Cation transport and cell volume changes in maturing rat reticulocytes

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Mairbaurl, Heimo, Susanne Schulz, and Joseph F. Hoffman. Cation transport and cell volume changes in maturing rat reticulocytes. Am J Physiol Cell Physiol 279: C1621–C1630, 2000.—During maturation, reticulocytes lose membrane material, including transporters, and this is accompanied by a loss of cell water and volume. Here we determined a possible role of ion transport in adjusting cell volume during maturation. Reticulocytes and red blood cells of different ages were prepared from erythropoietin-treated rats by density gradient fractionation. Cell volume and ion transport were measured in freshly prepared cells and in reticulocytes during in vitro maturation. Reticulocytes had an increased K content and cell volume, whereas intracellular Na was decreased. All parameters approached whole blood values after 2 days in culture. Na-K pump was elevated in reticulocytes and decreased during maturation. Na-K-2Cl cotransport (NKCC) activity was lower in reticulocytes and was activated 8- and 20-fold by shrinkage and okadaic acid, respectively, whereas stimulation was barely detectable in high-buoyant density red blood cells. The ouabain- and bunetamide-insensitive Na flux in reticulocytes decreased on maturation. Most of it was inhibited by amiloride, indicating the presence of Na/proton exchange. Our results show that, although the Na-K-pump activity in reticulocytes is very much increased, the enhanced capacity of NKCC is essentially cryptic until stimulated. Both types of capacities (activities) decrease during maturation, indicating a possible loss of transport protein. The decrease was constrained to the period of reticulocyte maturation. Loss of transport capacity appears to exceed the loss of membrane surface area. Reticulocyte age-related changes in the net electrochemical driving force indicate that the increasing NKCC activity might contribute to the reduction in cell water.

DURING DIFFERENTIATION OF RED BLOOD CELLS significant functional and structural changes occur (for review see Ref. 38). Structural changes include enucleation, loss of organelles, and changes in composition and membrane surface area with subsequent changes in cellular metabolism, e.g., loss of the capability of protein synthesis and oxidative metabolism. The decrease in the membrane surface area (38, 42) has been explained by a “shedding” of membrane material as exosomes, which are vesicles that can be collected by high-speed centrifugation from the supernatant of in vitro maturing reticulocytes (22) and from plasma after phlebotomy (23). Exosome membranes show the typical composition of the red blood cell membrane but exhibit high concentrations of selected membrane proteins characteristic of immature red blood cells such as the transferrin receptor (24), which is barely detectable in plasma membranes of mature red blood cells. Therefore, a selective extrusion mechanism for the removal of certain membrane proteins has been proposed (9, 22, 23). Interestingly, exosomes do not seem to contain cytosolic proteins (22).

The decrease in cell volume during maturation of reticulocytes can be attributed in part to the loss of membrane and intracellular constituents. However, red blood cell volume is also determined by the cellular cation content that, in turn, is controlled by specific transport systems (for review see Ref. 39). In red blood cell subpopulations of different ages, separated according to their buoyant density (4, 18), an inverse relationship was found between cell density and cell volume as well as cation content (1, 7, 10, 18, 27). Under steady-state conditions, cell volume and cation content are set by a balance between the activity of the Na-K pump, cation leaks, and other transporters such as Na-K-2Cl cotransport (NKCC) (12, 35, 41). A decrease in the activity of the Na-K pump during reticulocyte maturation has been described in a variety of species. In sheep red blood cells, which show a dimorphism with regard to their cation content, Na-K pumps are inactivated to a greater extent from reticulocytes during maturation from low-K sheep than during maturation from their high-K counterparts. A significant amount of those lost Na-K pumps was found to be shed into exosomes or was degraded by energy-dependent mechanisms (3). Mature red blood cells from carnivores lack the Na-K pump (13, 19, 40), although it still can be found in their reticulocytes (40), again indicating specific inactivation or extrusion mechanisms. Changes in...
the Na-K-pump activity during reticulocyte maturation also have been described by others (e.g., Refs. 5 and 15). The activity of other transporters possibly involved in the control of red blood cell volume, such as NKCC, Na/Li exchange, Na/H exchange, and K-Cl co-transport, was also found to be increased in fractions of young red blood cells (5, 6, 8, 10, 16, 37).

