# Calculation of free $Mg^{2+}$ concentration from $^{31}$P-NMR spectra of ATP

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## Zusammenfassung

In MgATP-Lösungen, in Erythrocyten von Ratten, Schweinen und Rindern, in Menschen- und Rattenhirn sowie in Muskeln von Menschen wurde die Konzentration an freiem $Mg^{2+}$ ($[Mg^{2+}]_i$) anhand der $^{31}$P-NMR-Spektren des ATP bestimmt. Verschiedene Methoden wurden verwendet, um die Konzentration an freiem $Mg^{2+}$ zu ermitteln. Die $\alpha\beta$- und $\beta\gamma$-Differenzen der chemischen Verschiebungen ergaben entsprechend den Gleichungen von *Gupta* und *Moore* (J. Biol. Chem. 255 (1980) 3987–3993) und *Golding* und *Golding* (Mag. Res. Med. 33 (1995) 467–474) vernünftige Werte. Die Berechnung von $[Mg^{2+}]_i$ aus den Differenzen der chemischen Verschiebung von Phosphokreatin und der $\beta$-ATP-Resonanz ergaben höhere Werte und die Berechnung von $[Mg^{2+}]_i$ aus den $\beta/\alpha$- und $\gamma/\alpha$-Quotienten der Resonanzamplituden ergaben wesentlich niedrigere Werte als die Berechnungen nach *Guptas* und *Goldings* Gleichungen.
Wegen der Unsicherheit der $K_D^{MgATP}$ unter intrazellulären Bedingungen und wegen der Kompartmentierung des intrazellulären $Mg^{2+}$ liefert die Bestimmung der $[Mg^{2+}]_i$ mittels $^{31}$P-NMR nur räumlich integrale und keine absoluten Werte der $[Mg^{2+}]_i$.

## Summary

In MgATP solutions, in erythrocytes from rats, pigs and cattles, in human and rat brains, and in human muscles the concentration of free $Mg^{2+}$ ($[Mg^{2+}]_i$) was determined from the $^{31}$P-NMR spectra of ATP. Various methods were used to calculate the concentration of free $Mg^{2+}$. The $\alpha\beta$ and $\beta\gamma$ shift differences according to the equations given by *Gupta* and *Moore* (J. Biol. Chem. 255 (1980) 3987–3993) and by *Golding* and *Golding* (Mag. Res. Med. 33 (1995) 467–474) yielded conclusive results. Calculation of $[Mg^{2+}]_i$ from the phosphocreatine and $\beta$ ATP chemical shift differences yielded higher values and calculation of $[Mg^{2+}]_i$ from the $\beta/\alpha$ and $\gamma/\alpha$ peak height ratios revealed extremely lower values than the calculations using *Gupta*'s and *Golding*'s equations.
Because of the uncertainty of $K_D^{MgATP}$ under intracellular conditions and compartmentation of intracellular free $Mg^{2+}$, measurement of $[Mg^{2+}]_i$ by $^{31}$P-NMR can only give spatial integral and not absolute values of $[Mg^{2+}]_i$.



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## Introduction

In any tissue, magnesium ($Mg^{2+}$) is the most frequent intracellular divalent cation [1]. $Mg^{2+}$ is the essential activator for almost all ATP-dependent reactions. It is thus involved in some hundred enzymatic reactions [1, 2]. $Mg^{2+}$ stabilizes the structure of nucleic acids [1, 2] and is involved in the permeability of plasma and sarcoplasm membranes by blocking various ion channels [3–5].
The major part of intracellular $Mg^{2+}$ is bound to various negatively charged ligands such as ATP, ADP, citrate, proteins, membrane phospholipids etc. [2]. Free and reversibly bound $Mg^{2+}$ represent an Mg buffer [2, 6]. Its concentration of free $Mg^{2+}$ ($[Mg^{2+}]_i$) determines the quantitative effects of $Mg^{2+}$ on intracellular Mg-dependent reactions which usually express a bell-shaped function [1]. In order to define the effects of $[Mg^{2+}]_i$ and of alterations in $[Mg^{2+}]_i$, the absolute values of the intracellular concentration of free $Mg^{2+}$ must be known.
Now, several methods are used to measure $[Mg^{2+}]_i$ such as $Mg^{2+}$-sensitive microelectrodes, fluorescent dyes and $^{31}$P-NMR. $^{31}$P-NMR is important because, e.g. in erythrocytes the fluorescent dyes cannot be used due to the high quenching of fluorescence by hemoglobin. Moreover, $^{31}$P-NMR offers an advantage over other methods since it can be used as a non-invasive method for surviving tissues and living animals and may become important in human physiology and medicine.
Different methods are used to infer $[Mg^{2+}]_i$ from $^{31}$P-NMR data, which unfortunately deliver different results. Mostly, $[Mg^{2+}]_i$ is calculated according to eq. 1:

$$[Mg^{2+}]_i = K_D^{MgATP} \cdot \left(\frac{1}{\phi} - 1\right) \qquad (1)$$

$\phi$ is usually calculated from the chemical shift differences of the $\alpha\beta$, P peaks of ATP due to the binding of $Mg^{2+}$ whereby binding of other ionic species to ATP such as $H^+$ or $K^+$ were neglected or considered. Since the usage of the $\alpha\beta$ shift differences will lead to erroneous estimates of $\phi^{-1}$ near the saturation of ATP with $Mg^{2+}$, the shift differences of the $\beta\gamma$ peaks of ATP [7, 8] or the arithmetic mean of the $\alpha\beta$ P and $\beta\gamma$ P peaks of ATP were taken for calculation [9].
However, in a previous experiment using the αβ resonance shift differences, 12 % higher values were received than when using the arithmetic means of $\alpha\beta$ and $\beta\gamma$ P chemical shift differences [10]. Other authors used the shift differences of $\beta$ P of ATP and phosphocreatine (PCr) since the $\alpha$ ATP peak is less accurate due to unresolved resonances of $NADP^+$, NADPH

and $\alpha$ ADP underlying the $\alpha$ ATP peak [11].

