intracellular ionized magnesium concentrations ([Mg++]i) were measured in erythrocytes by 31P nuclear magnetic resonance (NMR) and zero-point titration in 14 controls and seven patients with renal magnesium loss. The mean intracellular ionized magnesium concentration in controls measured by 31P NMR was 0.20 (SD 0.03) mmol/L cell water, compared with 0.55 (SD 0.12) mmol/L cell water by zero-point titration. Total erythrocyte magnesium content measured with the lysate method was 0.63 mmol/L cell water higher than estimated by 31P NMR, probably because not all magnesium complexes are fully visible to the NMR technique. We found a positive correlation between plasma ultrafiltrable magnesium and [Mg++]i irrespective of the [Mg++]i assay used. [Mg++]i measured with 31P NMR correlated modestly but significantly with [Mg++]i, determined by zero-point titration (r = 0.58, P < 0.02). Washing erythrocytes before the zero-point titration decreased the ATP content and the cell water fraction, which led to overestimation of [Mg++]i by zero-point titration. Although absolute values for [Mg++]i differ with the assay used, both methods determined significantly lower values for [Mg++]i in patients with isolated renal magnesium loss.

Magnesium, the second most abundant intracellular cation, is present in bound and free (Mg2+) form. Several methods for the measurement of total cellular magnesium content have been described, but the concentration of intracellular free magnesium ([Mg2+]i) is physiologically relevant and therefore of special interest. Using different techniques, several investigators have reported a wide range of [Mg2+]i for various cell types. For example, in deoxygenated erythrocytes a threefold increase in [Mg2+]i was seen with no change in total intracellular magnesium content (2). On the other hand, [Mg2+]i in hepatocytes appeared to be very sensitive to variations in total magnesium, with small increases or decreases in total magnesium resulting in large variations of [Mg2+]i (3).

Differences were also reported between measurement of [Mg2+]i by 31P NMR spectroscopy, as developed by Gupta et al. (2), and by zero-point titration (ZPT), as described by Flatman and Lew (4). We are not aware of a study in which both techniques were used simultaneously.

The aim of this study is to compare 31P NMR, an “indirect” method, with the “direct” zero-point titration method, for the measurement of [Mg2+]i in human erythrocytes. We used both methods to measure [Mg2+]i in oxygenated erythrocytes from healthy controls and from patients with isolated renal magnesium loss (5).

Materials and Methods

Plasma magnesium was measured by atomic absorption spectrophotometry (Model 5000; Perkin-Elmer, Norwalk, CT). Ultrafiltrates were obtained after ultrafiltration with Amicon tubes (Amicon Corp., Danvers, MA) (6). Erythrocyte total magnesium content was measured in lysed erythrocytes, as described previously (7), and expressed in mmol/L cell water. Hemoglobin content, hematocrit, number of erythrocytes, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were measured with an H1-Jr (Technicon Instruments Corp., Tarrytown, NY).

31P NMR

Freshly drawn heparinized venous blood (10 mL) was centrifuged and 10 mmol of glucose was added to the plasma. The plasma was mixed with the erythrocytes and incubated for 10 min at 20°C. The blood was centrifuged and we obtained an erythrocyte suspension with a hematocrit of 90%. Complete oxygenation was ensured by treating the samples with oxygen/CO2 (95/5 by vol) in a tonometer (Instrumentation Laboratory, Milan, Italy). The sample was transferred to a 10-mm (o.d.) glass NMR tube with a 4-mm (o.d.) coaxial insert containing D2O for a deuterium lock.

31P NMR spectra were recorded at 81 MHz and 37°C with a Bruker WM 200 spectrometer (Bruker, Karlsruhe, F.R.G.) operating in the Fourier transform mode. Pulses of 55° in combination with gated proton decoupling were applied with a repetition time of 0.78 s; the samples were spun at 10 Hz during the entire recording. Concentrations of ATP and 2,3-diphosphoglycerate (DPG) in the erythrocytes were determined with commercially available kits (Sigma Chemical Co., St. Louis, MO). The calculations of the concentrations of ATP and DPG complexed to Mg2+ and (or) hemoglobin were made.

