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# Calculation of Free-$Mg^{2+}$ Concentration in Adenosine 5′-Triphosphate Containing Solutions in Vitro and in Vivo†

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ABSTRACT: We have attempted to resolve the differences between the levels of free $Mg^{2+}$ in muscle calculated by Wu et al. [Wu, S. T., Pieper, G. M., Salhany, J. M., & Eliot, R. S. (1981) *Biochemistry 20*, 7399–7403] (2.5 mM in guinea pig heart) and by Gupta and Moore [Gupta, R. K., & Moore, R. D. (1980) *J. Biol. Chem. 255*, 3987–3993] (0.6 mM in frog *skeletal* muscle) on the basis of substantially identical measurements by $^{31}P$ NMR of the phosphate peaks in the spectrum of $MgATP^{2-}$. The differences depend on the methods of calculation, including which reactions in which multiple equilibria are being considered. Biochemists and physical chemists customarily use different working definitions of the stability constant for $MgATP^{2-}$ in particular. Wu et al. used in their calculations, without reconciliation, methods involving three different operational definitions of the chelation equilibria involved. An algorithm for calculating $Mg^{2+}$ and total ATP, which can be carried out with a hand calculator, is described here. With it, we calculated $Mg^{2+}$ levels that agree with those determined by Gupta et al. [Gupta, R. K., Benkovic, J. L., & Rose, Z. B. (1978) *J. Biol. Chem. 253*, 6165–6171] with their in vitro systems. We therefore agree with the finding of Gupta and Moore that the $Mg^{2+}$ level in skeletal and cardiac muscle is 0.6 mM.

Recently, Wu et al. (1981) published a determination of free $Mg^{2+}$ in perfused guinea pig heart based on the known shift in the $^{31}P$ NMR spectrum of ATP due to $Mg^{2+}$. They calculated a $Mg^{2+}$ level of 2.5 mM, in disagreement with the value of 0.6 mM found by Gupta & Moore (1980) for skeletal muscle in frog. Since the NMR spectra reported by both groups were nearly identical, this disagreement arises from different interpretations of the data. Gupta and Moore used

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an apparent stability constant for MgATP that was evaluated from NMR measurements in vitro. (Gupta et al., 1978a). Wu et al. evaluated free cytosolic $Mg^{2+}$ with a computer program that solved the multicomponent equilibrium involving $Mg^{2+}$, $ATP^{4-}$, $K^+$, and $H^+$ and other components. They assumed that 85% of the $Mg^{2+}$ present in the heart was in equilibrium with all of the ATP and other Mg-binding metabolites. They then argued that Gupta and Moore were using the wrong apparent stability constant for $MgATP^{2-}$. While attempting to check the work of Wu et al., we found that they were using three functional definitions for the stability constant of $MgATP^{2-}$ and that one of their tables was calculated erroneously. These errors cast doubt on the validity of their computed value.

Our interest in this subject stems from its relevance to our simulations of cardiac metabolism (Achs et al., 1982). In our work, we have calculated cytosolic levels of free $Mg^{2+}$ that were less than 1 mM, more nearly in agreement with the findings of Gupta and Moore. We concluded that there was metabolic control by $Mg^{2+}$, which would not be operative at the high $Mg^{2+}$ levels reported by Wu et al. The precise value of free $Mg^{2+}$ is important in determining the degree to which Mg-binding ligands are in their active or inhibitory forms.

Cohn & Hughes (1962) showed that the positions of the three $^{31}P$ NMR peaks of ATP are shifted when ATP is complexed to $Mg^{2+}$. Gupta et al. (1978b) used the shifts to measure noninvasively in intact cells the proportion of ATP complexed to $Mg^{2+}$, and from this, the free intracellular $Mg^{2+}$ was calculated. The calculation required the use of a dissociation constant for MgATP that was appropriate under intracellular conditions, and this was obtained by observing with NMR the titration of ATP with $Mg^{2+}$ at pH 7.2 in 150 mM KCl to simulate physiological conditions. Gupta et al. (1978a) used the expression