In the present study changes in cell volume, cation content, and cation transport were evaluated during maturation of rat reticulocytes to evaluate a possible role of changes in transport activity in determining the cell volume during reticulocyte maturation and the role of matured red blood cells. A method suitable to enhance reticulocyte production in rats by injection of erythropoietin (EPO) and their isolation by density gradient centrifugation is described for confirmation of preliminary results. It was found that the Na-K-pump capacity decreased with maturation. NKCC activity was low in normal reticulocytes and increased slightly with maturation and cell aging. In contrast, reticulocyte NKCC capacity evidenced by maximal activation with okadaic acid was lost during in vivo and in vitro maturation. Preliminary results have been presented in abstract form (30, 31, 33).

MATERIALS AND METHODS

Treatment of animals and preparation of red blood cells with different buoyant density. Male Sprague-Dawley rats with an average weight of 450 g were injected intraperitoneally with a single dose of 200 U/kg recombinant human EPO (Recormon; Boehringer Mannheim, Mannheim, Germany). Control rats were injected with an equivalent volume of 150 mM NaCl solution. Three to five days later, the rats were anesthetized with ether and exsanguinated by cardiac puncture. The blood of up to six animals was pooled for one experiment. Aliquots (5 ml) of whole blood were layered on 26-ml density gradient medium composed of 10% 10-fold concentrated phosphate-buffered saline (GIBCO-BRL, Grand Island, NY), 68% Percoll (Sigma), and 22% deionized water in polycarbonate centrifuge tubes. This medium had a density of 1.095 g/ml and an osmolality of 281 mosmol/kg (34). The tubes were centrifuged for 30 min at 20,000 g at 12°C in a Sorvall RC-5B or R28S centrifuge using the SS-34 Rotor (Sorvall, Du Pont). Several fractions of cells were collected by aspiration, beginning at the top after the supernatant plasma and buffy coat were removed. Contaminating leukocytes were removed from each fraction of red blood cells by filtration through a mixture of a-cellulose and microcrystalline cellulose. The top fraction, formed of red blood cells of the lowest buoyant density, usually contained >90% reticulocytes; a fraction taken from the middle of the tube contained 2.5% reticulocytes; and the bottom fraction, formed of red blood cells with the highest buoyant density, usually had <1% reticulocytes (see Table 1).

In vitro maturation of reticulocytes. Red blood cells from the top fraction were suspended [hematocrit (Hct) 0.3%] in culture medium (RPMI 1640; GIBCO-BRL) substituted with 7% fetal calf serum, 2 mM glutamine, 10 mM HEPES (pH 7.3 at room temperature), and antibiotics and were incubated for several days under tissue culture conditions in a humidified 5% CO2-balance air atmosphere.

Flux measurements. For the measurement of the unidirectional efflux of 22Na, the concentrations of intracellular Na (Na) and K (K) were optimized by adjustment to ~50 mmol per liter of cells with the nystatin technique as described previously (32), except that all steps were carried out at room temperature. During this procedure the cells were also loaded with trace amounts of 22Na. The loading medium was composed of (in mM) 70 NaCl, 70 KCl, 1 Na2HPO4, 1 MgSO4, 23 sucrose, and 20 HEPES (pH 7.2 at room temperature). The content of NaCl and KCl of the loading medium was altered when different Na and K concentrations were required. After the cells were loaded, nystatin was removed by seven washes with loading medium containing 0.3% bovine serum albumin (Sigma).

The flux medium contained (in mM) 100 NaCl, 5 KCl, 10 glucose, 80 sucrose, and 20 HEPES (pH 7.4 at 37°C). The sucrose concentration in the flux medium was modified to adjust the cell volume. Fluxes (Hct 2%) were measured over a period of 90 s in the absence of ouabain but over a period of 5 min in the presence of ouabain. Fluxes were started by adding cells to the flux medium and were terminated by packing the cells in a microfuge for 10 s. Radioactivity was determined in the original cell suspension and in the supernatant medium after centrifugation. Fluxes were calculated from the initial Na concentration and the efflux rate constant (kNa). The fluxes were linear over the indicated time periods as determined in separate experiments.

The activity of the Na-K pump was taken as the portion of 22Na efflux inhabitable with 5 mM ouabain (22Naouba), whereas NKCC activity was the portion inhibited with 10 mM bumetanide in the presence of ouabain (22Naouba). Okadaic acid (Calbiochem, La Jolla, CA) was used to inhibit protein-phosphatase activity to maximize NKCC activity. The effectiveness of concentrations of inhibitors used in flux experiments was tested with dose-response curves.

