Results from one of two experiments with similar findings are shown (subject 2 in Fig. 2). Ouabain-sensitive Na efflux and Rb influx were measured on each set of red cells as part of the same experiment. We do not know the basis for the variations in the Na/K pump rates between the two types of cell preparations nor the extent to which the variations depend on the external conditions. The variation coefficient of triplicate measurements was 1.5 ± 1.8%. Fluxes are given in millimoles per liter cells per hour; the stoichiometry of the pump is calculated as the ratio between ouabain-sensitive Na efflux and Rb influx.

MCV with increasing cell density (Chalfin, 1956; Hoffman, 1958; Bernstein, 1959; Haidas et al., 1971; Astrup, 1974; Lee et al., 1984), but the mechanisms underlying these changes are not known at present. When the same kind of studies as discussed in connection with Figs. 1 and 2 were carried out in fractionated populations of red cells from the group of selected individuals whose whole unfractionated red cell population had already been characterized (numbered symbols in Figs. 1 and 2), the

results also show a direct relationship between MCV and K$_i$ (Fig. 3 *A*) and an inverse relationship between MCV and Na$_i$ (Fig. 3 *B*) and MCHC (Fig. 3 *C*). The regression statistics for these results are presented in Table I. A direct relationship was also found between MCV and Na + K$_i$ (r = 0.725; $P \leq$ 0.00001; Na$_i$ + K$_i$ [in millimoles per liter cells] = 27.43 + 0.770 × MCV; data not shown). In contrast to Lee et al. (1984), we did not see any significant change (increase) in MCH with increasing cell density (data not shown).

Additional parameters (Table III) were determined in density-fractionated samples of red cells taken from six other individuals different from those referred to in Fig. 3.
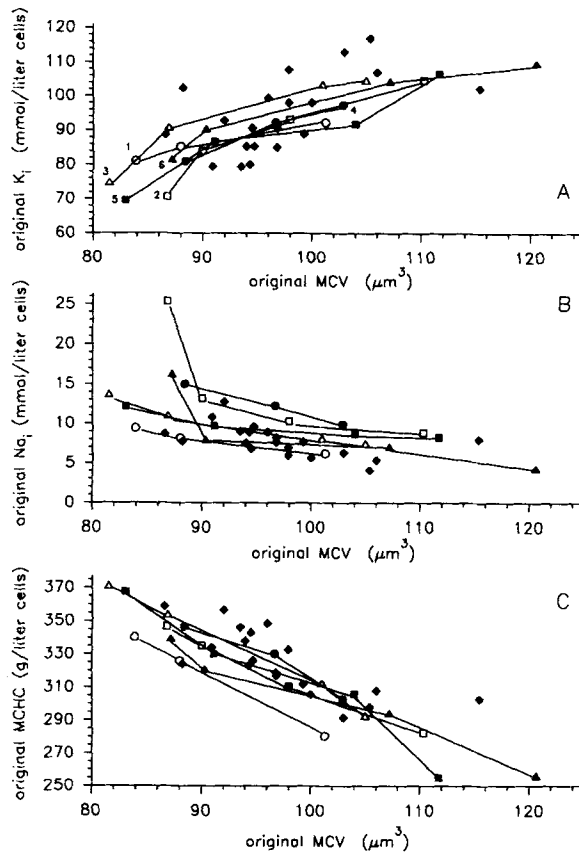

FIGURE 3. The relationship between MCV and (*A*) K$_i$, (*B*) Na$_i$, and (*C*) MCHC in unmodified red cells after fractionation by density gradient centrifugation (see Methods). The same values of MCV on the abscissa refer to the same samples of fractionated cells in all panels. The symbols connected by lines represent results from blood samples of donors numbered 1–6 shown in Figs. 1 and 2. Note that the numbered bloods were separated into either three or four fractions. The filled diamonds are samples from other bloods treated similarly to the numbered bloods. Regression statistics are presented in Table I. See text for discussion.

Each person's red cells were separated into three fractions with different buoyant density. The cells in the top fraction represented 12% of the total population. The middle fraction contained 73% of all cells, and the bottom fraction contained the cells with the highest density, which amounted to an average of 15% of the whole population. Fractional cell water was higher in the cells with the lowest buoyant density than in the most dense cells. Note the trend that chloride ratios were highest in low density red cells and decreased with increased density. This would imply a slight acidification of the cytoplasm, calculated from r_{Cl} (pH$_{i,calc}$), with increasing cell

density, which was found by Murphy et al. (1974) and Schmidt et al. (1987) but which was not confirmed by direct pH_i measurements (at an external pH of 7.4 in the absence of HCO_3) as indicated in Table III.

On the basis that unfractionated red cells differed in $^oM_{Na}^{bumet}$ and MCV, while the pump and leak fluxes were similar (see Fig. 2), we studied the same donors as characterized in Fig. 3 (numbered 1–6 in Fig. 1) to see to what extent the inverse correlation between MCV and cotransport would also hold within the fractionated population of cells of individual donors. The cells were modified in order to carry out the flux measurements at optimal levels for Na_i and K_i as before. It is evident in Fig. 4A that $^oM_{Na}^{bumet}$ was inversely related to MCV, mirroring the results found in unfractionated red cells (Fig. 2A). It can also be seen in the subjects whose cotransport rates were high in unfractionated red cells that they were also elevated in all subpopulations with varying MCV. The correlation between cotransport and MCV for the fractionated cells was stronger ($r = -0.730$; $P \leq 0.0001$; flux [millimoles per liter cells per hour] = 2.594 − 0.020 × MCV) than that seen for the unfractionated