$$K_D^{MgATP} = \frac{\delta_{\alpha\beta} - \delta_{\alpha\beta}^{MgATP}}{\delta_{\alpha\beta}^{ATP} - \delta_{\alpha\beta}} \left( [Mg]_T - \frac{\delta_{\alpha\beta}^{ATP} - \delta_{\alpha\beta}}{\delta_{\alpha\beta}^{ATP} - \delta_{\alpha\beta}^{MgATP}} [ATP]_T \right) \quad (1)$$

where $K_D^{MgATP}$ is the apparent dissociation constant of MgATP, $\delta_{\alpha\beta}$ is the separation between the $\beta$-P and $\alpha$-P resonances of ATP, $\delta_{\alpha\beta}^{ATP}$ and $\delta_{\alpha\beta}^{MgATP}$ are the values of $\delta_{\alpha\beta}$ with $Mg^{2+}$ absent or saturating, respectively, $[Mg]_T$ is the concentration of total Mg, and $[ATP]_T$ is the concentration of total ATP.

Their experimentally determined value for the dissociation constant, 0.045 mM, corresponds to a stability constant of 22 000 $M^{-1}$. Levels of free $Mg^{2+}$ in unknown samples were then calculated without inclusion of the concentration of total $Mg^{2+}$ according to

$$[Mg]_f = K_D^{MgATP} \left( \frac{1}{\phi} - 1 \right) \quad (2)$$

$$\phi = \frac{[ATP]_f}{[ATP]_T} = \frac{\delta_{\alpha\beta}^{cell} - \delta_{\alpha\beta}^{MgATP}}{\delta_{\alpha\beta}^{ATP} - \delta_{\alpha\beta}^{MgATP}} \quad (3)$$

where $ATP_f$ is the sum of all ATP species not chelated to $Mg^{2+}$, i.e., $[KATP^{3-}]$, $[HATP^{3-}]$, and $[ATP^{4-}]$.

Gupta et al. evaluated the levels of free $Mg^{2+}$ in intact human erythrocytes (Gupta et al., 1978b), ascites tumor cells (Gupta & Yushok, 1980), and frog muscle (Gupta & Moore, 1980). In all cases, the free $Mg^{2+}$ calculated was less than 1 mM (0.6 mM for frog muscle) and was concluded to be rate-limiting for key metabolic reactions. Gupta et al. did not offer any independent evidence for the levels of free $Mg^{2+}$ in any of their cellular samples. They relied on approximate agreement with published values for this stability constant and with similar findings from other methods including EPR to validate this method for red blood cells. Gupta et al. (1983) have since confirmed their values for free $Mg^{2+}$ in solutions containing physiological levels of ATP by monitoring $Mg^{2+}$ with the dye antipyrylazo III optically in the same solutions as those studied with NMR.

Wu et al. (1981) subsequently attempted to evaluate free $Mg^{2+}$ by applying the same experimental technique to a guinea pig heart that was perfused inside an NMR tube. Although they observed the same chemical shift for ATP as Gupta and Moore, they interpreted their results differently and concluded that the free $Mg^{2+}$ level was 2.5 mM instead of 0.6 mM. They based their conclusions partly on computations with a computer program written by Storer & Cornish-Bowden (1976) for calculating the complex equilibria involving $Mg^{2+}$, ATP, and other ligands in purified systems. Wu et al. applied the program to a whole heart basically by running the program with total heart metabolite levels under the assumption that all of the ATP was in equilibrium with 85% of the total $Mg^{2+}$. They did not report the NMR spectrum of any in vitro system whose $Mg^{2+}$ composition corresponds to what they were postulating in their perfused heart. They assumed that the water content of their heart was constant during 35 min of ischemia, even though it is known to change under such circumstances (Polimeni & Al-Sadir, 1975). They assumed that the $Mg^{2+}$ composition of the heart was constant, even though ischemic heart is known to lose $Mg^{2+}$ (Alta & Dhalla, 1979; Polimeni & Al-Sadir, 1975).

## Experimental Procedures

In an effort to determine the source of difference between Gupta et al. and Wu et al., we recalculated the results of Wu et al. In this process, we had to investigate their claim that the constants reported by many investigators were equivalent when differences in experimental conditions were considered. While checking these calculations, we found that the literature descriptions of apparent vs. intrinsic constants were generally incomplete and complicated by the use of different standard reference conditions. It was therefore necessary to find definitions that applied to the present problem. Since Gupta et al. did not offer any independent evidence for the free $Mg^{2+}$ concentration in the in vitro solutions they studied with NMR, we calculated the free $Mg^{2+}$ with a hand calculator by procedures that were equivalent to the program of Storer & Cornish-Bowden (1976). Wu et al. had used this program and the constants published with it to calculate the free $Mg^{2+}$ in their perfused guinea pig hearts. We generally used the constants given by Storer and Cornish-Bowden in our calculations. We used different values for the stability constant of MgATP only when testing their effect on the $Mg^{2+}$ concentration.