Hct was determined after microcentrifugation, and the hemoglobin concentration was measured spectrophotometrically after conversion to cyanmethemoglobin. Reticulocytes were counted on air-dried smears of cells after they were stained (reticulocyte stain, Sigma). Na and K concentrations were determined by flame photometry (Corning 410) in cell lysates after being packed to a Hct 97% in narrow-bore 300-l microcentrifuge tubes. The distribution ratio of Cl (rCl) was determined after red blood cells were incubated with tissue culture medium to which trace amounts of 36Cl were added (20). The activity of 36Cl was measured in a beta counter (model TR2100, Packard) after the medium and the cells were separate by microcentrifugation. In some experiments cell water content was determined from the wet weight-to-dry weight ratio, and the volume of water in the cells was calculated according to Tosteson and Hoffman (41). For presentation, cell water was calculated from mean cellular hemoglobin concentration (MCHC) (36).

The activity of the Na-K-ATPase was measured according to Forbush (14) on membranes prepared from red blood cells after lysing in and four washes with 5 mM Tris-phosphate buffer (pH 7.3 at room temperature). Na-K-ATPase activity was taken from the time course of phosphate release that was sensitive to inhibition by 5 mM ouabain.

Statistical evaluation. Results are presented as means ± SD and are from a series of experiments in which individual parameters were determined. Not all measurements could be made on each set of samples because of the small number of cells obtained in individual preparations. All measurements were performed in duplicate or triplicate. Differences among parameters determined in fractions of red blood cells of different buoyant density and among reticulocytes kept in culture for various periods of time were evaluated by one-way
The level of statistical significance was $P < 0.05$.

**RESULTS**

Treatment of rats with EPO caused an increase in whole blood reticulocyte counts from $\sim 2.5\%$ up to $10\%$. In the fraction of cells with the lowest buoyant density, reticulocytes could be enriched to a purity $>90\%$ by density gradient centrifugation compared with preparations from the blood of untreated rats in which the yield was only $\sim 50\%$. Reticulocytes obtained after EPO treatment had a lower buoyant density than those obtained from untreated rats, and the density of new methylene blue-stained material was higher than, and often showed patterns similar to, those seen in red blood cell precursors just after enucleation. These observations probably indicate that reticulocytes obtained after EPO treatment are on average younger than normal and may represent reticulocytes that have undergone accelerated release (38).

When cells from the top fraction ($>95\%$ reticulocytes) were kept in culture for in vitro maturation, the number of reticulocytes decreased by $\sim 5\%$–$15\%$ within the first 24–36 h (Fig. 1A). A rapid drop to $<10\%$ was then seen over the following 2 days, whereas barely any reticulocytes were detectable after 4 days in culture. Loss of stained material was associated with an increase in MCHC by $\sim 25\%$ (Fig. 1B).

**Cell water and cation content.** As a measure of changes in the cell volume of reticulocytes, the change in cell water content was estimated from the changes in MCHC (Table 1). This assumes that the amount of hemoglobin per cell was the same in the different fractions of red blood cells separated by density gradient centrifugation and does not change on subsequent incubation. MCHC was $\sim 25\%$ lower initially in the reticulocyte-rich fractions than in the unfractonated cell population (Table 2) but approached the latter level during the 3 days of incubation.

The concentration of $\text{Na}_i$ per liter of cells was lower in reticulocytes than in unfractonated cells, whereas the concentration of $\text{K}_i$ was elevated (Table 1). These results are similar to those observed in pig reticulocytes (26) but are in contrast to the results of Furuwaka et al. (15), who found that $\text{Na}_i$ and $\text{K}_i$ in rat reticulocytes obtained after phenylhydrazine treatment were essentially the same as those in mature erythrocytes of control rats. Our results show that $\text{Na}_i$ increases and $\text{K}_i$ decreases to values similar to those found in unfractonated red blood cells as MCHC increases in red blood cells of increasing buoyant density (Table 1). The small difference in $\text{K}_i$ between red blood cells from the middle and bottom fractions was not statistically significant, although $\text{Na}_i$ was significantly increased in cells from the bottom fraction. There was no consistent reticulocyte age-related change in $r_{\text{cl}}$, but in the most dense (bottom) red blood cell population $r_{\text{cl}}$ was slightly higher ($P < 0.15$) than in the reticulocyte-rich (top) fraction (results not shown).