As another method to calculate $[Mg^{2+}]_i$, the $\beta/\alpha$ and $\gamma/\alpha$ peak height ratios were applied to perfused rat hearts [12]. The $\beta$ and $\gamma$ peak is broadened due to the chemical exchange ($MgATP \rightleftharpoons ATP + Mg^{2+}$), with a constant area under the curve dependent on ATP concentration.

For comparing and testing, $[Mg^{2-}]_i$ was calculated from the same $^{31}P$-NMR spectra according to various methods. In order to have a comprehensive survey, $[Mg^{2+}]_i$ was determined by $^{31}P$-NMR in a solution of MgATP and in various cell types and tissues such as erythrocytes from pig, rat and cattle, rat and human brain and human skeletal muscles.

## Materials and methods

### In vitro measurements

Measurements of ATP and MgATP solutions and of erythrocytes (blood) were carried out on a Bruker AMX500 spectrometer with a $^{31}P$ resonance frequency of 202 MHz. The characteristics of the individual ATP signals were determined by means of a Lorentzian deconvolution.

The ATP solutions contained (in mM): 5 $Na_2ATP$ or 5 $Na_2ATP$ plus 5 $MgCl_2$ or 5 $Na_2ATP$ plus 12.5 $MgCl_2$ dissolved in 140 KCl, 20 TrisCl buffer (pH 7.2). $Na_2ATP$ was neutralized with Tris. To 2 ml of the solutions, 0.7 ml $D_2O$ was added to permit heteronuclear field frequency locking on deuterium.

A one-pulse sequence (90° pulse) without proton decoupling was used. 128 acquisitions were recorded with a pulse repetition delay of ls and a sweep width of 20 KHz. The probe head temperature was held constant at 310K. Prior to the fast Fourier transformation (FFT) an exponential multiplication (line broadening factor lb = 1.2 Hz) of the 64 Kbyte time domain data points was carried out and yielded a digital resolution of 0.3 Hz/point. The resonances were referenced to external $H_3PO_4$.

Heparinized blood from rats, pigs and cattles was taken directly without the addition of $D_2O$ to establish physiological conditions. For rat and pig blood, 1000 acquisition and for cattle blood 4000 scans were averaged because of their low ATP content [13]. Line broadening of 12 Hz and manual baseline correction were applied to the spectra.

### In vivo measurements

Wistar rats (age 12–21 d) were anesthetized with Nembutal (50.0 mg/kg body weight i.p.). The $^{31}P$-NMR spectra were measured on a 2.35 T Bruker Biospec 24/40 system using an FID sequence. The spectra were obtained from about 1 ml of the rat brain volume using a home-built two turn surface coil (diameter: 1.4 cm). With a pulse length of 60 µs 512 transients with 1024 datapoints were recorded with a repetition delay of 3 s and a sweep width of 2000 Hz.

For measurement with human, $^{31}P$-NMR spectra were taken from brain of healthy volunteers aged up to 3 years, 11.1 ± 3.3 years and 31.7 ± 6.6 years. Muscle spectra (M. gastrocnemius, M. vastus medialis) from juveniles (12.2 ± 0.3 years) and adults (29.1 ± 6.7 years) were estimated. Informed consent was obtained from all volunteers or from the parents respectively.

The spectra were obtained with a Siemens Magnetom SP63 whole body scanner operating at 1.5 T. Using a surface coil (diameter: 5 cm) and an FID sequence the 1024 time domain data were measured. In all cases 256 scans were recorded with a repetition delay of 3 s and a sweep width of 2000 Hz. The pulse length (500 µs) was chosen to suppress signal contribution from superficial tissue. Prior to the FFT the acquired time domain data were processed using an exponential multiplication (1b = 15 Hz for the rat brain spectra; 1b = 1.5 Hz for the human spectra) and a zerofilling to 2048 datapoints. The spectra were manually baseline corrected and the characteristics of the resonances (frequency, area, half line width, amplitude) were determined with a Marquardt-Levenberg [14] algorithm.

## Calculations

In the sequel, the determination of $[Mg^{2+}]_i$ will use the dissociation constants given in Tab. 1. These constants were derived from standard reaction quantities given by *Alberty* and *Goldberg* [15]. They were converted to the temperature of T = 310K using van't Hoff's equation and to the appropriate ionic strength I = 0.15 M using the extended Debye-Hückel theory to mimic physiological conditions.