A wide variety of values for the stability constant of MgATP have been reported in the literature (Phillips et al., 1966). The values are sensitive to experimental conditions, especially pH, ionic strength, and the presence of interfering components. Many of the early measurements were made at ionic strengths that were too high to be physiologically relevant or in the presence of interacting cations or buffers. The more recently reported values for the intrinsic constant have ranged from 20 000 to 100 000 $M^{-1}$ (O'Sullivan & Smithers, 1979).

## Results and Discussion

Wu et al. (1981) attribute the difference between their calculation and Gupta's to differences in the apparent binding constant for $MgATP^{2-}$. They suggested that Gupta had failed to consider the formation of $KATP^{3-}$. However, this suggestion

neglects the fact that Gupta's observed stability constant reproduces the observed NMR spectrum. Wu et al. then gave an equation for what they consider the correct constant:

$$K' = K_{MgATP}/(1 + [H^+]K_{HATP} + [K^+]K_{KATP}) \quad (4)$$

Wu et al. furthermore attempted to show that the value of Gupta et al. for the stability constant for $MgATP^{2-}$ was incorrect by comparing it with others reported in the literature, all corrected to the same conditions: 25 °C, pH 7.0, $[K^+]$ = 0.15 M, $\mu$ = 0.15 M. These corrections supposedly made according to procedures recommended by O'Sullivan & Smithers (1979) were applied by Wu et al. as if the corrections for $K^+$ and $H^+$ were independent of each other. If they had actually followed these procedures, they would have been using

$$K' = K_{MgATP}/[(1 + [H^+]K_{HATP})(1 + [K^+]K_{KATP})] \quad (5)$$

and not the widely accepted eq 4. As O'Sullivan and Smithers only considered measurements at alkaline pH where the pH effect is small, eq 5 does not introduce serious errors into their examples. We can only match the calculations of Wu et al. by using eq 6. When we calculated stability constants with

$$K' = K_{MgATP}/[(1 + [H^+]K_{HATP}) \times (1 + [H^+]K_{HATP} + [K^+]K_{KATP})] \quad (6)$$

eq 2 using the measured $\phi$ of 0.06 and the calculated free $Mg^{2+}$ concentrations of Wu et al., we obtained the values given in column 10 of Table I. These differed from the "normalized stability constants" (given in column 7 of Table I) by a factor of 4.1–4.4, not by a factor of 3.1 that they claim to have used. Thus, the corrections were made incorrectly. As a result, all of the constants measured in the absence of $K^+$ were decreased erroneously, making the results of Gupta et al. appear to deviate very much from other measured values.

Since Wu et al. had thus calculated their $Mg^{2+}$ values incorrectly, we recalculated their results, which are shown in Table I. Table IV of Wu et al. is reproduced in columns 2–7. Column 7 gives the values cited by Wu et al. for various literature determinations of the stability constant corrected to 25 °C, pH 7, and $\mu$ = 0.15 M in the absence of $K^+$; their determination and that of Gupta et al. are included. It was necessary to calculate column 8 in order to correct the errors Wu et al. introduced with the extra term involving $H^+$. This column gives these constants corrected to a high pH, where there is very little $HATP^{3-}$ and no $K^+$. They were calculated from eq 4. Column 9 gives the values of these constants at pH 7 in the presence of 0.15 M $K^+$. The corresponding values of Wu et al. are given in column 10. The corrected concentrations for free $Mg^{2+}$ calculated with our constants from column 9 are given in column 11. The values of free $Mg^{2+}$ reported by Wu et al. are shown in column 12.