Reticulocyte maturation-related changes of $\text{Na}_i$, $\text{K}_i$, MCHC, and cell water are summarized in Table 2. In the course of 3 days of in vitro maturation, the percentage of reticulocytes decreased from initially $>95\%$ to $\sim 2.2\%$. As the percentage of reticulocytes decreased, both MCHC and $\text{Na}_i$ increased, whereas $\text{K}_i$ decreased. No significant change in $r_{\text{cl}}$ was found. It is also evident that, as cells matured in culture (i.e., day 3 compared with fresh whole blood), there was a lower MCHC and higher values of $\text{Na}_i$ and $\text{K}_i$.

**Na-K pump.** To assess whether the Na-K pumps of reticulocytes compared with those of matured erythrocytes had the same or different sensitivities to ouabain, dose-response curves were performed (Fig. 2). The results indicate that the sensitivity of the Na-K pump to inhibition by ouabain was similar in the reticulocyte-rich fraction (top) and in the fraction with mature erythrocytes (middle and bottom fractions). The Na-K-pump activity was measured at an extracellular K concentration of 2.5 mM. It should be recognized that...
Table 1. Composition of unfraccionated and density-fractionated red blood cells

<table>
<thead>
<tr>
<th></th>
<th>Whole Blood</th>
<th>EPO</th>
<th>Fractionated cells</th>
<th>Top</th>
<th>Middle</th>
<th>Bottom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retics</td>
<td>1.5 ± 0.2</td>
<td>6.1 ± 1.9</td>
<td>91.9 ± 4.8</td>
<td>25.4 ± 2.0</td>
<td>0.8 ± 0.7</td>
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<tr>
<td>MCHC</td>
<td>336.0 ± 10.0</td>
<td>328.6 ± 8.0</td>
<td>251.0 ± 10.0</td>
<td>330.0 ± 17.0</td>
<td>358.0 ± 14.0</td>
<td></td>
</tr>
<tr>
<td>Nai</td>
<td>7.1 ± 1.6</td>
<td>7.5 ± 1.5</td>
<td>4.4 ± 0.7</td>
<td>5.8 ± 2.0</td>
<td>9.4 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Ki</td>
<td>117.7 ± 7.3</td>
<td>119.7 ± 8.7</td>
<td>129.3 ± 8.3</td>
<td>119.5 ± 22.1</td>
<td>114.7 ± 9.7</td>
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</tr>
<tr>
<td>Cw</td>
<td>0.721</td>
<td>0.727</td>
<td>0.787</td>
<td>0.744</td>
<td>0.712</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD from 4 different preparations of unfraccionated red blood cells from control and erythropoietin (EPO)-treated rats as well as density-fractionated red blood cells from EPO-treated rats. Top cells have the lowest buoyant density; bottom cells have the highest buoyant density. Cell water was estimated from MCHC (see MATERIALS AND METHODS). Retics, reticulocyte counts (%); MCHC, mean cellular hemoglobin concentration (g Hb/l red blood cells); Nai and Ki, cellular concentrations of Na and K, respectively (mmol/l packed cells); Cw, cell water (ml/ml packed cells).

pump inhibition in all red blood cell fractions was incomplete even at an ouabain concentration of 5 mM. Higher concentrations of ouabain were avoided because of the possibility of nonspecific effects. However, the main conclusion concerning changes in pump capacity would not be statistically altered had full pump inhibition been obtained.

In freshly isolated reticulocytes, when Nai had been increased, the activity of the Na-K pump [maximum volume (V max) for Nai] in the top fraction was 3–5 times higher than in the middle or bottom red blood cell populations (Fig. 3A), confirming the results indicated in the dose-response experiments shown in Fig. 2. Similar observations were made previously on reticulocytes from high- and low-K+ sheep (3), rats (15, 31), pigs (26), and humans (32). In addition, during in vitro maturation of reticulocytes, the magnitude of the Na-K pump, studied at V max, decreased with the disappearance of reticulocytes (Fig. 3B) and reached values representative of unfraccionated cells (data not shown) on day 2 in culture. These results indicate the time course of pump capacity found in maturing reticulocytes. Results shown in Fig. 3, A and B, were obtained in different series of experiments, which explains the difference in the magnitude of the pump fluxes between cells in the top fraction of fractionated cells (Fig. 3A) and day 0 in unfraccionated cells in culture (Fig. 3B).