Tab. 1: Dissociation constants of ATP species (in µM). Dissociation constants of ATP species were scaled to the temperature of 310 K, an ionic strength of I = 0.15 M and pH = 7.2. These constants are based on values given by *Alberty* and *Goldberg* [15].

| | | |
|---|---|---|
| $K_{a4}$ | $(HATP^{3-} \rightleftharpoons ATP^{4-} + H^+)$ | 0.17 |
| $K_D^{MgATP}$ | $(MgATP^{2-} \rightleftharpoons ATP^{4-} + Mg^{2+})$ | 44.30 |
| $K_D^{MgHATP}$ | $(MgHATP^- \rightleftharpoons HATP^{3-} + Mg^{2-})$ | 5430.00 |

The intracellular pH was calculated from the NMR spectra using eq. 2 published by *Petroff* et al. [16]:

$$pH = pK_a + \log\left(\frac{\delta^x - 3.29}{5.68 - \delta^x}\right) \qquad (2)$$

where $\delta^x$ equals the chemical shift difference between the PCr and $P_i$ resonances. The $PK_a$ for the reaction $H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$ is 6.77. The two constants (3.29 and 5.68) are the chemical shift differences for the acid and base species of this reaction. The dissociation constant at pH = 7.2 was corrected for intracellular pH of brain and muscle according to the individual NMR spectra, using the formula of *Bock* et al. [17] by multiplication $K_D^{MgATP}$ with factor f:

$$f = \frac{1 + 10^{pK\ pH}}{1 + 10^{pK-7.2}} \qquad (3)$$

The free $Mg^{2+}$ concentrations were calculated according to eq. 1:

$$\phi = \frac{[ATP]_{free}}{[ATP]_{free} + [MgATP]} \qquad (4)$$

$\phi$, which represents the ratio of free ATP to the total amount of ATP, was calculated from the chemical shift differences of $\alpha$-, $\beta$-, $\gamma$-phosphoryl group resonances of ATP (eqs. 5–7):

$$\phi = \frac{\delta^{x}_{\alpha\beta} - \delta^{MgATP}_{\alpha\beta}}{\delta^{ATP}_{\alpha\beta} - \delta^{MgATP}_{\alpha\beta}} \quad (5)$$

$$\phi = \frac{\delta^{x}_{\beta\gamma} - \delta^{MgATP}_{\beta\gamma}}{\delta^{ATP}_{\beta\gamma} - \delta^{MgATP}_{\beta\gamma}} \quad (6)$$

$$\phi = \frac{1}{2} \cdot \left(\frac{\delta^{x}_{\alpha\beta} - \delta^{MgATP}_{\alpha\beta}}{\delta^{ATP}_{\alpha\beta} - \delta^{MgATP}_{\alpha\beta}} + \frac{\delta^{x}_{\beta\gamma} - \delta^{MgATP}_{\beta\gamma}}{\delta^{ATP}_{\beta\gamma} - \delta^{MgATP}_{\beta\gamma}}\right) \quad (7)$$

From eqs. 5–7, it is assumed that ATP has pK values stated in [15] and that protonated ATP ($HATP^{3-}$) has a negligible affinity for $Mg^{2+}$. However, under intracellular conditions, there may be several species of ATP, e.g. $HATP^{3-}$, $H_2ATP^{2-}$, $MgHATP^{-}$, $MgH_2ATP$, $Mg_2ATP$, etc. [18, 19]. The interference of four of these ATP species was considered in eq. 8:

$$[Mg^{2+}]_i = K_D^{MgATP} \cdot \frac{\delta^{x} \cdot (1 + a) - (\delta_1 + a \cdot \delta_2)}{(\delta_3 + b \cdot \delta_4) - \delta^{x} \cdot (1 + b)} \quad (8)$$

where $a = [H^+]/K_{a4}$ and $b = a \cdot (K_D^{MgATP}/K_D^{MgHATP})$. The parameters $\delta_1$, $\delta_2$, $\delta_3$ and $\delta_4$ correspond to the $^{31}P$ chemical shifts of the four species $ATP^{4-}$, $HATP^{3-}$, $MgATP^{2-}$ and $MgHATP^{-}$, respectively. $K_D^{MgATP}$ is the dissociation constant for the $Mg^{2+}/ATP^{4-}$ equilibrium as in eq. 1, $K_{a4}$ is the dissociation constant for the $H^+/ATP^{4-}$ equilibrium and $K_D^{MgHATP}$ is the dissociation constant for the $Mg^{2+}/HATP^{3-}$ equilibrium. Tab. 1 shows the constants used.

A similar approach was published by *Leclerc* et al. [20]. Here the parameter $\delta^{x}_{\alpha\beta}$ was expanded to summarize all significant exchange processes and $[Mg^{2+}]_i$ was calculated by eq. 1.

Using the chemical shift of the $\beta$ ATP peak referenced to the PCr resonance frequency leads to eq. 9 for $\phi$:

$$\phi = \frac{\delta^{x}_{PCr\beta} - \delta^{MgATP}_{PCr\beta}}{\delta^{ATP}_{PCr\beta} - \delta^{MgATP}_{PCr\beta}} \quad (9)$$

Finally free $Mg^{2+}$ concentration was calculated from the $\beta/\alpha$ and the $\gamma/\alpha$ peak height ratios according to eqs. 10 and 11 [12]:

$$[Mg^{2+}]_i = \frac{2.5 \cdot K_D^{MgATP} \cdot (1 + 10^{pK_{a4} - pH}) \cdot h_{(\beta/\alpha)}}{h_{(\beta/\alpha)max} - h_{(\beta/\alpha)}} \quad (10)$$

$$[Mg^{2+}]_i = \frac{K_D^{MgATP} \cdot (1 + 10^{pK_{a4} - pH}) \cdot h_{(\gamma/\alpha)}}{h_{(\gamma/\alpha)max} - h_{(\gamma/\alpha)}} \quad (11)$$

For $K_D^{MgATP}$ and $K_{a4}$ the values 44.30 $\mu$M and 0.17 $\mu$M from Tab. 1 were used. The constants for $h_{(\alpha/\beta)max}$ and $h_{(\gamma/\alpha)max}$ were determined to 1.53 and 0.9 according to Ref. [12].