TABLE III

Characteristics of Unmodified, Fractionated Red Cells

	Top	Middle	Bottom	p values
Cumulative percentage	0–12	12–85	85–100	
Percentage cell water	0.677 ± 0.017	0.652 ± 0.008	0.635 ± 0.006	0.002
r_{Cl}	0.779 ± 0.021	0.746 ± 0.034	0.717 ± 0.037	0.057
$pH_{i,calc}$	7.291 ± 0.014	7.278 ± 0.020	7.263 ± 0.020	0.057
$pH_{i,meas}$	7.263 ± 0.048	7.244 ± 0.030	7.281 ± 0.029	0.239
Mg_i^{tot}, mmol/liter cells	2.31 ± 0.24	2.48 ± 0.25	2.73 ± 0.15	0.057

Whole populations of unmodified red cells were fractionated by density gradient centrifugation as described in Methods and represent the results on blood samples taken from six different individuals. The cumulative percentage of cells refers to the proportion of the total cell population in each of the fractions taken from the gradient beginning from the top. In these cells original cell water, r_{Cl}, as well as $pH_{i,calc}$ (red cell pH calculated from r_{Cl} at an external pH of 7.4) measured pH_i, and Mg_i^{tot} were determined. The results represent means ± SD. The p values were calculated by analysis of variance.

cell population (Fig. 2A). Calculation of the cotransport fluxes measured in fractionated red cells based on a constant cell number (10^{13}) also showed a statistically significant correlation although the slope of the regression line was reduced ($r = -0.629$; $P \leq 0.0017$; flux [millimoles per 10^{13} cells] = 1.893 − 0.013 × MCV). These results are similar to those reported by Chipperfield and Mangat (1986) and Duhm (1987), but differ from those of Canessa et al. (1987), who found no consistent differences in bumetanide-sensitive K efflux between fractions of red cells with different density (and presumably MCVs) from donors with reticulocytosis. The basis for this reported difference with the present results is not clear. Nevertheless it is clear that the inverse relation between cotransport and MCV of the unfractionated populations of red cells (Fig. 2A) can at least be qualitatively accounted for by the findings on the subpopulations of cells (Fig. 4A), even though quantitatively the differences in cotransport for the bulk of the cell populations and MCV (ranging approximately between 88 and 110 μm^3) appear too small.

It should be noted in Fig. 4B that in fractionated red cells there was a direct

correlation of $^oM_{Na}^{ouab}$ with MCV, which was statistically significant ($r = 0.871$; $P \leq 0.00001$; flux [millimoles per liter cells per hour] $= 12.62 + 0.205 \times$ MCV). This might be explained by a cell density–related decline in pump activity (Furukawa et al., 1981; Blostein et al., 1983). In addition, as shown in Fig. 4 C, $^oM_{Na}^{leak}$ was higher in cells with high MCV ($r = 0.752$; $P \leq 0.00006$; flux [millimoles per liter cells] $= -1.349 + 0.029 \times$ MCV). It should be mentioned that the correlations between the Na/K pump and the leak fluxes with MCV become even stronger when the flux calculations are based on a constant cell number (not shown).
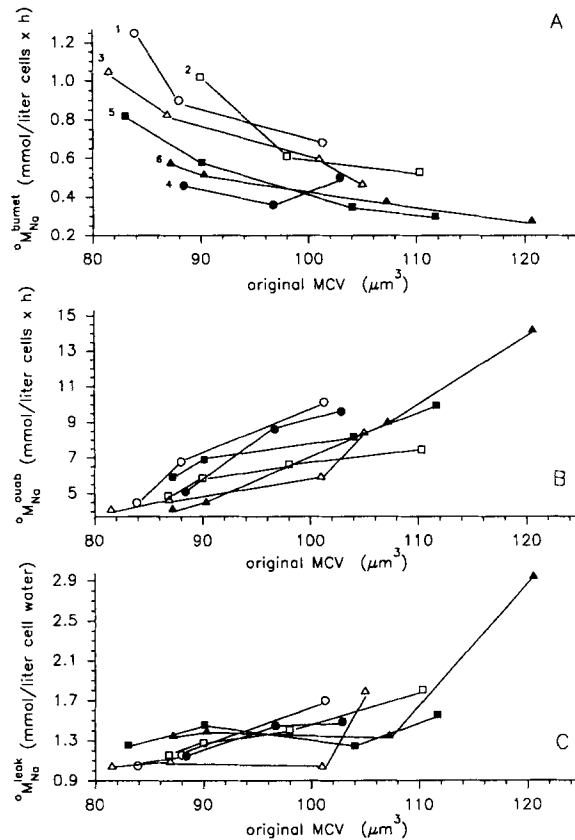

FIGURE 4. The relationship between MCV and (A) $^oM_{Na}^{bumet}$, (B) $^oM_{Na}^{ouab}$, and (C) $^oM_{Na}^{leak}$ in red cells modified after density fractionation. Red cells from donors numbered 1–6 (see Figs. 1–3) were separated according to density as described in Methods. The modification of Na_i and K_i as well as flux measurements were carried out as described in the legend to Fig. 2. Each point represents the mean value of triplicate measurements. The same value of MCV on the abscissa as well as the same symbols refer to the same samples of fractionated cells in all three panels of this figure. The symbols for blood samples correspond to samples of individuals numbered 1–6 shown in the Figs. 1 and 2.