During the course of these experiments we realized that, when cells were loaded with nystatin to achieve V max conditions with concentrations of Nai and Ki of ~50 mM, the Nai of reticulocytes decreased during preparations for the flux measurements, reaching ~35 mM. This was probably caused by the high activity and capacity of the Na-K pump despite storage at 12°C. Because we wanted to obtain an estimate of Na-K-pump capacity rather than actual activity, it was important to know the dependency of the pump-mediated Na efflux on Nai. Figure 4 shows that V max was achieved at values of Nai of ~25 mmol per liter of packed cells (31). Obviously the decrease in Nai did not significantly affect the results on pump capacity measurements as presented in Fig. 3B.

NKCC. In freshly fractionated red blood cells, the ouabain-insensitive 22Na efflux was highest in reticulocytes and decreased with increasing cell density. This kind of increased activity of ion transport has also been found in human red blood cells from patients with reticulocytosis (44). NKCC, measured in the presence of ouabain as the bumetanide-sensitive efflux at optimized values of Nai and Ki, was somewhat higher in density-fractionated red blood cells taken from the middle and bottom fractions compared with that in the reticulocyte-rich top fraction (the context values in Fig. 5A and Fig. 8). During the culture of reticulocytes for 4 days, the bumetanide-sensitive efflux of 22Na appears to have decreased slightly (Fig. 5B).

To test whether the capacity of NKCC was different in different portions of red blood cells, 22Na efflux was also measured in these cells after treatment with okadaic acid to achieve maximal NKCC stimulation by inhibition of the phosphatase activity (29). Figure 5A indicates that NKCC activity of reticulocytes (top portion) can be stimulated ~10-fold by okadaic acid. In contrast, an approximately threefold activation of transport was found in the middle and a twofold activation in the bottom fraction. Measurements on cultured reticulocytes show that the activation of NKCC

Table 2. Composition of reticulocytes matured in tissue culture

<table>
<thead>
<tr>
<th></th>
<th>Whole Blood</th>
<th>EPO</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retics</td>
<td>5.6 ± 1.8</td>
<td>96.8 ± 2.2</td>
<td>91.2 ± 3.0</td>
<td>7.8 ± 5.8</td>
<td>2.2 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>MCHC</td>
<td>333.0 ± 9.0</td>
<td>257.0 ± 13.0</td>
<td>275.0 ± 18.0</td>
<td>288.0 ± 18.0</td>
<td>318.0 ± 12.0</td>
<td></td>
</tr>
<tr>
<td>Nai</td>
<td>8.9 ± 1.2</td>
<td>5.3 ± 1.3</td>
<td>7.6 ± 1.9</td>
<td>8.8 ± 2.5</td>
<td>9.6 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Ki</td>
<td>106.7 ± 8.5</td>
<td>126.3 ± 9.6</td>
<td>121.8 ± 10.0</td>
<td>123.4 ± 20.3</td>
<td>117.3 ± 11.7</td>
<td></td>
</tr>
<tr>
<td>Cw</td>
<td>0.724</td>
<td>0.787</td>
<td>0.771</td>
<td>0.754</td>
<td>0.735</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD from 3 different preparations of fresh unfraccionated red blood cells and cultured reticulocytes. Cell water was estimated from MCHC (see MATERIALS AND METHODS).
by okadaic acid decreased significantly during maturation of reticulocytes (Fig. 5B). Freshly isolated reticulocytes (Fig. 5A, top fraction) and reticulocytes prepared for maturation studies were from different experimental series, which explains the difference in the magnitude of NKCC fluxes between the types of experiments.

To test the responsiveness of NKCC to changes in cell volume, we treated freshly fractionated red blood cells of different density (age) with nystatin to increase Nai and then incubated them in media with different sucrose concentrations to alter cell volume. The results summarized in Fig. 6A show that NKCC activity was very low in the reticulocyte-rich fraction when the cells swelled in hypotonic medium (255 mosmol/kg). By contrast, cell shrinkage in medium of 385 mmol/kg caused a 25-fold activation relative to the that in the swollen state. In swollen red blood cells of high buoyant density (bottom fraction), NKCC transport activity was much higher than that seen in swollen reticulocytes but was less responsive to cell shrinkage where the transport rate was less than doubled.