The in vivo spectra were corrected for the saturation effects due to the incomplete relaxation caused by the repetition time (TR). Assuming a 90° excitation pulse, the $\alpha$, $\beta$ and $\gamma$ peak heights were multiplied by the factor

$$g = [1 - \exp(-\frac{TR}{T_1})]^{-1}$$

for the individual resonances in vivo spectra. The used values for the spin - lattice relaxation time $T_1$ (in s) are shown in Tab. 2.

Tab. 2: Spin-lattice relaxation times $T_1$ for $\alpha$, $\beta$, $\gamma$P in ATP. $T_1$ values were used to circumvent the saturation effects due to incomplete relaxation [38].

| | $T_1$ (s) | | |
|---|---|---|---|
| | $\gamma$ | $\alpha$ | $\beta$ |
| rat brain | 2.00 | 1.50 | 1.60 |
| human brain | 0.65 | 0.85 | 0.80 |
| human muscle | 4.76 | 3.60 | 4.31 |

## Results

Tab. 3 summarizes the values for the concentration of free $Mg^{2+}$ in MgATP solutions and various cell types, which were calculated from the same $^{31}P$-NMR spectra by different methods (eqs. 5–11). For better comparison of these various methods in Tab. 4, the absolute values from Tab. 3 are given as relative ones, taken those derived from eq. 5 as 100 %.

As shown from the means in Tab. 4, the use of eq. 6 ($\beta\gamma$ shift differences) yielded 12.5 % lower values for $[Mg^{2+}]_i$ compared to the results of eq. 5. The values for $[Mg^{2+}]_i$ calculated from the mean of the $\alpha\beta$ and $\beta\gamma$ shift differences (eq. 7) showed a decrease of 7.2 %. This latter value is in agreement with the theoretically expected arithmetical mean. Furthermore, these computed values and their small differences demonstrate the reliability of these methods.

Calculation of $[Mg^{2+}]_i$ taking into account several species of ATP complexes (eq. 8) revealed 100.6 % and thus nearly the same values as obtained by eq. 5.

Using the $\beta$ ATP peak referenced to the PCr resonance frequency as stated by eq. 9 resulted in 163.2 % of the $[Mg^{2+}]_i$ obtained from eq. 5.

Computations of free $Mg^{2+}$ concentration from the $\beta/\alpha$ and $\gamma/\alpha$ peak height ratios according to eqs. 10 and 11 yielded only 36.7 and 32.2 % of $[Mg^{2+}]_i$ as compared to eq. 5.

## Discussion

From these results it can be concluded that the calculations using eqs. 5 to 8 yield the most probable values for $[Mg^{2+}]_i$. Thus, the values obtained from eq. 5 which was used by most authors yielded reasonable values for $[Mg^{2+}]_i$. However, these results must be considered with respect to the used $K_D^{MgATP}$ in eq. 1 (see below).

The calculation of $[Mg^{2+}]_i$ by eqs. 6 and 7 resulted only in minor different values compared to those obtained by eq. 5. These small differences may be explained by the various complexes which are formed by $Mg^{2+}$ and ATP.

$[Mg^{2+}]_i$ measurement by resonance shifts in $^{31}P$-NMR is based on $Mg^{2+}$ chelate formation with ATP, which

Tab. 3: Free $Mg^{2+}$ concentration in 3.7 mM MgATP solutions and $[Mg^{2+}]_i$ in erythrocytes, brains and muscles of various species determined by $^{31}$P-NMR and calculated by various methods. For details see Materials and methods. Values (in µM) are means ± SEM of n measurements. Significant differences to eq. 5 by unpaired Student's t-test; a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.

| | Calculation according to: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | Eq. 5 | Eq. 6 | Eq. 7 | Eq. 8 | Eq. 9 | Eq. 10 | Eq. 11 | n |
| MgATP | 502.8 | 383.2 | 435.5 | 410.0 | | 45.7 | 28.7 | 2 |
| **Erythrocytes** | | | | | | | | |
| Pig | 192.9 ± 0.1 | 165.7 ± 2.1[c] | 178.5 ± 1.2[c] | 205.3 ± 0.1[c] | | 25.5 ± 0.1[c] | 46.0 ± 0.1[c] | 2 |
| Rat | 193.0 ± 3.4 | 197.4 ± 5.7 | 195.0 ± 1.0 | 205.4 ± 3.0 | | 14.9 ± 0.1[c] | 31.5 ± 0.1[c] | 2 |
| Cattle | 176.8 ± 1.5 | 146.8 ± 7.1 | 160.6 ± 4.7 | 191.2 ± 1.3[a] | | 13.3 ± 0.1[c] | 43.5 ± 0.1[c] | 2 |
| **Brain** | | | | | | | | |
| Rat | 474.1 ± 13.0 | 344.7 ± 15.8[c] | 390.7 ± 12.4[c] | 392.7 ± 8.3[c] | 445.9 ± 25.4 | 113.1 ± 2.4[c] | 61.4 ± 1.3[c] | 74 |
| Children (< 3 years) | 276.4 ± 4.3 | 238.6 ± 4.1[c] | 256.1 ± 3.9[c] | 323.8 ± 3.9[c] | 369.4 ± 7.5[c] | 232.1 ± 7.0[c] | 188.8 ± 7.5[c] | 31 |
| Juveniles (11.1 ± 3.3 years) | 283.8 ± 7.2 | 286.0 ± 16.9 | 283.9 ± 13.6 | 331.9 ± 10.8[c] | 402.1 ± 22.7[c] | 212.3 ± 12.3[c] | 168.3 ± 12.1[c] | 11 |
| Adults (31.7 ± 6.6 years) | 346.0 ± 23.7 | 346.4 ± 37.9 | 344.9 ± 30.9 | 385.7 ± 19.4 | 396.3 ± 28.9 | 181.6 ± 10.4[c] | 120.7 ± 8.8[c] | 10 |
| **Muscle** | | | | | | | | |
| **M. vastus medialis** | | | | | | | | |
| Juveniles (12.2 ± 0.3 years) | 508.4 ± 13.7 | 390.7 ± 9.9[c] | 441.4 ± 10.2[c] | 464.6 ± 9.0[c] | 983.5 ± 51.0[c] | 163.6 ± 9.8[c] | 126.0 ± 5.4[c] | 11 |
| Adults (29.1 ± 6.7 years) | 413.7 ± 7.5 | 326.2 ± 9.3[c] | 365.0 ± 8.0[c] | 398.7 ± 5.2 | 677.1 ± 37.7[c] | 266.1 ± 42.9[c] | 255.1 ± 43.0[c] | 11 |
| **M.gastrocnemius** | | | | | | | | |
| Juveniles | 522.5 ± 11.0 | 457.6 ± 19.4[b] | 485.5 ± 12.8[a] | 479.7 ± 7.9[b] | 1169.1 ± 70.4[c] | 160.9 ± 3.5[c] | 116.6 ± 2.6[c] | 12 |
| Adults | 472.1 ± 13.7 | 467.2 ± 17.4 | 468.8 ± 12.8 | 453.8 ± 8.2 | 1135.0 ± 64.2[c] | 188.5 ± 6.1[c] | 150.1 ± 8.5[c] | 11 |