Departments of 1 Pediatrics (P.O. Box 9101), 2 Clinical Chemistry, and 3 Physiology, University Hospital Nijmegen, University of Nijmegen, 6500 HB Nijmegen, The Netherlands.
Department of Biophysical Chemistry and SON National HP NMR Facility, University of Nijmegen.
5 Department of Hematology, Laboratory of Medical Enzymology, University Hospital Utrecht, Utrecht, The Netherlands.
6 Nonstandard abbreviations: [Mg2+]i, ionized free intracellular magnesium concentration; [MgT], intracellular total magnesium concentration; [Mg4+]o, ionized magnesium concentration in the medium at equilibration; NMR, nuclear magnetic resonance; ZPT, zero-point titration; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; DPG, 2,3-diphosphoglycerate; and Φf, fraction of free (noncomplexed) ATP.

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essentially according to Gupta et al. (2) and expressed in mmol/L cell water. We calculated the free fraction of ATP not complexed to magnesium (\(\Phi\)) from the NMR spectrum by means of the equations listed by Gupta et al. (2), solved with the aid of Newton's iteration method with a program for IBM-compatible computers.

\[
\Phi = \frac{[ATP]_F + [HbATP]}{[ATP]_T}
\]

(1)

where ATP\(_F\) is free ATP and ATP\(_T\) is total ATP.

\[
\Phi = \frac{(\delta_{\text{cell}} - \delta_{\text{MgATP}})}{(\delta_{\text{ATP}} - \delta_{\text{MgATP}})}
\]

(2)

in which \(\delta_{\text{cell}}\) is the measured resonance position of the \(\beta\)-resonance of ATP with respect to the \(\alpha\)-resonance, which has a nearly constant position.

\[
[Mg^{2+}]_i = K_d^{\text{MgATP}} [(1/\Phi) - 1]
\]

(3)

where \(K_d^{\text{MgATP}}\) is the dissociation constant for MgATP. \(K_d^{\text{MgATP}} = 3.8 \times 10^{-5}\) mol/L at 37°C and pH 7.2 (2).

The total magnesium concentrations are calculated as the sums of the concentrations of all magnesium-containing complexes and free magnesium, neglecting the possible amount of magnesium complexed to other red cell constituents.

Zero-Point Titration

Freshly drawn heparinized venous blood (10 mL) was centrifuged. The plasma was removed and the erythrocytes were washed three times in Medium I (per liter, 75 mmol of KCl, 75 mmol of NaCl, and 10 mmol of Tris - Cl, pH 7.4, 37°C) containing ethylene glycol bis(\(\beta\)-aminoethyl ether)-\(N,N,N',N'\)-tetraacetic acid (EGTA), 0.1 mmol/L, to chelate contaminant calcium and extracellular magnesium (4). The cells were then washed twice in Medium I containing 10 \(\mu\)mol of EGTA per liter and oxygenated with oxygen/CO\(_2\) (95/5 by vol) in a tonometer. Packed cells were then added to the incubation medium (Medium I with 10 \(\mu\)mol of EGTA and 10 mmol of inosine per liter) containing different concentrations of magnesium (hematocrit ~15%). After 10-min incubation at 37°C with constant stirring in plastic vials, samples were taken for the measurement of the initial magnesium content of the cells.

At \(t = 0\), ionophore A23187 was added to a final concentration of 10 \(\mu\)mol/L. At \(t = 10\) and 20 min, 0.5-mL samples were taken to measure the magnesium content of the cells. Samples were ejected into an Eppendorf microcentrifuge tube containing 0.8 mL of ice-cold inactivation Medium I (with 10 \(\mu\)mol/L EGTA) and 0.4 mL of di-\(\alpha\)-butylphthalate (density 1.042 kg/L) and immediately centrifuged at 8000 \(\times\) g for 1 min. The medium and oil were removed and the tube was carefully cleaned with a bevel-cut disposable Sample Cells and Chamber Cleaner (Advanced Instruments, Needham Heights, MA). The cell pellet was lysed in 400 \(\mu\)L of distilled water and frozen at \(-20^\circ\)C until assay.