Alteration of Mg_i

We were interested to measure Mg_i in red cell populations of varying MCV in order to determine the relationship between Mg_i and $^oM_{Na}^{bumet}$ because it has been previously reported that cotransport was sensitive to the concentration of Mg_i (Ellory et al., 1983; Flatman, 1988; Starke and McManus, 1988; Haas, 1989; Starke, 1989). The average Mg_i^{tot} concentration (measured in 9 of the 19 individuals referred to in Fig. 1) was 2.30 ± 0.12 mmol/liter cells (ranging from 2.08 to 2.63 mM Mg_i^{tot}/liter cells) with a trend ($r = -0.290$) for a small increase in Mg_i^{tot} with decreasing MCV in this

group of subjects. This trend is presumably reflected in fractionated red cells, since, as shown in Table III, Mg_i^{tot} was found to be lowest in the largest cells (top fraction) and highest in the smallest cells (bottom fraction). Because it was also known (Haidas et al., 1971; Schmidt et al., 1987; Mairbäurl et al., 1990) that 2,3-DPG, and to a lesser extent ATP, decreased with increasing cell density, it was important to know the relationship between the concentration of 2,3-DPG and MCV in whole cell populations. As shown in Fig. 5 A, a direct correlation between these two parameters was
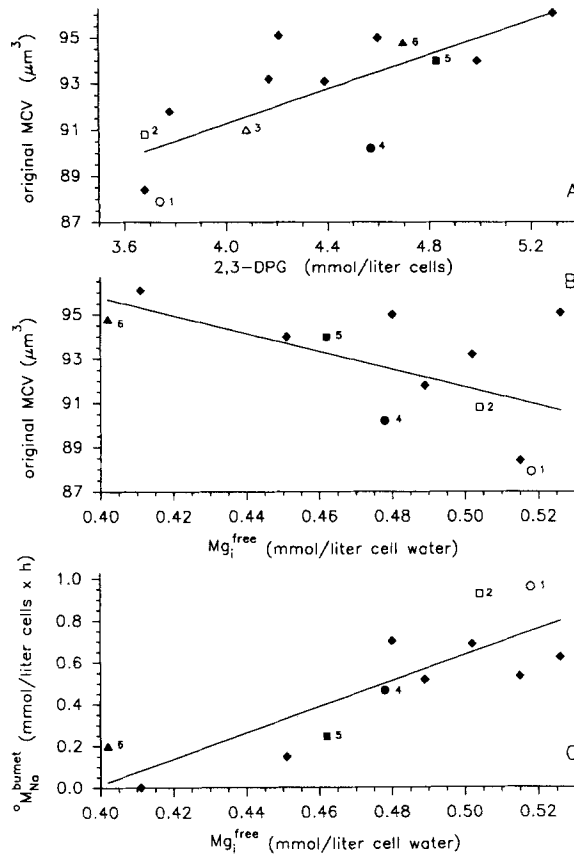

FIGURE 5. The relationship between MCV and (A) 2,3-DPG from unmodified red cells from blood samples of 13 different donors including those numbered 1–6 shown in Figs. 1–4. The indicated values of 2,3-DPG and the measured values of Mg_i^{tot} (data not shown) were used to estimate the values of Mg_i^{free} shown in B and C. In three separate experiments each, Mg_i^{tot} in individuals 1 and 6 was 2.58 ± 0.11 and 2.12 ± 0.13 mmol/liter cells, respectively. Binding constants (see Methods) and the methods reported by Gerber et al. (1973), Gupta et al. (1978), and Chiancone et al. (1972) were used for the calculations of Mg_i^{free}. These calculations were also based on assumed concentrations of 1.5 mmol ATP/liter cell water (the population mean in our subjects was 1.52 ± 0.15 mmol/liter cell water) and 105 mmol Cl_i/liter cell water and full saturation of hemoglobin with oxygen. B presents the relationship between this calculated Mg_i^{free} and MCV. In C the relation between the calculated Mg_i^{free} and measured $^oM_{Na}^{bumet}$ is shown. The cotransport and MCV values used are the same as presented in Figs. 1 C and 2 A for corresponding samples.

found ($r = 0.762$; $P \le 0.0018$; MCV $= 76.10 + 3.775 \times 2,3$-DPG [millimoles per liter cells]).

Since, as already noted, Mg_i^{tot} was found to vary only slightly with MCV among the different individual bloods, it could be inferred from the latter result that Mg_i^{free} could be expected to vary inversely with the concentration of 2,3-DPG. Therefore Mg_i^{free} was calculated as described in Methods. These computations were carried out using the measured values of Mg_i^{tot}, 2,3-DPG, and Hb assuming, respectively, a

concentration (per liter of cell water) of 105 mM Cl_i and 1.5 mM ATP together with full oxygen saturation of hemoglobin. (The variation of the cellular concentration of ATP for the individuals studied (i.e., Fig. 1) was 1.52 ± 0.15 (SD), ranging from 1.2 to 1.67 mmol/liter cell water). The results of these calculations are shown in Fig. 5 *B*. It is clear that there is an inverse correlation of Mg_i^{free} with MCV ($r = 0.604$, $P \leq 0.015$, MCV $= 111.26 - 41.158 \times Mg_i^{free}$). When the measured values of $^oM_{Na}^{bumet}$ are then replotted as a function of Mg_i^{free} (Fig. 5 *C*) it becomes clear that a direct correlation between both parameters exists ($r = 0.829$, $P \leq 0.005$, $^oM_{Na}^{bumet} = -2.496 + 6.275 \times Mg_i^{free}$). Because of these findings and the reports on activation of $^oM_{Na}^{bumet}$ by Mg_i in red cells from ferrets (Flatman, 1988) and Pekin ducks (Starke and