During these experiments we observed that the efflux that remained after exposure to both ouabain and bumetanide, that is, the ouabain + bumetanide-insensitive (OBI) flux, or \( \text{OBI} \), was markedly stimulated by cell shrinkage in the reticulocyte fraction (Fig. 6B). It is also evident that, in shrunken reticulocytes, the OBI flux exceeded considerably the NKCC activity shown in Fig. 6A. In the older, more dense cells, the OBI efflux was lower and displayed only a modest increase with decreasing cell volume. Preliminary results indicate that activation of OBI by cell shrinkage was almost completely inhibited by 100 µM amiloride and may therefore represent Na/proton exchange (29). Figure 7 shows that okadaic acid, in addition to stimulating NKCC, also dramatically stimulates the OBI Na efflux in the reticulocyte-rich (top) fraction, while stimulation was only two- to fourfold in more dense cells (bottom fraction). Thus cell shrinkage (Fig. 6B) and okadaic acid treatment (Fig. 7) of cells at their normal volume

Fig. 2. Ouabain sensitivity of \( \text{Na}^{22} \) efflux in rat red blood cells of different buoyant density. Rats were treated with EPO, and red blood cells of different buoyant density were prepared by density gradient centrifugation (see MATERIALS AND METHODS). Three fractions of cells were prepared: the top fraction contained 98.2%, the middle fraction contained 6%, and the bottom fraction contained 0.1% reticulocytes. Intracellular Na (Na\(_i\)) and K (K\(_i\)) were adjusted with the nystatin technique. \( \text{Na}^{22} \) efflux was measured in a medium containing (in mM) 140 choline-Cl, 2.5 KCl, 9 glucose, 20 HEPES (pH 7.4 at 37°C), and varying concentrations of ouabain. Each flux was measured in duplicate. The ouabain-sensitive flux (\( \text{OBI Na}^{22} \)), taken as the Na\(^{-}\)K\(^{+}\) pump flux, represents the difference in Na\(^{-}\) efflux obtained in the absence and presence of ouabain at each concentration. Data are from 1 of 2 experiments with similar results.

Fig. 3. Activity of the Na\(^{-}\)K\(^{+}\) pump at maximal cell volume (\( V_{max} \)) for Na\(_i\) of density fractionated red blood cells (A) and reticulocytes in culture (B). Density-fractionated red blood cells (A) with increased Na\(_i\) were prepared as described in MATERIALS AND METHODS. The top, middle, and bottom fractions had the same approximate reticulocyte content as that indicated in Table 1. Reticulocyte measurements, as used in Fig. 1B, were similar to those referred to in Table 2, when the fraction used contained \( >90\% \) reticulocytes. \( \text{Na}^{22} \) efflux was measured in the absence and presence of 5 mM ouabain, with the difference representing the ouabain-sensitive or Na-K pump component of the efflux. Date are means ± SD from triplicate flux measurements from 1 of 4 experiments with similar results. *P < 0.05 compared with values from top fraction (A) and day 0 (B).
both resulted in stimulation of the OBI flux similar to their stimulation of NKCC activity (Figs. 5A and 6A). Whether the underlying mechanism(s) controlling these fluxes are the same or related is not known.

Because NKCC can be stimulated by either okadaic acid (Fig. 5A) or cell shrinkage (Fig. 6A), the question can be asked, what are the combined effects of okadaic acid and cell shrinkage? Figure 8 shows that the activation of NKCC by both shrinkage and okadaic acid was highest in reticulocytes (top fraction) and decreased with increasing cell density (middle and bottom fractions). However, in reticulocytes the activation by okadaic acid exceeded considerably the activation by shrinkage, whereas in the bottom cell fraction both okadaic acid and shrinkage caused the same degree of stimulation. Shrinkage did not affect NKCC activity significantly in okadaic acid-treated cells, indicating that okadaic acid caused maximal transport activation in all cell fractions. It should be added that the protein kinase inhibitor staurosporine markedly reduced the NKCC activity of reticulocytes at normal cell volume but had no effect on the middle and bottom fractions of cells (results not shown).

**DISCUSSION**

The purpose of this study was to examine changes in the cation transport activity, ion content, and volume during the maturation of reticulocytes to determine at which stage of cell maturation these changes occur and to test whether the measured changes in transport might account for the reticulocyte maturation-related changes in cell volume. The results show that the capacity of cation transport systems such as the Na-K pump, NKCC, and, possibly, Na/H exchange is highest in reticulocytes and decreases with increasing red blood cell buoyant density. Concomitantly, Na increases, whereas K and cell volume decrease. Results from experiments on cultured reticulocytes indicate that changes in transport and ionic composition are constrained to the period of reticulocyte maturation because no further changes were found in mature cells. These results are in line with other studies on maturation-dependent changes of various ion trans-
port systems of red blood cells (e.g., Refs. 2, 3, 10, 16, and 25) but add new insight into a possible contribution of NKCC in maturation-dependent changes in cell volume.