At \(t = 10\) min, besides taking samples for the magnesium content of the erythrocytes, we also took two 0.1-mL samples for measuring the hematocrit, two 0.1-mL samples for measuring the water fraction and density, and two 0.4-mL samples for measuring the chloride distribution ratio (R).

Other Procedures

Water fraction, dry weight, and density. The wet and dry weights of a 0.1-mL erythrocyte suspension of known hematocrit were measured and the water fraction and density of the erythrocytes were calculated. No correction for entrapped water was necessary because the H1-Jr calculates hematocrit as the number of erythrocytes multiplied by their mean corpuscular volume.

Chloride distribution ratio. To an Eppendorf tube containing a 0.4-mL cell suspension we added 50 \(\mu\)L of \(^{36}\)Cl tracer (5 mCl/L; Amersham International plc, Amersham, Bucks., U.K.) and incubated the contents at 37°C for 10 min. We then added di-\(\alpha\)-butylphthalate (0.4 mL) and centrifuged the sample at 8000 \(\times\) g for 5 min. A 100-\(\mu\)L sample was taken from the supernate, and the rest was carefully and completely removed before 0.150 mL of trichloroacetic acid (50 g/L) was added to the cell pellet. \(^{36}\)Cl activities in the supernate and the cell pellet were determined in duplicate by liquid scintillation counting (Mark III liquid scintillation counter; Searle Analytic Inc., Des Plaines, IL).

\[
R = [\text{Cl}^-]_0/[\text{Cl}^-]_i
\]

(4)

where \([\text{Cl}^-]_0\) and \([\text{Cl}^-]_i\) are the \(^{36}\)Cl activities in 100 \(\mu\)L of supernate and cell water, respectively.

Ionized magnesium concentration. The concentration of ionized magnesium in the medium at equilibrium \((Mg^{2+})_0\) was extrapolated from the initial and final magnesium concentrations of the erythrocytes and the magnesium concentrations in the corresponding medium. \((Mg^{2+})_i\) was then calculated according to the following formula:

\[
(Mg^{2+})_i = R^2 (Mg^{2+})_0
\]

(5)

Statistics. Spearman's rank correlation coefficients were used to detect a possible relation between variables, and the signed rank test was taken to test for significant differences between different variables. Results were considered to be significant when \(P < 0.05\).

Results

Table 1 lists the relevant data obtained from the blood of controls and patients with renal magnesium loss. In comparison with controls, patients have significantly lower values for plasma ultrafiltrable magnesium and intracellular \((Mg^{2+})\), measured either with NMR and ZPT. In patients with renal magnesium loss, \(\Phi\) is higher than in controls (0.265 and 0.193, respectively), so relatively more magnesium is complexed with ATP. No differences between the two groups were found for

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concentrations of ATP and DPG, water fraction, chloride distribution ratio, and density. The total erythrocyte magnesium contents measured in the lysate by atomic absorption spectrometry were not lower in patients than in controls. However, when total erythrocyte magnesium content was measured by $^{31}$P NMR, the values were significantly lower for patients than for controls.

Figure 1 shows the $^{31}$P NMR spectra of one control and one patient with renal magnesium loss. A difference in the $\beta$-ATP peak position results in $\delta_{\text{ap}}$ of 709.00 Hz in the control and 723.30 Hz in the patient with renal magnesium loss, whereas $[\text{Mg}^{2+}]_{i}$ was 0.20 and 0.14 mmol/L cell water, respectively.