TABLE IV

Alteration of Red Cell Mg_i by Varying Mg_o in the Presence of A23187

Mg_o	Mg_o^{A23}		r_{Cl}		Mg_i^{free}	
	1	6	1	6	1	6
0.0*	<.01[‡]	<0.1[‡]	0.781	0.769	—	—
0.10	0.15	0.15	0.725	0.724	0.29	0.28
0.20	0.25	0.25	0.723	0.723	0.48	0.48
0.50	0.54	0.53	0.727	0.736	1.02	0.98
1.50	1.50	1.55	0.728	0.748	2.83	2.77

The relationship between medium and cellular Mg (Mg_o^{A23} and Mg_i, respectively) was determined after incubation of modified red cells for 15 min in a shaking water bath at 37°C at a Hct of 6% in the presence of the indicated concentrations of Mg_o and 5 µM A23187. The nystatin technique was used to modify Na_i and K_i (each to ~50 mmol/liter cells) as described in Methods. The cellular free Mg (Mg_i^{free}) was estimated from the measured values of Mg_o^{A23}, after incubation with A23187 and r_{Cl} according to the procedure described by Flatman and Lew (1980). The results of two experiments on bloods 1 and 6 are shown. These measurements were carried out as part of the same experiments on these bloods as presented in Fig. 6. The concentration of Mg_o and Mg_o^{A23} is given in millimolar and that of Mg_i^{free} in millimoles per liter cell water. All determinations were performed in duplicate.

*Nominally Mg_o-free medium containing 5 mM EDTA; at this EDTA concentration the free Mg_o concentration is estimated to be <1 µM (see Yingst and Hoffman, 1983). 10 µM EDTA was present in all Mg_o-containing media. [‡]This is the estimated value of free Mg in the medium in the presence of A23187 at 5 mM EDTA.

McManus, 1988; Starke, 1989) it was of interest to test directly the effects of varying Mg_i^{free} on cotransport under conditions when Na_i and K_i had been optimized as before.

The red cell concentration of Mg_i^{free} was changed by varying Mg_o in the presence of the ionophore A23187 (Reed, 1976; Yingst and Hoffman, 1978; Flatman and Lew, 1977, 1980). The result of two such experiments are shown in Table IV. The values of Mg_i^{free} were estimated (see Methods) to vary from nominally 0 to 1.5 mmol/liter cell water. When $^oM_{Na}^{bumet}$ was measured in cells treated in this way, with A23187 present in the flux medium, it was evident in the experiments shown in Fig. 6 that cotransport increases with increasing Mg_o (and therefore Mg_i), reaching saturation at ~1 mM. The small amount of cotransport that persists at zero Mg_o in the presence of 5 mM
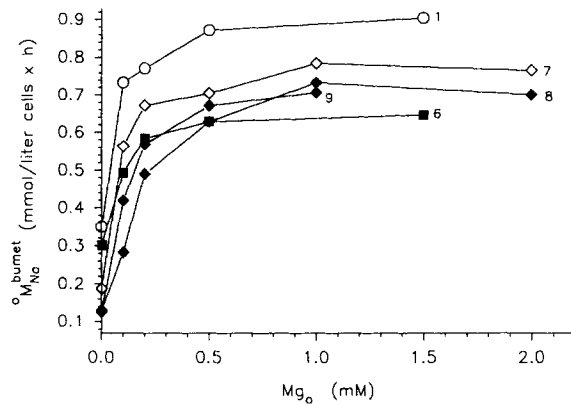
FIGURE 6. The effect of varying Mg_i on cotransport activity. Before varying Mg_i, both Na_i and K_i were modified as described in the legend to Fig. 2 to ~50 mmol/liter cells each with the nystatin technique. Na effluxes were measured as the bumetanide-sensitive component ($^oM_{Na}^{bumet}$) in media composed of (mM): 135 NaCl, 5 KCl, 5 glucose, 1 NaH_2PO_4, 20 HEPES-Tris, pH 7.4 at 37°C, and 5 μM A23187 (from a stock of 2 mM in ethanol). Mg_o in the medium was varied as indicated with choline-Cl added to keep the medium osmolality constant. Variations in Mg_i were thus brought about by varying Mg_o in the presence of A23187. 5 mM EDTA was added to the nominally Mg_o-free medium in the presence of A23187 to deplete the cells of Mg_i. The data points represent the mean values of triplicate flux measurements on red cells from five different donors, including donors 1, 6, and 7 in Figs. 1 and 2. The table below shows, on the left-hand side, the cotransport rates ($^oM_{Na}^{bumet}$) and MCVs for the different bloods before modification of Mg_i with the A23187 maneuver. Therefore these cells contained their original Mg_i^{tot}. The right-hand side of the table shows estimates by three different methods of values for Mg_i^{free} that presumably exist in the cells at their original Mg_i^{tot}. Methods A and C both depend on varying Mg_i by varying Mg_o as depicted in the graph together with measurements of the Donnan ratio for Cl as well as Mg_i^{tot} at each Mg_o concentration used. By plotting either the $^oM_{Na}^{bumet}$ flux or the Mg_i^{tot} (data not shown) against the calculated values of Mg_i^{free} (Flatman and Lew, 1980) the approximate estimates of Mg_i^{free} could be interpolated for the original values of either $^oM_{Na}^{bumet}$ (method A) or Mg_i^{tot} (method C), respectively. In method B, Mg_i^{free} was calculated from the original Mg_i^{tot}, total 2,3-DPG, ATP, hemoglobin, and cell chloride by an algorithm that utilizes appropriate complexation constants (see Methods and Fig. 5).