The results of Wu et al. and Gupta et al. are now in much closer agreement: the range of values for all the corrected entries in Table I is now smaller. Wu et al. obtained their incorrect values by artifactually lowering the stability constants in the literature with eq 6, which is erroneous. In our work (Kohn et al., 1977), we used a stability constant of 90 $mM^{-1}$, which was taken from the work of Asai & Morales (1962), who made their measurements at 23 °C., pH 9, and $\mu \sim 0.1$ M. When this stability constant is corrected to the conditions of Wu et al., it assumes a value of 21 $mM^{-1}$, in complete agreement with the results of Gupta et al. In our work, we obtained even lower values for $Mg^{2+}$ because we assumed a lower level of $K^+$ and a higher pH.

The three methods cited by Wu et al. are not equivalent or equally valid, despite their claims. We have considered the

Table I: Corrected Calculation of Free $Mg^{2+}$ from NMR Spectrum of Perfused Guinea Pig Heart Using Equilibrium Constants Cited by Wu et al.

| method | supporting electrolyte | pH | ionic strength (M) | temp (°C) | apparent stability constants[a] reported ($M^{-1}$) | "normalized", Wu et al. ($M^{-1}$) | cor to pH 8, $\mu$ = 0.15 M, no $K^+$, 25 °C ($M^{-1}$) | cor to pH 7, $\mu$ = 0.15 M, 0.15 M $K^+$, 25 °C: this study ($M^{-1}$) | cor to pH 7, $\mu$ = 0.15 M, 0.15 M $K^+$, 25 °C: Wu et al. ($M^{-1}$) | calcd $Mg^{2+}$ ($\phi$ = 0.06): this study (mM) | calcd $Mg^{2+}$ ($\phi$ = 0.06): Wu et al. (mM) | ref |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| competition with 8-hydroxyquinoline | *N*-ethylmorpholine buffer | 8.0 | 0.05 | 30 | 73 000 | 26 000 | 53 800 (a) | 12 900 (c) | 6 200 | 1.24 | 2.6 | O'Sullivan & Perrin (1964) |
| | triethanolamine buffer | 8.4 | 0.1 | 25 | 38 000 | 20 300 | 42 000 (a) | 10 000 (c) | 4 900 | 1.60 | 3.3 | Burton (1959) |
| | triethanolamine buffer | 8.2 | 0.05 | 25 | 80 000 | 30 400 | 62 900 (a) | 15 100 (c) | 7 300 | 1.06 | 2.2 | Watanabe et al. (1963) |
| resin competition | $(C_3H_7)_4$, NBr | 8.7 | 0.1 | 25 | 42 700 | 23 000 | 47 600 (a) | 11 400 (c) | 5 500 | 1.39 | 2.9 | Phillips et al. (1966) |
| pH titration | $(C_2H_5)_4$, NBr | 7.0 | 0.1 | 25 | 26 700 | 26 000 | 53 800 (a) | 12 900 (c) | 6 200 | 1.24 | 2.6 | Nanninga (1961) |
| $^{31}P$ NMR | KCl | 7.2 | 0.15 | 25 | 22 000 | 59 000 | 83 300 (b) | 20 000 (c) | 14 100 | 0.80 | 1.1 | Gupta & Moore (1980) |
| 8-hydroxyquinoline-5-sulfonate | KCl in 25 mM Hepes buffer | 7.1 | 0.15 | 25 | 9 800 | 29 000 | 40 000 (b) | 9 000 (c) | 7 000 | 1.6 | 2.2 | Wu et al. (1981) |

[a] Stability constants for KATP and HATP were taken from Table II. Equations a and b are variants of eq 4; eq b reduces to eq a in the absence of $K^+$. The letters in parentheses correspond to the following equations: eq a, $K_{pH8} = K_{pH7}(1 + [H^+]K_{HATP}) = 2.07K_{pH7}$; eq b, $K_{pH8} = K_{pH7}(1 + [H^+]K_{HATP} + [K^+]K_{KATP}) = 4.17K_{pH7}$; eq c, $K_{pH7,0.15M K^+} = K_{pH8}/(1 + [H^+]K_{HAPT} + [K^+]K_{KATP}) = K_{pH8}/4.17$.