Figure 2 shows the relation between plasma ultrafiltrable magnesium and NMR-determined $[\text{Mg}^{2+}]_{i}$, or ZPT-determined $[\text{Mg}^{2+}]_{i}$. Plasma ultrafiltrable Mg is positively correlated with NMR $[\text{Mg}^{2+}]_{i}$ and with ZPT $[\text{Mg}^{2+}]_{i}$.

Total erythrocyte magnesium concentration ($[\text{MgT}]_{i}$) is significantly correlated with $[\text{Mg}^{2+}]_{i}$ as measured with both methods (Figure 3). In 12 of 14 controls, NMR $[\text{Mg}^{2+}]_{i}$ was nearly constant (0.20 mmol/L cell water), whereas total magnesium varied between 2.5 and 3.7 mmol/L cell water (Figure 3, top).

When we compared the results obtained by NMR and ZPT, we observed that NMR $[\text{Mg}^{2+}]_{i}$ remained nearly constant for a wide range of ZPT $[\text{Mg}^{2+}]_{i}$ values (Figure 4) in controls and showed lower concentrations in samples taken from patients with renal magnesium loss. The correlation of NMR $[\text{Mg}^{2+}]_{i}$ and ZPT $[\text{Mg}^{2+}]_{i}$ was modest but significant ($r = 0.58$, $P < 0.01$) for the samples from controls and patients taken together.

To explain the considerable differences in $[\text{Mg}^{2+}]_{i}$ values determined by the two methods, we investigated the differences in ATP, DPG, water fraction, and dry weight before and after the washing procedure in the ZPT experiments. Concentrations of total erythrocyte magnesium and DPG (not shown) did not change; however, ATP decreased considerably during washing and was partly restored during incubation with inosine before the addition of the ionophore. The final decrease in ATP concentration after washing ranged between 6% and 15%.

The water fraction and dry weight were measured before and immediately after washing and after 10-min incubation with 0.1 or 0.3 mmol/L magnesium and inosine (10 mmol/L) in the presence of the ionophore. The water fraction decreased after the washing and incubation procedure (Table 2). The dry weight was the same before and after the ZPT washing procedure.

To test the influence of dissociation constants on the $^{31}$P shifts measured in the NMR technique, we chose a value at random for every dissociation constant, from all values between the mean ± 2 SD, then calculated NMR $[\text{Mg}^{2+}]_{i}$ and NMR $[\text{MgT}]_{i}$. We repeated this procedure 1000 times for every control and patient. With this approach we found that NMR $[\text{Mg}^{2+}]_{i}$ was 0.19 (SD 0.08) mmol/L in controls and 0.13 (SD 0.04) mmol/L in patients with renal magnesium loss, whereas NMR $[\text{MgT}]_{i}$ was 2.36 (SD 0.25) and 1.92 (SD 0.17) mmol/L, respectively.
Discussion

For the determination of [Mg\(^{2+}\)] in erythrocytes, no "golden standard" is available, and the two methods, \(^{31}\)P NMR and ZPT, lead to different [Mg\(^{2+}\)] values.

\(^{31}\)P NMR

The mean \([\text{MgT}]_0\), the result of the sum of the concentrations of magnesium-containing complexes and the free magnesium concentration, was ~0.6 mmol/L cell water lower when determined by NMR than when measured via the lyseate method. It has been suggested that this difference of ~25% might be caused by the binding of magnesium to the cell membranes (8). In our calculations of the \([\text{MgT}]_0\) from the NMR data, all possible complexes considered by Gupta et al. (2) were accounted for, including the hemoglobin–ATP complex. The dissociation constant of the latter complex may vary considerably, i.e., \(K_d = 3.43 \pm 0.86\) mmol (2). Also, not all ATP may be available for binding to magnesium, a situation not recognized in the calculations.