| Person | Values at original Mg_i^{total} | | Mg_i^{free} | | |
	$^oM_{Na}^{bumet}$	MCV	A	B	C
			mmol/liter$_{cw}$		
1	0.76	89.1	0.40	0.39	0.67
6	0.43	93.0	0.24	0.19	0.46
7	0.27	95.5	0.09	—	—
8	0.43	91.8	0.35	—	—
9	0.54	90.7	0.37	—	—

EDTA is presumably due to the residual Mg_i^{tot} that remains after treatment. These results indicate that the cotransport rate can, at optimal levels of Na_i and K_i, be further increased by increasing Mg_i. In addition, it should be pointed out that the range of variation in $^oM_{Na}^{bumet}$ noted before among red cells from the different donors (i.e., Fig. 2 A) becomes considerably reduced after maximizing Mg_i (Fig. 6). Note that the results in Fig. 6 include measurements in red cells from donors 1, 6, and 7, which represent the extremes in Fig. 2 A, together with donors 8 and 9 (not included in Fig.

2 A) whose values for $^oM_{Na}^{bumet}$ and MCV (before modifications of Mg$_i$) are given in the table in the legend to Fig. 6. (It should be stressed that all of the results presented in Fig. 6 are from experiments carried out 18 mo after those depicted in Fig. 2 A, which could be responsible for the small differences in the values of $^oM_{Na}^{bumet}$ and MCV for donors 1 and 6). Thus the variation in Mg_i^{free} that underlies the variation in $^oM_{Na}^{bumet}$ noted before in Fig. 2 A evidently occurs below saturation and in the steep portion of the curve as altered by changes in Mg$_o$ as seen in Fig. 6. This is discussed further below.

Another possible implication of the differences in red cell 2,3-DPG concentration noted in Fig. 5 A is the resulting consequence of 2,3-DPG's effect on the Donnan equilibrium (Duhm, 1971) with regard to differences in pH$_i$. This could be important since Duhm (1987) also found that the cotransport V_{max} was sensitive to changes in pH, decreasing by a factor of about two from pH 8.0 to pH 6.7. However, we did not observe any significant correlation between MCV and pH$_i$ or r_{Cl} for different whole blood cell populations with different mean concentrations of 2,3-DPG when washed and suspended at equilibrium at pH$_o$ 7.40 in a buffered (HEPES-Tris) medium. Additionally, the results on fractionated red cells presented in Table III also show no statistically significant difference in pH$_i$ among unmodified cells of different density despite a small decrease in the r_{Cl} with increasing cell density. Furthermore, we found that $^oM_{Na}^{bumet}$ under our standard conditions was not changed significantly (data not presented) by altering pH$_i$ within the range (7.24–7.29) shown in Table III. Thus the results taken together minimize the role of pH$_i$ as a factor contributing to an inverse relationship between MCV and $^oM_{Na}^{bumet}$.

DISCUSSION

The two most important results of the work presented in this paper are, first, the inverse correlation between the original MCVs of different individuals' red blood cells and the cotransport rate as shown in Fig. 2 A, and second, the crucial role that Mg_i^{free} plays in this correlation as shown in Fig. 5 C. The former result implies that the cotransport system is an important contributor to the in vivo steady-state cell volume, whereas the latter indicates the basis for a possible regulatory mechanism. In terms of precautionary measures that were followed throughout these studies, it should be understood that although the cells were modified to increase Na$_i$ and K$_i$ with regard to maximizing the cotransport rate (see legend to Fig. 2), the experimental protocol was carried out in a manner that preserved, as closely as possible, the MCV of the original red cells characteristic of the population at the time of blood withdrawal. This emphasizes the fact that we were focused on measuring cotransport rate under circumstances where cell volume per se was minimally perturbed, if at all, in order to avoid alterations in cotransport rates (see below) due to cell swelling (inhibition) or cell shrinkage (stimulation). This of course also applies to cells treated with nystatin and/or A23187. In addition, there were no measurable changes in cell volume that took place during the time course of the flux determinations, even in the presence of ouabain, presumably because the outward leakage of K$_i$ would act to offset any inward movement of Na given that the ratio P_K/P_{Na} is > 1 (see Tosteson and Hoffman, 1960). It should also be noted that, since the cotransport rates are sensitive to the cellular concentrations of 2,3-DPG as well as Mg_i^{tot}, these, together

with ATP, were not altered by application of the nystatin technique (data not shown). And because Mg_i^{free} varies inversely with the degree of hemoglobin oxygenation (Bunn et al., 1971), all flux determinations were carried out where the saturation of hemoglobin was >95%. (The measured Po_2 was always >120 torr.)