Table II: Calculation of Equilibria (Including Weak Interactions of ATP and $Cl^-$)[a]

| equilibria considered | $K$ ($M^{-1}$) | ref |
|---|---|---|
| $H^+ + ATP^{4-} = HATP^{3-}$ | $1.09 \times 10^7$ | Phillips et al. (1966) |
| $K^+ + ATP^{4-} = KATP^{3-}$ | $1.4 \times 10$ | O'Sullivan and Perrin (1964) |
| $Mg^{2+} + ATP^{4-} = MgATP^{2-}$ | $3.48 \times 10^4$ | Phillips et al. (1966) |
| $Mg^{2+} + HATP^{3-} = MgHATP^-$ | $5.42 \times 10^2$ | Phillips et al. (1966) |
| $Mg^{2+} + MgATP^{2-} = Mg_2ATP$ | $4.0 \times 10$ | Noat et al. (1970) |
| $Mg^{2+} + Cl^- = MgCl^+$ | 3.4 | Blair (1970) |

Algorithm Using Apparent Constants[b]

$K_3' = ([MgATP] + [MgHATP] + [Mg_2ATP])/([ATP] + [HATP] + [KATP])([Mg^+] + [MgCl^+])] = ["MgATP"]/(["ATP"]["Mg^{2+}"])$
$K_3' = 6.09 \times 10^3\ M^{-1}$
$["ATP"] = [ATP^{4-}] + [KATP^{3-}] + [HATP^{3-}] = [ATP^{4-}](1 + [K^+]K_{KATP} + [H^+]K_{HATP})$
$["Mg^{2+}"] = [Mg^{2+}] + [MgCl^+] = [Mg^{2+}](1 + [Cl^-]K_{MgCl})$
$["MgATP"] = [MgATP] + [MgHATP] + [Mg_2ATP] + [Mg_2ATP]$
$["MgATP"] = [Mg^{2+}](1 + [ATP^{4-}]K_{MgATP} + [HATP^{3-}]K_{MgHATP} + [MgATP^{2-}]K_{Mg_2ATP})$

[a] Stability constants taken from Storer & Cornish-Bowden (1976). [b] Conditions assumed: pH 7.2, $\mu$ = 0.15 M, $[K^+]$ = 0.15 M, $[Cl^-]$ = 0.15 M.

most widely used constant, the one defined by eq 4, first. We found that the proper apparent constant is specific for the species thought to be present in a given system. The calculated concentrations are the same whether one uses an iterative method such as Storer and Cornish-Bowden's or an appropriately defined apparent constant.

Traditionally, physical chemists and biochemists handle the equilibrium problem involving ATP and its interacting cations, $Mg^{2+}$, $K^+$, and $H^+$, differently. The program of Storer & Cornish-Bowden (1976) is an example of the physical chemists' approach. Biochemists have attempted to simplify these calculations by defining these constants so that the thermodynamic constants are corrected for the presence of interfering ions and then solving a single equilibrium problem with these constants. Unfortunately, there is an ambiguity in the definition of the term "apparent constant". When physical chemists define an apparent constant, they are only considering the effects of nonreacting electrolytes on the equilibrium constant they are measuring. Phillips et al. (1966) have studied the effect of ionic strength on the stability constants of $MgATP^{2-}$ and similar compounds. Biochemists are more concerned with the effects of interacting ions. As O'Sullivan & Smithers (1979) and Adolfsen & Moudrianakis (1978) have indicated, the effects of ionic strength and interfering ions need to be considered when evaluating an apparent constant.

*Methods of Calculation with Storer and Cornish-Bowden's Program.* The concentration of MgATP and other ATP chelates present in a given solution is a function of temperature and ionic strength as well as concentrations of all ions that can form complexes: $Mg^{2+}$, $H^+$, $K^+$, and $Cl^-$. Calculating the concentrations of the different ionic species involves finding iteratively the levels of free magnesium, $[Mg^{2+}]$, and unchelated ATP, $[ATP^{4-}]$, that will satisfy the relevant equilibria and yield the correct concentrations of total magnesium and total ATP. Storer & Cornish-Bowden (1976) have written a program that calculates equilibrium concentrations iteratively. In a solution containing these constituents at pH 6–8, an ionic strength of 0.15 M, and a nonreacting buffer, the chemical reactions listed in Table II are in equilibrium. Reactions occurring at low pH (i.e., $HATP^{3-} + H^+ \rightarrow H_2ATP^{2-}$) have been eliminated here. Storer and Cornish-Bowden chose these reactions to represent this system under a wide range of concentrations. Several weak interactions that are not normally considered are listed. These include the formation of $MgCl^+$ and $Mg_2ATP$. Table III lists the equilibria that correspond to the apparent constant defined by eq 4. The results of calculations of free $Mg^{2+}$ with the methods described in Tables II and III are given in Table IV.