induces a resonance shift. MgATP can in principle have seven different structural isomeres ($\alpha$-, $\beta$-, $\gamma$-monodendate, $\alpha\beta$-, $\beta\gamma$-, $\alpha\gamma$-bidendate and $\alpha\beta\gamma$-tridentate). The $\alpha$ and $\beta$ P atoms are prochiral centers. Therefore, MgATP can be a mixture of 17 different stereoisomers [21].

The $\alpha\beta$ and $\beta\gamma$ resonance shifts may be caused by different structural isomers of MgATP. These may have different $K_D$'s. However, in the calculations of $[Mg^{2+}]_i$ the overall dissociation constant is taken. The differences in $[Mg^{2+}]_i$ would be eliminated if the individual $K_D$'s were used. Since the macroscopic $K_D^{MgATP}$ is the weighted mean of all $K_D$'s, the real value of $[Mg^{2+}]_i$ must be different from values calculated by using a $K_D^{MgATP}$ = 44.3 µM (Tab. 1), which represents the $\beta\gamma$ bidentate chelate.

However, in some cases, probably by incorrect determination of the resonance frequency, due to a low signal to noise ratio and digital resolution, the values for $[Mg^{2+}]_i$ from eqs. 5–7 were similar. This may be the reason, why *Gupta* et al. [9] did not find a significant difference of $[Mg^{2+}]_i$ calculated from the $\alpha\beta$ resonance shifts and the arithmetic means of the $\alpha\beta$ and $\beta\gamma$ shifts. Also, in some of our measurements (rat erythrocytes and brain of juveniles and adults) eqs. 5, 6 and 7 yielded the same values.

When 4 ATP species, listed in Tab. 1 ($ATP^{4-}$, $HATP^{3-}$, $MgATP^{2-}$, $MgHATP^{-}$), were taken into account in the calculation of $[Mg^{2+}]_i$ from $\alpha\beta$ chemical shift resonances (eq. 8), the same values for $[Mg^{2+}]_i$ were obtained as by eq. 5, indicating that the other ATP species did not play a significant role. $[Mg^{2+}]_i$ as determined from the PCr-$\beta$ ATP resonance shift differences revealed up to twice higher values than $[Mg^{2+}]_i$ calculated from the $\alpha\beta$ and/or $\beta\gamma$ shift differences. We have no explanation for these high values. Only in rat brain was $[Mg^{2+}]_i$ (eq. 9) the same as by the $\alpha\beta$ resonance method (eq. 5).

Values of $[Mg^{2+}]_i$ estimated by $\beta/\alpha$ and $\gamma/\alpha$ peak height ratios were considerably lower than $[Mg^{2+}]_i$ from all other methods, although the same value for $K_D^{MgATP}$ was used. Therefore, this method does not yield reliable and reasonable results. Also other authors [22] reported incorrect values for $[Mg^{2+}]_i$ using eq. 10. Since the measured peak height ratio strongly depends on the field homogeneity, inhomogeneity of the field may be a reason for the unrealistic low values by peak height ratios. Very recently, it was found, that the determination of $[Mg^{2+}]_i$ using MgATP solutions based on the peak-height ratio of the $\beta$ and $\alpha$ resonances of ATP ($h_{\beta/\alpha}$) according to eq. 10 has numerous problems. These problems include a flaw in the basic multiple equilibrium analysis, the inapplicability of the calibration curve to in vivo studies, and a lack of sensitivity of the measured ratio to changes in $[Mg^{2+}]_i$ at clinical field strength [23].

Invisibility of ATP in $^{31}$P NMR as another reason for unrealistic $[Mg^{2+}]_i$ values by peak height ratios can be excluded. Intracellular metabolites may be unobservable by $^{31}$P-NMR as a consequence of binding to macromolecular structures, localization in compartments with high viscosity or association to paramagnetic ions. In erythrocytes about 65 % of ATP may be invisible in $^{31}$P-NMR spectra irrespective of oxygen saturation. This effect was explained by binding of ATP to erythrocyte membranes [24].