The inverse correlation between the activity of the cotransport system and MCV, as presented in Fig. 2 A, confirms previous such relationships as reported by Duhm and Göbel (1984) and Stewart (1988) for whole populations of unmodified human red cells as well as for subpopulations of normal cells (cf. Fig. 4 A) as found by Chipperfield and Mangat (1986) and Duhm (1987). Because these various studies utilized normal (unmodified) red cells it was not clear to what extent the differences in the cotransport rates were due to inherent differences and/or possible alterations in the steady-state values of Na_i and MCVs before or during the course of the various measurements. It was because of these uncertainties that we chose to compare the cotransport rates of different batches of red cells at their maximal values of Na_i and K_i (which also acted to normalize any prior differences in the chemical gradients for Na and K) while preserving their respective original MCVs. Whether or not Duhm and Göbel (1984), for instance, would have obtained the same relationships had they measured bumetanide-sensitive Na efflux rather than furosemide-sensitive Rb uptake (which, by the way, correlated inversely with K_i) cannot be answered without a direct assessment. We also felt that if the interindividual differences in MCVs (range of variation ~7–8%) were in fact due to differences in cotransport rates (which varied four to fivefold) they would best be compared on a background when the magnitudes of the various cells' Na/K pump activities (assessed at V_{max}) and electrodiffusion leaks were similar. This was reasonably well achieved in the former instance, at least among the numbered bloods (Fig. 2 B, where the range of variation in activity was ~13%) and to a lesser extent with the latter (Fig. 2 C). Unfortunately, the leak permeabilities of nystatin-treated red cells are variable, being several-fold higher than their untreated counterparts (our unpublished results). Thus the above considerations set the rationale and experimental bases for the selection and characterization of the different individuals and parameters as studied in this paper. Nevertheless it is also clear that, since the same qualitative inverse correlation between cotransport rates and MCVs were observed both in this and in the previously mentioned studies that were carried out under less stringent conditions, other factors that modulate cell volume must be involved. The fact that the activity of the cotransport system had been found to be sensitive to variations in Mg_i (Flatman, 1988; Haas, 1989) promoted a detailed examination of this cofactor.

In considering the critical role of Mg_i, it was clear that its importance emerged from a convergence of correlations that resulted in focusing on the interrelationship between the interindividual variations in MCV (range, 88–96 μm^3) and 2,3-DPG content (range, 3.6–5.3 mmol/liter cells), as presented in Fig. 5 A. Since the various cell populations contained approximately the same concentrations of Mg_i^{tot} and ATP, the variation in 2,3-DPG content necessarily results in a variation in Mg_i^{free}, as depicted in Fig. 5 B. Fig. 5 C highlights the important correlation between Mg_i^{free} and the cotransport rate. Thus cells with small MCVs have low concentrations of 2,3-DPG but high cotransport rates due to high Mg_i^{free}, the converse applying to cells with large MCVs. Maximation of Mg_i^{tot}, when Na_i and K_i were already optimized,

minimizes the interindividual variability of the cotransport rates, as shown in Fig. 6. This is evident from a comparison in Fig. 6 of the different individual maximal cotransport rates ($^oM_{Na}^{bumet}$) achieved after modification of Mg_i^{tot} (graph) with their respective cotransport rates before modification of Mg_i^{tot} (table in Fig. 6, legend). It should be noted that the same inverse correlation between cotransport rate and MCV depicted in Fig. 2 *A* is upheld with the data presented in the inset table. Thus these results emphasize that the differences in the cotransport rates, displayed by different populations of red cells with different MCVs, are dependent on their modulation rather than their total cotransport capacity (number of transporters per cell). The modulatory role of Mg_i is tracked by differences in Mg_i^{free}, which in turn is defined by the relative cellular content of 2,3-DPG, as previously discussed. Given this indicator role of Mg_i^{free}, we have estimated by three different methods (A, B, and C) this parameter regarding the results presented in Fig. 6 as seen in the table legend. Methods A and C are similar (see legend) since they both depend on an interpolated value of Mg_i^{free} that obtained when either the original cotransport flux (method A) or the Mg_i^{tot} (method C) was measured; method B presents computed values based on the cells' original contents of Mg_i^{tot}, ATP, 2,3-DPG, and Cl_i. Even though these data are limited, there is a clear indication that all three methods yield results that show that a high cotransport rate is associated with a high value for Mg_i^{free}, as in Fig. 5 *C*. On the other hand, more information is needed to decide what the actual Mg_i^{free} values may be, or what the basis is for the discrepancy in the values estimated by methods A and C.

From the foregoing discussion it would appear that Mg_i^{free} is a more important determinant of the relative cotransport rate than Na_i. This would provide a rational explanation for the fact that the same correlation between cotransport rate and MCV that was observed in cells unmodified with regard to their content of Na, K_i, or Mg_i (e.g., Duhm and Göbel, 1984) is the same as that seen in the present work (Fig. 2 *A*) where Na_i was maximized. Consistent with this inference, we found that unmodified cells from donors 1 and 5 contained, at the time of the flux measurement, 11.4 and 8.4 mmol Na/liter cells, respectively (cf. Fig. 1 *C*), and that the cotransport rates ($^oM_{Na}^{bumet}$) were 0.71 and 0.25 mmol/liter cells per h, respectively (cf. Fig. 2 *A*, where Na_i is set at its maximal value). Thus, while it is clear that the cotransport rate is sensitive to the level of Na_i (see also Canessa et al., 1986), the control by 2,3-DPG of the availability of Mg_i to the cotransport system would appear, on a relative basis, to be the more critical variable, as mentioned before.

Given the key role exerted by Mg_i in modulating the steady-state cotransport rate, it is important to ask not only what membrane mechanisms regulate Mg_i^{tot} but also what glycolytic mechanisms regulate the cellular level of 2,3-DPG. While Mg transport systems have only recently begun to be analyzed (see review by Flatman, 1991) it appears, at least in human red cells, that Mg is transported across the membrane by two different mechanisms. One mechanism is by an ATP-dependent Mg_i/Na_o exchanger and the other by passive diffusion (Feray and Garay, 1986; Lüdi and Schatzmann, 1987; Frenkel et al., 1989). The maximal rate for Mg_i efflux (measured in the unsteady state) is ~0.2 mmol/liter cells per h, with the concentrations of Mg_i^{free} and Na_o giving half-maximal rates being ~1 and 8 mM, respectively (Lüdi and Schatzmann, 1987; Frenkel et al., 1989). The evidence indicates that Mg