A number of other workers have applied computers to the calculation of the concentrations of ionic species involved in

Table III: Calculation of Equilibria (Not Including Weak Interactions of ATP and $Cl^-$)

| multiple equilibria | $K$ ($M^{-1}$) |
|---|---|
| $H^+ + ATP^{4-} = HATP^{3-}$ | $1.09 \times 10^7$ |
| $K^+ + ATP^{4-} = KATP^{3-}$ | $1.4 \times 10$ |
| $Mg^{2+} + ATP^{4-} = MgATP^{2-}$ | $3.48 \times 10^4$ | 
| $Mg^{2+} + HATP^{3-} = MgHATP^-$ | $5.42 \times 10^2$ |

Algorithm Using Apparent Constants[a]

$K_3' = ([MgATP] + [MgHATP])/[([ATP] + [HATP] + [KATP])[Mg^{2+}]] = ["MgATP"]/(["ATP"][Mg^{2+}]) = 9.2 \times 10^3\ M^{-1}$
$["ATP"] = [ATP^{4-}](1 + [H^+]K_{HATP} + [K^+]K_{KATP})$
$["MgATP"] = [Mg^{2+}]([ATP^{4-}]K_{MgATP} + [HATP^{3-}]K_{MgHATP})$

[a] Conditions assumed: pH 7.2, $\mu$ = 0.15 M, $[K^+]$ = 0.15 M, $[Cl^-]$ = 0.15 M.

complex equilibria (Perrin, 1965). Kuby & Noltmann (1962) have applied this methodology to the equilibria involving the interactions of the substrates of creatine kinase with the enzyme and various ions. The program of Storer & Cornish-Bowden (1976) uses the values of a number of constants reported in the elegant work of Phillips et al. (1966) and solves for the equilibria with a rapidly converging iterative algorithm.

*Calculations with "Apparent Constants".* Since these calculations are rather tedious and time consuming without computers, biochemists have resorted to a number of expedients to avoid them. One involves including large excesses of $Mg^{2+}$ in reaction mixtures in an attempt to reduce the concentration of interfering ionic species ($KATP^{3-}$, $HATP^{3-}$, etc.). Another is using "apparent constants" to simplify the calculations. Such constants have been discussed above. Unfortunately, these apparent constants are not defined completely or consistently, although there seems to be general agreement that they should be based on total concentrations of interacting components.

In order to compare the concentrations of components obtained by different methods of calculation, we calculated the concentrations of $Mg^{2+}$ and other ions in the in vitro systems studied by Gupta et al. (1978a). Column A of method 2 (Table IV) was calculated by solving the six equilibria given in Table II interatively with the constants given in Table II. $MgCl^+$ and $Mg_2ATP$ are components in this system. Column A of method 3 was calculated in the same way except that the four equations given in Table III were used. $MgCl^+$ and $Mg_2ATP$ are assumed to be absent from the system in Table III.

We then compared the solutions to this problem obtained with the iterative calculations with the apparent constants. It was necessary to define these apparent constants precisely. Unfortunately, the most useful definitions are given in such obscure sources as miniprint supplements or very specialized

Table IV: Calculated Free $Mg^{2+}$ Concentrations of Solutions Studied by Gupta et al. (1978a)