In previous studies reticulocytes were prepared by long-term centrifugation of whole blood of healthy donors and patients with reticulocytosis (7). In animal experiments reticulocytosis was induced by repeated bleeding (3) or treatment with phenylhydrazine (26). Both treatments put considerable strain on the studied animals due to oxidative stress and hypoxia caused by decreased oxygen transport capacity. In the present study reticulocytes were separated from the whole blood of rats after erythropoiesis was stimulated by the injection of human recombinant EPO. This treatment, which was very well tolerated by the animals, caused an increase in reticulocyte counts from ~2.5 to almost 10% within the 4 days between EPO application and blood sampling but did not increase Hct. Density gradient fractionation resulted in reticulocyte fractions with a purity of >90%, whereas the lowest density fractions of red blood cells from untreated animals contained only ~50% reticulocytes. Morphological studies of reticulocytes from EPO-treated rats showed premature stages indicated by ~5% state 0 reticulocytes (17). These cells also had the highest staining intensity as judged from gray-scale measurements of digitized images (not shown). In the course of 3–4 days of in vitro culturing, almost all material stained by new methylene blue had disappeared, which is indicative of mature red blood cells.

An increased activity of transporters in reticulocyte-enriched fractions of red blood cells has been observed repeatedly (for review see Ref. 38). Yet, neither the mechanisms causing the transport activity to decrease nor their consequences on red blood cell function are understood. In addition, it is important to study reticulocytes that are formed under physiological conditions without possible side effects of treatment. During re-

![Graph A](image1)

Fig. 6. Effects of changes in cell volume on the activity of NKCC (A) and on the activity of ouabain + bumetanide-insensitive (OBI) $^{22}$Na efflux (B) in freshly prepared reticulocytes (top fraction) and dense (bottom fraction) red blood cells. The reticulocyte-enriched fraction contained ~97% reticulocytes, whereas the most dense (bottom) fraction had ~0.2% reticulocytes. Cells were treated with nystatin to increase $N_a$. Na effluxes ($\nu_{M_{\text{Na}}}$) were measured in media with varying sucrose concentration to adjust the osmolality to the indicated values and contained 5 mM ouabain ± 10 μM bumetanide. A: bumetanide-sensitive (NKCC) efflux of $N_a$. B: efflux that remains after inhibition of the Na-K pump and NKCC with ouabain + bumetanide ($\nu_{M_{\text{OBI}}}$) as a function of flux medium osmolality. Cells are at their normal cell volumes at medium osmolality between 300 and 310 mosmol/kg. Data are mean values from 1 (triplicate flux measurements, maximal experimental error ± 10%) of 3 experiments with similar results. OBI flux rates measured in reticulocytes at the highest osmolalities were at best estimates because the flux rate was outside the linear range.

![Graph B](image2)

![Graph C](image3)

Fig. 7. Activation of OBI $^{22}$Na efflux by okadaic acid of red blood cells of different buoyant density. The reticulocyte-enriched top fraction contained 95%, the middle fraction contained 6.5%, and the bottom fraction <0.1% reticulocytes. Fluxes were measured after $N_a$ and $K$ were optimized by adjustment with the nystatin technique as described in MATERIALS AND METHODS. The concentration of okadaic acid was 1 μM. Data are means ± SD from 3 experiments. *$P < 0.05$ compared with fluxes measured in freshly prepared reticulocytes (Top) at equivalent experimental conditions; #$P < 0.05$ between untreated (control) and okadaic acid-treated cells.
ticulocyte maturation the total cell surface area decreases by \(20\text{–}30\\%\) \((38, 42)\), indicating a loss of membrane material. Some of this material that was shed appears to be liberated in nanometer-sized vesicles called "exosomes" \((22)\), composed similarly to the red blood cell membrane but distinctly differ in their content of certain proteins \((21, 22, 24)\). It was therefore speculated that liberation of exosomes represents a mechanism for active extrusion of "unnecessary" membrane components \((23)\).

Our results confirm earlier findings of a significant number of Na-K pumps being lost during transition from the stage of reticulocyte to mature red blood cell \((3, 15)\). A portion of the lost pumps was found in exosomes \((22)\), and the remaining ones were probably proteolyzed \((43)\). Our results show further that reticulocytes prepared after EPO treatment undergo changes similar to those obtained with phenylhydrazine treatment or bleeding. This result might indicate that treatment effects seem not to affect the equipping of cells with certain transporters or their fate during reticulocyte maturation. However, in addition to confirming previous findings on the decrease in pump capacity, which indicates a decrease in the number of pump copies during reticulocyte maturation \((10)\), our results provide no further insight into mechanisms of removal or inactivation.