can only be moved in the outward direction since either increasing $Mg_o > Mg_i$ or reversing the Na gradient does not result in a detectable net increase in Mg_i (Lüdi and Schatzmann, 1987). Thus it is not clear what mechanisms exist to control the steady-state level of Mg_i^{tot} (or Mg_i^{free}) or how, if applicable, the Mg_i/Na_o transporter senses Mg_i. This is an important issue because we have no explanation for the maintenance of the steady-state variation in Mg_i^{free} as we noted above. Thus if changes in the levels of 2,3-DPG can vary the distribution of bound Mg_i in the different bloods as indicated by variations in Mg_i^{free} (Fig. 5 B), what is the mechanism that maintains Mg_i^{tot} in the different bloods approximately constant? This problem is additionally emphasized by our results on fractionated cells where an increased Na_i (Fig. 3 B) is associated with a decreased level of Mg_i^{tot} (Table III). Whether or not in vivo differences in the average degree of oxygenation obtain among the bloods from the various donors is not known, but this possibility offers another approach to understanding differences in the modulation of Mg_i^{free}.

The type of variability in 2,3-DPG content seen among our various donors has also been noted before by others (e.g., Brewer, 1974). But the metabolic basis for this variability in normal individuals, all living essentially at sea level, is not at all known. One possibility is that there are genetic differences in the activities of the 2,3-DPG mutase/phosphatase activities that result in differences in the pool size of 2,3-DPG (Gilroy et al., 1980, Yu et al., 1990). Another (see Rose and Warms, 1970; Rose, 1971) is that the level of 2,3-DPG is a reflection of the relative activities of the enzymes, phosphoglycerate kinase and pyruvate kinase (PK), that, in controlling the concentration of 1,3-DPG, necessarily control the concentration of 2,3-DPG (see also Inoue et al., 1987). But this explanation also depends on genetic variability in the relative activities of various glycolytic enzymes, studies that have yet to be carried out in the context of variation in 2,3-DPG content in normal individuals. On the other hand, high levels of 2,3-DPG have been reported in red cells that are genetically deficient in PK (Bowdler and Prankerd, 1964).

Another question to be addressed concerns the mechanism by which Mg_i (as indexed by Mg_i^{free}) determines the relative cotransport rate. We have extended our previously mentioned results (Fig. 6), where the activity of the cotransport system could be increased by increasing Mg_i^{free}, by evaluating the effects of okadaic acid, a protein phosphatase inhibitor (Cohen et al., 1990), and other maneuvers. We found, in a separate study (Mairbäurl and Hoffman, 1991) in cells at their normal MCV containing their original Mg_i^{free}, that okadaic acid stimulates the cotransport system to operate at a rate that exceeds the rate that was seen when Mg_i was maximally raised, as in the experiments characterized in Fig. 6. This new cotransport rate presumably represents a true maximum since increasing Mg_i over its original level does not result in a further increase in rate in the presence of okadaic acid. In addition, pretreatment of the red cells with the protein kinase inhibitor K-252a (Kase et al., 1986) can reduce or completely prevent cotransport in cells at their normal MCV. These results strongly suggest that the mechanism that regulates the cotransport activity (and also senses cell volume and/or changes in shape; see below) may involve a protein phosphatase that dephosphorylates serine and/or threonine residues (Lytle and Forbush, 1990; Pewitt et al., 1990). Thus Mg_i^{free}, in defining the relative steady-state phosphorylated level of the cotransport system, would in turn

determine the extent to which the steady-state cell volume is modulated by the relative cotransport rate, as depicted in Fig. 5 *C*. This of course raises the question of how the kinase/phosphatase system is positioned in the membrane relative to the cotransporter protein. A similar type of kinase/phosphatase cycle has been previously invoked to underlie swelling-dependent KCl cotransport in human (Kaji and Tsukitani, 1991) and rabbit (Jennings and Schulz, 1991) red cells and may be involved in shrinkage-activated Na/proton exchange in dog red blood cells (Parker et al., 1990). We have also evaluated (Mairbäurl and Hoffman, 1991) effects of alterations in Mg_i and in the kinase/phosphatase cycle on osmotically induced cell volume changes on the cotransport activity in human red cells (a maneuver known to change the cotransport rate of a variety of cells [see Kregenow, 1981; Siebens, 1985; Hoffmann and Simonsen, 1989]). Thus the cotransport rate of cells at their normal cell volume, which had been optimized by changes in Na_i, K_i, and Mg_i, could be further increased by cell shrinkage. On the other hand, the okadaic acid–stimulated increase in the cotransport rate of normal cells could not be further increased by cell shrinkage. In addition, pretreatment of the cells with K-252a inhibits cotransport in shrunken as well as in normal cells (see Mairbäurl and Hoffman, 1991).

Lastly, it is instructive to consider how the cotransport system might be involved in the modulation of cell volume. We assume, of course, that the sum Na_i + K_i, in defining the relative content of cellular water, also defines the mean cell volume of a population of cells (Fig. 1 *A*; Tosteson and Hoffman, 1960; Funder and Wieth, 1966*b*; Lee et al., 1984). Therefore, for cells in the steady state, where the sum Na_i + K_i is constant with time, it is possible to calculate the net driving force, $\Delta\mu_{net}$, for cotransport from the chemical potential gradients of Na,K, and Cl that exist across the cell membrane, using the formula:

$$\Delta\mu_{net} = RT \cdot \ln \frac{[Na_0][K_0][Cl_0^2]}{[Na_i][K_i][Cl_i^2]}$$