Tab. 4: Relative values for the concentration of free $Mg^{2+}$ (in %) in 3.7 mM MgATP solutions, in erythrocytes, brain and muscle of various species determined by $^{31}P$ NMR and calculated by various methods. Values were taken from Tab. 3, those determined by eq. 5 were taken as 100 %.

| | Calculation according to: | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sample** | Eq. 5 | Eq. 6 | Eq. 7 | Eq. 8 | Eq. 9 | Eq. 10 | Eq. 11 |
| MgATP | 100.0 | 76.2 | 86.6 | 81.5 | | 9.1 | 5.7 |
| **Erythrocytes** | | | | | | | |
| Pig | 100.0 | 85.9 | 92.5 | 106.4 | | 13.2 | 23.8 |
| Rat | 100.0 | 102.3 | 101.0 | 106.4 | | 7.7 | 16.3 |
| Cattle | 100.0 | 83.0 | 90.8 | 108.1 | | 7.5 | 24.6 |
| **Brain** | | | | | | | |
| Rat | 100.0 | 72.7 | 82.4 | 82.8 | 94.1 | 23.9 | 13.0 |
| Children (< 3 years) | 100.0 | 86.3 | 92.7 | 117.2 | 133.7 | 84.0 | 68.3 |
| Juveniles (11.1 ± 3.3 years) | 100.0 | 100.8 | 100.2 | 117.0 | 141.9 | 75.3 | 59.3 |
| Adults (31.7 ± 6.6 years) | 100.0 | 100.1 | 99.7 | 111.5 | 114.5 | 52.5 | 34.9 |
| **Muscle** | | | | | | | |
| **M. vastus medialis** | | | | | | | |
| Juveniles (12.2 ± 0.3 years) | 100.0 | 76.9 | 86.8 | 91.4 | 193.5 | 32.2 | 24.8 |
| Adults (29.1 ± 6.7 years) | 100.0 | 78.9 | 88.2 | 96.4 | 163.7 | 64.3 | 61.7 |
| **M.gastrocnemius** | | | | | | | |
| Juveniles | 100.0 | 87.6 | 92.9 | 91.8 | 223.8 | 30.8 | 22.3 |
| Adults | 100.0 | 99.0 | 99.3 | 96.1 | 240.4 | 39.3 | 31.8 |
| Mean ± SEM | 100.0 | 87.5 ± 3.1 | 92.8 ± 1.8 | 100.6 ± 3.6 | 163.2 ± 18.4 | 36.7 ± 7.8 | 32.2 ± 5.9 |

Also, in ischemic liver a part of ATP particularly mitochondrial ATP was invisible by $^{31}P$-NMR. This was concluded from the lesser decrease of ATP in ischemia as measured by chemical methods than by NMR [25]. In contrast, *Desmoulin* et al. [26] did not find a decrease of the visibility of ATP in liver cells. However, invisible ATP compartments may not affect $[Mg^{2+}]_i$ determination because chemical shift differences or peak height ratios are used. Only if the individual $\alpha$, $\beta$ and $\gamma$ peaks of ATP showed a different relaxation behavior would an effect be seen. Moreover, invisibility of ATP can also be excluded since the peak height ratio yielded unrealistic values for free $[Mg^{2+}]$ in MgATP solutions (Tab. 3, [23]).

In mitochondria-containing cells only single shift differences of ATP are seen. This may be due to the result that $[Mg^{2+}]_i$ in cytosol and matrix of mitochondria is not significantly different [27, 28].

There are further problems in determining the absolute value of $[Mg^{2+}]_i$. All equations for calculation of $[Mg^{2+}]_i$ based on complexation of $Mg^{2+}$ with ATP need the dissociation constant of the MgATP complex. This constant was reported to be within a wide range between 10.0 and 145.0 µM [29]. (See also Ref. [30]). $K_D^{MgATP}$ is measured in solutions adapted to the intracellular concentrations of $K^+$, $Na^+$ and $H^+$. However, all these solutions contain $Cl^-$ as the anion. Therefore, in erythrocytes which contain a high intracellular $Cl^-$ concentration amounting to 84.4 mM [31], the $K_D$ of MgATP in these cells is most adapted to the in vitro measured $K_D^{MgATP}$ value. In all other cell types, the intracellular $Cl^-$ concentration is low (1–3 mM [32] or 10–20mM [39]) and other anions predominate. When $K_D$ of MgATP was determined in the presence of 3.5 mM inorganic phosphate, a higher value of $K_D^{MgATP}$ was received [33] than in phosphate-free solutions. This may be due to the lower activity coefficient of $Mg^{2+}$ in the presence of other anions (phosphate) in addition to $Cl^-$. Recently, $[Mg^{2+}]_i$ values from the literature were recalculated using newly measured and recalculated $K_D^{MgATP}$ values [30]. All these recalculated values for $[Mg^{2+}]_i$ were higher by the factor 2–3 and reached values around 1 mM, corresponding to an intracellular pMg = 3, which is in the range of the pMg optimum of most Mg-dependent enzymes [2].

The same problem of the real $K_D$ of Mg complexes is involved in the determination of $[Mg^{2+}]_i$ by means of fluorescent dyes, e.g. mag-fura-2. Also, in this case the dissociation constant for the Mg-mag-fura-2 chelate estimated under in vivo conditions was about 3 times higher than under in vitro conditions (5.4 mM versus 1.5 mM) [34]. Therefore, also $[Mg^{2+}]_i$ measured by mag-fura-2 would be in the range of 1 mM, when using the higher $K_D$ for intracellular conditions.