| total concn (mM) | | chemical species | free $Mg^{2+}$ concn (mM) | | | | |
|---|---|---|---|---|---|---|---|
| | | | method 1 | method 2 | | method 3 | |
| Mg | ATP | | Gupta | A | B | A | B |
| 4.47 | 4.33 | "$Mg^{2+}$" | 0.50 | 0.81 | 0.85 | 0.72 | 0.71 |
| 1.34 | 1.08 | | 0.375 | 0.528 | 0.52 | 0.45 | 0.465 |
| 3.87 | 4.33 | | 0.235 | 0.529 | 0.55 | 0.41 | 0.42 |
| 0.59 | 0.73 | | 0.22 | 0.33 | 0.33 | 0.31 | 0.297 |
| 4.47 | 4.33 | $Mg^{2+}$ | | 0.536 | 0.56 | | |
| 1.34 | 1.08 | | | 0.35 | 0.34 | | |
| 3.87 | 4.33 | | | 0.35 | 0.36 | | |
| 0.59 | 0.73 | | | 0.22 | 0.22 | | |
| 4.47 | 4.33 | "MgATP" | 3.98 | 3.61 | 3.63 | 3.76 | 3.80 |
| 1.34 | 1.08 | | 0.96 | 0.824 | 0.82 | 0.87 | 0.875 |
| 3.87 | 4.33 | | 3.73 | 3.31 | 3.37 | 3.46 | 3.44 |
| 0.59 | 0.73 | | 0.50 | 0.398 | 0.395 | 0.437 | 0.433 |
| 4.47 | 4.33 | $MgATP^{2-}$ | | 3.501 | 3.51 | | |
| 1.34 | 1.08 | | | 0.804 | 0.816 | | |
| 3.87 | 4.33 | | | 3.227 | 3.34 | | |
| 0.59 | 0.73 | | | 0.39 | 0.383 | | |
| 4.47 | 4.33 | "$ATP_f$" | 0.36 | 0.709 | 0.71 | 0.568 | 0.57 |
| 1.34 | 1.08 | | 0.115 | 0.249 | 0.26 | 0.209 | 0.205 |
| 3.87 | 4.33 | | 0.695 | 1.00 | 1.01 | 0.91 | 0.98 |
| 0.59 | 0.73 | | 0.095 | 0.193 | 0.19 | 0.151 | 0.157 |
| 4.47 | 4.33 | $ATP^{4-}$ | | 0.187 | 0.187 | 0.15 | 0.15 |
| 1.34 | 1.08 | | | 0.066 | 0.069 | 0.055 | 0.054 |
| 3.87 | 4.33 | | | 0.265 | 0.266 | 0.24 | 0.23 |
| 0.59 | 0.73 | | | 0.051 | 0.05 | 0.042 | 0.04 |

journals. Generally, only partial definitions are given; e.g., Berger et al. (1973) and Wu et al. have given partial definitions.

O'Sullivan & Smithers (1979) have defined such constants clearly, without explaining more than that they are a convenience and that they may or may not equal the intrinsic constant. Unfortunately, they considered factors influencing the apparent constant one at a time and did not specify corrections in the presence of several such factors. Wu et al. erred in applying these corrections singly, without considering how the different effects interact.

Adolfsen & Moudrianakis (1978) have defined these constants but only specified a limited set of conditions under which they are valid. Nanninga (1961) has defined apparent constants for a simpler system where only a few of the reactions considered by Storer & Cornish-Bowden (1976) are considered. Their constant corresponds to the system we consider in Table III. Macfarlane et al. (1974) have given a rigorous definition of these constants and have even written a computer program for deriving them. Johnson has discussed general equilibrium reactions where the concentrations are given in terms of total concentrations rather than reactive species. According to Johnson (1960), equilibrium constants can be expressed in several ways. While the basic forms using concentrations of reactive species are more widely applicable, constants based on total concentrations are sometimes a convenience.

The algorithm chosen for an apparent equilibrium constant for the systems of six reactions considered in the top half of Table II is an extension of the expressions given by Johnson (1962), Berger et al. (1973), and Adolfsen & Moudrianakis (1978). The terms defined in this expression are those that would be calculated if this equilibrium were considered as a simple system. The concentrations labeled A were calculated by solving the multiple equilibrium problem defined by the chemical equations iteratively. The concentrations labeled B were obtained with the apparent constant and other variables defined in method 2B. The calculations in the algorithm of Table IIB are equivalent to those performed when the problem is solved iteratively. Once an apparent $K$ is defined for a given system, it can be used to solve for the concentration of MgATP, ATP, and Mg as if only those ligands are present. The resulting concentrations can then be corrected as needed for the presence of interfering ions, e.g., $K^+$, $H^+$, or $Cl^-$. The $K'$ given in Table II is specific for this system under the given conditions of temperature, ionic strength, pH, and concentrations of $K^+$ and $Cl^-$. Each different condition requires evaluation of a different apparent constant. If other interactions occur to a significant extent in a given system, then these interactions must be considered in a corresponding manner. The iterative method is more generally applicable, but additional reactions may have to be included if other components such as inorganic phosphate ions are present. This type of calculation does not necessarily apply to cells and tissues where the metabolites are not uniformly distributed.