NKCC activity at optimized Na was lowest in reticulocytes but increased as the buoyant density of cells increased. The highest cotransport activity was found in the most dense cells. Duhm \((10)\) also reported an increase in furosemide-sensitive Rb uptake with increasing density of human red blood cells whose Na and K had not been modified but did not specify the reticulocyte content of the samples studied. In this \((10)\) and other studies \((11, 12)\) the dependency of NKCC activity on the steady-state cell volume of red blood cells of humans and rats was pointed out. A similar relation was found in unfractionated cells of Na and K modified to optimize NKCC activity \((32)\). Together, these results indicate a direct relation between the activity of NKCC and cell volume that is valid not only when unfractionated red blood cells from different donors are compared \((12, 32)\) but also when the red blood cell age-related variation in cell volume is considered \((this\ study\ and\ Refs.\ 10\ and\ 32)\). In contrast, when NKCC capacity was measured after full stimulation with okadaic acid, its activity was highest in reticulocytes but decreased with maturation. Also, the degree of NKCC stimulation by okadaic acid was much higher in freshly prepared reticulocytes than in fresh red blood cells of high buoyant density and in reticulocytes matured in culture. Shrinkage activation of NKCC showed a similar pattern, although maximal shrinkage stimulation was smaller than stimulation by okadaic acid. This result indicates that the capacity of NKCC decreases as reticulocytes mature, reflecting a decrease in the number of copies of NKCC protein. Another possibility is, of course, that the protein kinase-protein...
phosphatase system controlling NKCC activity undergoes a maturation-dependent inactivation so that the NKCC phosphorylation potential decreases. NKCC phosphorylation and activity also depend on cell volume and cytosolic ion concentrations (28). Since cell volume decreases with maturation of reticulocytes and red blood cell aging, cell volume-related effects cannot account for the decreased phosphorylation potential. The much smaller degree of stimulation by cell shrinkage than by okadaic acid supports the idea of an age-related loss of cell volume-sensitive NKCC phosphorylation systems. Another factor that might account for NKCC changes during reticulocyte maturation might be the cytosolic Mg concentration. However, although reticulocytes have a higher total Mg content than mature red blood cells (38), it is difficult to obtain a reasonable measure of intracellular Mg relevant for NKCC activation (32) because of its binding to organic phosphates and RNA, all of which change during reticulocyte maturation.

The maturation-dependent decrease in cell volume, together with the lost membrane material, cannot explain the change in cellular ion content (Tables 1 and 2). The ionic composition depends not only on the content of nondiffusible ions but also on the activity of the Na-K pump and of secondary active transporters such as NKCC and the leaks (32, 35, 41). The decrease in pump capacity during reticulocyte maturation might account for some of the increase in Na. The increased cell volume of reticulocytes might also explain their low NKCC activity and the slight increase in NKCC activity during reticulocyte maturation, when the cell volume decreases. A high cotransport activity has been associated with a decreased steady-state cell volume (12). This has been explained on the basis that an outwardly facing net electrochemical driving force, \( \mu_{\text{net}} \), results in an NKCC-mediated net solute extrusion that would tend to keep the cell volume small (12, 32). Figure 9 shows that \( \mu_{\text{net}} \) is directed inward in reticulocytes but outward in mature red cells. This could mean that, because of ion gradients, NKCC might also contribute to the decrease in cell water and volume that occurs during reticulocyte maturation.

In conclusion, it is clear that maturing reticulocytes rapidly adjust volume, ionic composition, and cation transport capacity to that characteristic of mature red blood cells. Changes in transport activity certainly contribute to the decrease in cell volume of reticulocytes. The mechanisms causing the loss of transport capacity during reticulocyte maturation are unclear. They appear to be independent of the loss of membrane surface area, judging from the disproportionate change of both parameters. Despite the maturation-dependent decrease in the surface area, the amount of hemoglobin per cell remains unchanged. This is of significance for maintaining a high oxygen transport capacity without an increase in the number of red blood cells that would be necessary if hemoglobin were also lost in this process. Without the decrease in reticulocyte cell volume during maturation, the Hct of whole blood would be significantly elevated, placing additional strain on the cardiovascular system.

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