Moreover, there is a fundamental problem when discussing the effects of $[Mg^{2+}]_i$ on intracellular functions. $[Mg^{2+}]_i$ is compartmentalized [35]. As found by confocal laser UV microspectrofluorometry with the fluorescent dye mag-indo-1 in single human tracheal gland cells there was a large spatial heterogeneity of cytosolic $[Mg^{2+}]_i$ varying from one subcellular region (0.34 mM) to another opposite region (up to 3 mM) in the same cell [36].

Thus, measurements of $[Mg^{2+}]_i$ by means of $^{31}P$-NMR must be seen under these limitations and restrictions. They can only give an spatial integral value of $[Mg^{2+}]_i$ for cytosolic $[Mg^{2+}]_i$ with respect to the used $K_D$ for MgATP. Its real value remains open, although *Alberty* [37] favors a lower value of $K_D^{MgATP}$. Therefore, these methods can only give relative values for $[Mg^{2+}]_i$

and the results of these maesurements must be related to their controls. The advantage of the $^{31}P$-NMR method is that it works under non-invasive conditions and can be applied to humans.

## References


[1] *Ebel, H.; Günther, T.:* Magnesium metabolism: A review. J. Clin. Chem. Clin. Biochem. **18** (1980) 257–270.

[2] *Günther, T.:* Biochemistry and pathobiochemistry of magnesium. Mg.-Bull. **3** (1981) 91–101.

[3] *Johnson, J.W.; Ascher, P.:* Voltage-dependent block by intracellular $Mg^{2+}$ of N-methyl-D-aspartate-activated channels. Biophys. J. **57** (1990) 1085–1090.

[4] *Agus, Z.S.; Morad, M.:* Modulation of cardiac ion channels by magnesium. Annu. Rev. Physiol. **53** (1991) 299–307.

[5] *Meissner, G.; Darling, E.; Eveleth, J.:* Kinetics of rapid $Ca^{2+}$ release by sarcoplasmic reticulum. Effects of $Ca^{2+}$, $Mg^{2-}$, and adenine nucleotides. Biochemistry **25** (1986) 236–244.

[6] *Günther, T.:* Über die Rolle des Mg bei der Regulation des Zellstoffwechsels. Z. Naturforsch. **21b** (1966) 1174–1177.

[7] *Doumen, C.; Ellington, W.R.:* Intracellular free magnesium in the muscle of an osmoconforming marine invertebrate: Measurement and effect of metabolic and acid-base perturbations. J. Exp. Zool. **261** (1992) 394–405.

[8] *Williams, G.D.; Mosher, T.J.; Smith, M.B.:* Simultaneous determination of intracellular magnesium and pH from the three $^{31}P$ NMR chemical shifts of ATP. Anal. Biochem. **214** (1993) 458–467.

[9] *Gupta, R.K.; Moore, R.D.:* $^{31}P$ NMR studies of intracellular free $Mg^{2+}$ in intact frog skeletal muscle. J. Biol. Chem. **255** (1980) 3987–3993.

[10] *Günther, T.; Vormann, J.; Konstanczak, P.; Schäfer, A.:* Interaction of polyamines in the measurement of free magnesium concentration by mag-fura-2 and $^{31}P$-NMR. Biochim. Biophys. Acta **1192** (1994) 281–285.

[11] *Iotti, S.; Frassineti, C.; Alderighi, L.; Sabatini, A.; Vacca, A.; Barbiroli, B.:* In vivo assessment of free magnesium in human brain by $^{31}P$ MRS. A new calibration curve based on a mathematical algorithm. NMR Biomed. **9** (1996) 24–32.

[12] *Clarke, K.; Kashiwaya, Y.; King, M.T.; Gates, D.; Keon, C.A.; Cross, H.R.; Radda, G.K.; Veech, R. L.:* The $\beta/\alpha$ peak height ratio of ATP. J. Biol. Chem. **271** (1996) 21142–21150.

[13] *Miseta, A.; Bogner, P.; Berenyi, E.; Kellermayer, M.; Galambos, C.; Wheatley, D.N.; Cameron, I.L.:* Relationship between cellular ATP, potassium, sodium and magnesium concentrations in mammalian and avian erythrocytes. Biochim. Biophys. Acta **1175** (1993) 133–139.

[14] *Marquardt, D.W.:* An algorithm for least squares estimation of nonlinear parameters. J. Soc. Indus. Appl. Math. **11** (1963) 431–441.

[15] *Alberty, R.A.; Goldberg, R.N.:* Standard thermodynamic formation properties for the adenosine 5'-triphosphate series. Biochemistry **31** (1992) 10610–10615.

[16] *Petroff, O.A.C.; Prichard, J.W.; Behar, K.L.; Alger, J.R.; den Hollander, J.A.; Shulman, R.G.:* Cerebral intracellular pH by $^{31}P$ nuclear magnetic resonance spectroscopy. Neurology **35** (1985) 781–788.

[17] *Bock, J.L.; Wenz, B.; Gupta, R.K.:* Changes in intracellular Mg adenosine triphosphate and ionized $Mg^{2+}$ during blood storage: Detection by $^{31}P$ nuclear magnetic resonance. Blood **65** (1985) 1526–1530.

[18] *Golding, E.M.; Golding, R.*M.: Interpretation of $^{31}P$ MRS spectra in determining intracelular free magnesium and potassium ion concentrations. Mag. Res. Med. **33** (1995) 467–474.