Table III describes calculations with a system that corresponds to eq 4 and the apparent constant advocated by Wu et al. In this system, there is no $MgCl^+$. Therefore, the apparent constant $K_3'$ of Table III is multiplied by a factor of 1.51. All of the $Mg^{2+}$ not chelated with ATP is now free $Mg^{2+}$.

The data in Table IV show that evaluation of apparent constants is somewhat more complex than is generally believed. The criterion for defining a constant correctly involves accounting for all the material present in a system. Although it is possible to define other apparent constants, whose values do apply to the solution in question, one cannot calculate system behavior with such partial constants. This is shown by the differences between the calculations with the constant defined by Gupta et al. (method 1) and the more complex calculations in method 2.

The results of all of these calculations are in much closer agreement than those where Wu et al. evaluated free $Mg^{2+}$ with various constants in Table I. In the in vitro systems, material balance constrains the concentrations and is a more important factor than the exact value of any individual equilibrium constant or method of calculation. This constraint is lacking in Gupta's equation. Additional information is required. The recent results of Gupta et al. (1983) provide such data. They have monitored free $Mg^{2+}$ with the dye

antipyrylazo III and found that their spectrophotometric measurements agree with values calculated from their stability constant. The quantity $\phi$, the ratio of free ATP to Mg-chelated ATP, does not furnish any information about the absolute value on any concentration. Therefore, free $Mg^{2+}$ may assume any value, especially if unreasonable values of the stability constant are used. It is to be hoped that further advances in NMR techniques will provide information about the absolute levels of ATP being observed in a given sample.

## Conclusions

We have found by checking their calculations that Wu et al. have incorrectly calculated their results. When the stability constants reported by different workers are calculated correctly, all of their values agree more closely with that of Gupta et al. There is evidence, in addition to the work of Gupta, that the stability constant of $MgATP^{2-}$ may be higher than most of the values cited in Table I. Mohan & Rechnitz (1974) found values of $1.15 \times 10^6$ $M^{-1}$ for zero ionic strength. Asai & Morales (1962) found a stability constant of 90 000 $M^{-1}$ by using an optical method; O'Sullivan & Perrin (1974) have reported values as high as 100 000 $M^{-1}$ even though they prefer lower values. Wu et al. have erroneously considered the three methods for evaluating this constant to be equivalent when they clearly are not.

In performing their multiple equilibrium calculations, Wu et al. assumed that 85% of the Mg and all of the other metabolites were located in the cystol and that these quantities are unchanged in ischemia. This first assumption contradicts the results of fractionation experiments. R. A. Altschuld (personal communication) has performed digitonin fractionation of myocytes from rat and found that only 40% of the total $Mg^{2+}$ was located in the cytosol. We have already discussed the changes in the content of water and $Mg^{2+}$ that occur during ischemia. As Wu et al. stated in their paper, their result contradicts what is known about the properties of contractile proteins and the conditions under which most glycolytic enzymes function in vitro. They have used the valid method of Storer and Cornish-Bowden inappropriately and then invoked the authority of the computer to lend credence to their results. Unfortunately, a computed result is only as good as the underlying assumptions and data. Wu et al. have relied completely on their calculations to establish an $Mg^{2+}$ level when they should have taken NMR spectra of their in vitro systems. If the model in vitro system representing the cytosol of the heart could not be studied by NMR, then spectra of the in vitro system at the appropriate pH containing $Mg^{2+}$, ATP, and $K^+$ could have been taken. Such spectra would at least provide a basis for comparison with the NMR spectrum for perfused heart. We have shown that the agreement obtained by Wu et al. with two of their methods was erroneous and that their result actually agrees fairly closely with Gupta et al. and, contrary to their conclusion, supports an important role for free $Mg^{2+}$ in regulation of metabolic processes. The stability constant reported by Gupta is at least based on the observed spectrum of MgATP in their systems. Any disagreement in the interpretation of the NMR spectrum of MgATP should be resolved by further experimentation, preferably with systems that are easier to interpret than the perfused heart.

**Registry No.** ATP, 56-65-5; Mg, 7439-95-4.

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