as previously proposed (Schmidt and McManus, 1977; see also Haas et al., 1982, and Duhm and Göbel, 1984). The calculation assumes a cotransport stoichiometry of 1 Na/1K/2Cl as found for human red cells by Garay et al. (1981) and Kracke et al. (1988) in line with other cell types (Haas, 1989), but it should be kept in mind that stoichiometries different from this have also been reported for human red cells (e.g., Brugnara et al., 1986; Parker and Dunham, 1989). The calculation of $\Delta\mu_{net}$ in Fig. 7 *A* for each donor's red cells (unmodified and unfractionated) is based on the data presented in Fig. 1 together with the donor's corresponding measured values of Na_0, K_0, Cl_i, and Cl_0 (data not shown). In addition, the averaged values of unmodified but fractionated cells, taken from Fig. 3, are represented by the open inverted triangles and connected by the solid line in Fig. 7, *A* and *B*. Fig. 7 shows the relationship between the net driving force and, respectively, the original Na_i (Fig. 7 *A*) and MCV (Fig. 7 *B*). The direction of the net driving force is inward (positive) above and outward (negative) below the horizontal dashed line in both figures. It is clear that there is a shift in the direction of $\Delta\mu_{net}$, being inward at low and outward at high steady-state values of Na_i (Fig. 7 *A*), whether or not the cells were fractionated. When the values of $\Delta\mu_{net}$ for each blood are plotted (Fig. 7 *B*) against their respective values
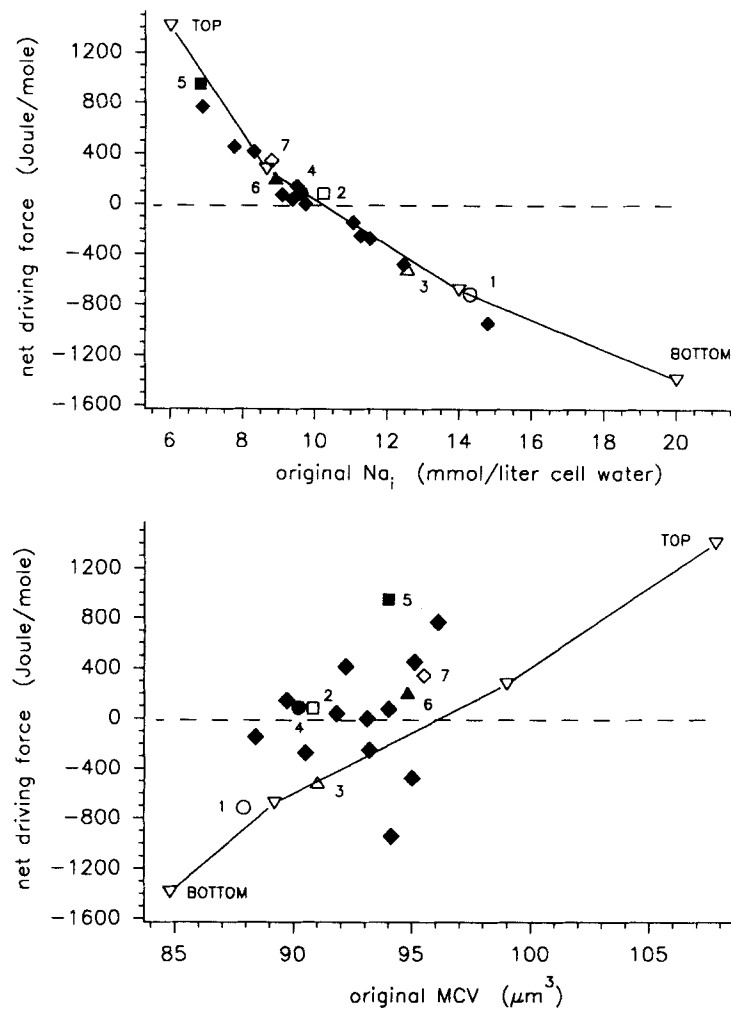
FIGURE 7.   Net chemical driving force ($\Delta\mu_{net}$) in joules per mole as a function of (A) Na_i and (B) MCV calculated according to Haas et al. (1982) and Duhm and Göbel (1984). The values of the driving force were calculated using individual Na_o, K_o, and Cl_o concentrations (data not shown) and a constant chloride ratio (0.72), as well as the respective values of Na_i and K_i indicated in Fig. 1. The symbols and numbers that identify individual bloods are the same as in previous figures. Open inverse triangles connected with lines indicate the averaged values of unmodified, density-fractionated red cells taken from Fig. 3. See text for discussion.

of MCV, it is clear that cell size decreases as $\Delta\mu_{net}$ shifts from inward to outward. Thus this analysis provides insight into the possible contribution of the cotransport system to the steady-state values of Na_i and K_i and consequently cell volume, and supports the conclusion drawn from the correlation presented in Fig. 2 A, as discussed before. But other evidence is needed before the conclusion is acceptable that the steady-state concentrations of Na and K across the membrane reflect the activity of the cotransport system superimposed on the distribution set by the Na/K pump–leak

balance. Thus direct measurements of *net* movements (of the appropriate size and direction) of Na and K by the cotransport system across the membrane of cells in the steady state would be required. This is an unlikely proposition at best, given the small size of the fluxes and the errors involved with their measurement. Another approach might be through the application of a formulation that extends the pump–leak model to include the contributions of any other type mechanism that results in net movements of Na and/or K such as the cotransport system (Milanick and Hoffman, 1986). But this approach also has its limitations because accurate assessment of the small leakage components are needed here as well. Nevertheless, appropriate analytical tools might be developed to make these types of measurements possible.

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