[19] *Golding, E.M.; Dobson, G.P.; Golding, R.M.:* A critical assessment of noise-induced errors in $^{31}P$ MRS: Application to the measurement of free intracellular magnesium in vivo. Mag. Res. Med. **35** (1996) 174–185.

[20] *Leclerc, J.H.J.; Wary, C.; Carlier, P.G.:* Determination of intracellular magnesium concentration by P-31 nuclear magnetic resonance spectroscopy – a new quantification technique based on the Wigner distribution. In: *Smetana, R.* (ed), Advances in Magnesium Research. John Libbey and Company LTD, London 1997, pp. 577–585.

[21] *Huang, S.L.; Tsai, M.*D.: Does the magnesium(II)ion interact with the α phospate of adenosine triphosphate? An investigation by oxygen-17 nuclear magnetic resonance. Biochemistry **21** (1982) 951–959.

[22] *Widmaier, S.; Jung, W.-I.; Dietze, G.l.; Lutz, O.:* Pitfall in the determination of free $[Mg^{2+}]$ by $^{31}P$ NMR when using the $\beta/\alpha$-ATP peak height ratio method. In: Proc. Int. Soc. Mag. Res. Med., Sydney 1998, pp. 1858.

[23] *Mulquiney, P.J.; Kuchel, P.W.:* Using the $\beta/\alpha$ peak-height ratio of ATP in $^{31}P$ NMR spectra to measure free $[Mg^{2+}]$: theoretical and practical problems. NMR Biomed **12** (1999) 217–220.

[24] *Petersen, A.; Kristensen, S.R.; Jacobsen, J.P.; Hørder, M.:* $^{31}P$-NMR measurements of ATP, ADP, 2,3-diphosphoglycerate and $Mg^{2+}$ in human erythrocytes. Biochim. Biophys. Acta **1035** (1990) 169–174.

[25] *Murphy, E.; Gabel, S.A.; Funk, A.; London, R.E.:* NMR observability of ATP: Preferential depletion of cytosolic ATP during ischemia in perfused rat liver. Biochemistry **27** (1988) 526–528.

[26] *Desmoulin, F.; Cozzone, P.J.; Canioni, P.:* Phosporous-31 nuclear-magnetic-resonance study of phosporylated metabolites compartmentation, intracellular pH and phosphorylation state during normoxia, hypoxia and ethanol perfusion, in the perfused rat liver. Eur. J. Biochem. **162** (1987) 151–159.

[27] *Günther, T.:* Die intrazelluläre Mg-Ionenkonzentration. Z. Naturforsch. **22b** (1967) 149–154.

[28] *Jung, D.W.; Panzeter, E.; Baysal, K; Brierley, G.P.:* On the relationship between matrix free $Mg^{2+}$ concentration and total $Mg^{2+}$ in heart mitochondria. Biochim. Biophys. Acta **1320** (1997) 310–320.

[29] *Sillén, L.G.; Martell, A.E.:* Stability Constants. Special Publication No. 25. The Chemical Society, Burlington House, London 1971.

[30] *Zhang, W.; Truttmann, A.C.; Lüthi, D.; McGuigan, J.A.S.:* Apparent $Mg^{2+}$-adenosine 5-triphosphate dissociation constant measured with $Mg^{2+}$ macroelectrodes under conditions pertinent to $^{31}P$ NMR ionized magnesium determinations. Anal. Biochem. **251** (1997) 246–250.

[31] *Zidek, W.; Losse, H.; Lange-Aschenfeldt, H.; Vetter, H.:* Intracellular chloride in essential hypertension. Clin. Sci. **68** (1985) 45–47.

[32] *Godt, R.E.; Maughan, D.W.:* On the composition of the cytosol of relaxed skeletal muscle of the frog. Am. J. Physiol. **254** (1988) C591–C604.

[33] *Malloy, C.R.; Cunningham, C.C.; Radda, G.K.:* The metabolic state of rat liver in vivo measured by $^{31}P$-NMR spectroscopy. Biochim. Biophys. Acta **885** (1986) 1–11.

[34] *Tashiro, M.; Konishi, M.:* Basal Intracellular free $Mg^{2+}$ concentration in smooth muscle cells of guinea pig tenia cecum: intracellular calibration of the fluorescent indicator furaptra. Biophys. J. **73** (1997) 3358–3370.

[35] *Günther, T.:* Functional compartmentation of intracellular magnesium. In: *Sigel, H.; Sigel, A.* (eds), Metal Ions in Biological Systems, Marcel Dekker, New York, **26** (1990) pp. 193–213.

[36] *Sébille, S.; Millot, J.-M.; Maizieres, M.; Arnaud, M.; Delabroise, A.-M.; Jacquot, J.; Manfait, M.:* Spatial and temporal $Mg^{2+}$ signaling in single human tracheal gland cells. Biochem. Biophys. Res. Commun. **227** (1996) 743–749.

[37] *Alberty, R.A.:* Change in the binding of hydrogen ions and magnesium ions in the hydrolysis of ATP. Biophys. Chem. **70** (1998) 109–119.

[38] *de Vré, R.M.; Delporte, C.:* Relaxation times of relevant nuclei in biological systems. In: *Certaines, J.D.D.; Bovée, W.M.M. J.; Podo, F.* (eds): Magnetic Resonance Spectroscopy in Biology and Medicine. Pergamon Press, Oxford 1992, pp. 77–96.

[39] *Hume, J.R.; Duan, D.; Collier, M.L.; Yamazaki, J.; Horowitz, B.:* Anion transport in heart. Physiol. Rev. **80** (2000) 31–81.



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