After critical evaluation (9, 10) of the dissociation constant for Mg–ATP (2), it was concluded that \(K_d\) of MgATP, as used in this paper, is correct. Recently, Petersen et al. (11) stated that NMR [Mg\(^{2+}\)] measurements derived solely from the separation of the \(\alpha\) and \(\beta\)-ATP peaks do not lead to "a true measurement" of intracellular concentrations of free magnesium and that the concentrations of the magnesium ligands ATP and DPG must be taken into account (11).

We found a considerable range for NMR [Mg\(^{2+}\)]i, but less variation for NMR [MgT]i. The observed spread in NMR [Mg\(^{2+}\)]i is not caused by variations in oxygenation and pH (11). Full oxygenation (\(S_{\text{aO}_2}\) > 98%) was achieved and the pH of the blood sample was 7.38 ± 0.01.
Zero-Point Titration

Values of $[\text{Mg}^{2+}]$, obtained with ZPT varied from 0.4 mmol/L cell water (4) to 0.75 mmol/L cells (12) (corresponding to 1.13 mmol/L cell water, assuming a cell water fraction of 0.66). However, in the study by Wehling and Theisen (12), $[\text{Mg}^{2+}]$ was estimated from the amount of magnesium released from magnesium-loaded erythrocytes.

In our study, water fraction values and chloride distribution ratios were similar to those reported by Flatman and Lew (4), but the mean ZPT $[\text{Mg}^{2+}]$, was greater, by 0.15 mmol/L cell water. The limited number of donors ($n=2$) investigated by Flatman and Lew may explain this difference.

Differences between $^{31}\text{P}$ NMR and ZPT

Although the results we derived by using both methods are close to the reported normal values, the two techniques yield a considerable mean difference in $[\text{Mg}^{2+}]$, in controls, 0.35 mmol/L cell water. Bock and Yusuf (13) suggested that the washing step used before application of the ZPT procedure might alter $[\text{Mg}^{2+}]$, and reported an increase of $[\text{Mg}^{2+}]$ after washing stored erythrocytes in buffer. The decrease in erythrocyte ATP found after washing the erythrocytes for ZPT and the partial restoration of the ATP concentration during the subsequent incubation in a magnesium–inosine–ionophore-containing buffer might be a possible source of the difference in $[\text{Mg}^{2+}]$. Flatman and Lew (4) pointed to a nearly 10-fold increase of $[\text{Mg}^{2+}]$ when the ATP concentration was lowered from normal (1.66 to 0.2 mmol/L cell water. Thus the decrease in intracellular ATP concentration of 6–15% that we found in erythrocytes used for ZPT might be at least partly responsible for the difference in $[\text{Mg}^{2+}]$, found between $^{31}\text{P}$ NMR and ZPT. Further investigations are necessary to explore this suggestion.

The changes in water fraction and dry weight that we observed during the washing procedure and the further changes in dry weight during ZPT in a medium containing higher magnesium concentrations indicate changes in cell volume and result in a 10% increase of $[\text{Mg}^{2+}]$ measured by ZPT.

Ultrafiltrable Magnesium

In controls, the ionized magnesium concentration in erythrocytes is ordinarily less than the electrochemical equilibrium concentration (14). The same is true for our patients with isolated renal magnesium loss. An active magnesium extrusion mechanism is present that depends on extracellular sodium for 10% and on net chloride efflux for charge compensation for 90% of this effect (15). Lower extracellular magnesium concentrations lead to lower intracellular ionized magnesium concentrations in magnesium-loaded erythrocytes (15).

It is striking that the intracellular ionized magnesium concentrations as measured by ZPT approach the value of ultrafiltrable Mg in plasma. However, the exact mechanism of magnesium transport and homeostasis in human erythrocytes is not yet established (14).

The zero-point titration method should be preferred because it is a direct method. Interpretation of $^{31}\text{P}$ NMR results depends on available $K_d$ values; deviations in the equilibrium constants or in the availability of the substrate may result in incorrect values of the calculated magnesium concentration. Although each method leads to different results, significant differences between controls and patients with renal magnesium loss are obtained, regardless of the method used.

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