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The β/α Peak Height Ratio of ATP

A MEASURE OF FREE [Mg^{2+}] USING ^{31}P NMR*

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From ^{31}P NMR measurements made *in vitro* at 38 °C, I = 0.25, pH 5.75–8.5, and calculated free [Mg^{2+}] from 0 to 5 mM, we show that, within the physiological range of cytosolic free [Mg^{2+}] from 0.25 to 1.5 mM, the chemical shift difference between the α- and β-ATP resonances, $\delta_{\alpha\beta}$, changes by only 0.6 ppm. Consequently, we developed new formalisms from known acid and Mg^{2+} dissociation constants by which the observed chemical shift of P_i, δ_{P_i}, and the peak height ratio of the β- and α-ATP resonances, $h_{\beta/\alpha}$, could be related to free [Mg^{2+}] by simultaneous solution of:

$$pH = pK_{a H_2PO_4^-} + \log \frac{(\delta_{P_i} - \delta_{H_2PO_4^-})}{(\delta_{HPO_4^{2-}} - \delta_{P_i}) + \frac{[Mg^{2+}]}{K_{D_{MgHPO_4}}}(\delta_{MgHPO_4} - \delta_{P_i})}$$

$$[Mg^{2+}] = \frac{2.5 \times K_{DMgATP^{2-}} \times (1 + 10^{pK_{a4}-pH}) \times h_{\beta/\alpha}}{h_{\beta/\alpha Max} - h_{\beta/\alpha}} \qquad \text{(Eq. A1)}$$

We found that $h_{\beta/\alpha}$ changed 2.5-fold as free [Mg^{2+}] varied from 0.25 to 1.5 mM, providing a more sensitive and accurate measure of free cytosolic [Mg^{2+}]. In working rat heart perfused with glucose, free [Mg^{2+}] was 1.0 ± 0.1 from $h_{\beta/\alpha}$ and 1.2 ± 0.03 from measured [citrate]/[isocitrate] but 0.51 ± 0.1 from $\delta_{\alpha\beta}$. Addition of ketone bodies to the perfusate decreased free [Mg^{2+}] estimated from $h_{\beta/\alpha}$ to 0.61 ± 0.02 and 0.74 ± 0.11 by [citrate]/[isocitrate] but the estimate from $\delta_{\alpha\beta}$ was unchanged at 0.46 ± 0.04 mM. Such differences in estimated free [Mg^{2+}] alter the apparent K_{eq} of the creatine kinase reaction and hence the estimated cytosolic free [ΣADP].

Intracellular free [Mg^{2+}] and [H^+] interact with numerous metabolites to alter the extent and direction of many of the key reactions of energy metabolism (1, 2) including the ΔG of ATP hydrolysis, the [PCr]/[ATP] ratio and hence the inferred free cytosolic [ADP] (3, 4), key steps in glycolysis and the tricarboxcylic acid cycle (5, 6) and the kinetics of a number of plasma membrane ion channels (7). Despite its importance in both energetics and kinetics, there has been no generally accepted method for estimation of intracellular free [Mg^{2+}] (8). Next to potassium, total magnesium is the most abundant of the intracellular cations, being about 6–7 μmol/g of wet weight in brain and perfused rat heart, 8 in kidney, and 10 in liver. The intracellular magnesium is, however, largely bound. Scatchard plot analysis of intracellular contents showed that Mg^{2+} binds to 2–3 μmol/g of wet weight of insoluble nucleic acid binding sites with a K_B of 3,700 M^{-1}, 5–10 μmol/g soluble protein binding sites with an apparent K_B of 530 M^{-1} and to a variety of intracellular phosphorylated and polycarboxylic acid metabolites (9). Using the appropriate amount of binding agents, their binding constants and knowing the amount of total magnesium present, the free intracellular [Mg^{2+}] has been calculated to be in the range of 0.5 to 1.3 mM in most cell types (9), save the red cell, where it has been estimated to be in the range of 0.15 to 0.25 mM (10, 11). In plasma, in addition to phosphate and carbonate, magnesium binds to serum proteins and some metabolites so that free [Mg^{2+}] is estimated to be about 0.5 mM (12), or essentially the same as intracellular free [Mg^{2+}] in spite of the negative electric potential of the intracellular, relative to the extracellular, phase of cells and in marked contrast to the large gradient of free [Ca^{2+}] existing between extra- and intracellular phases (13).

Estimation of intracellular free [Mg^{2+}] has been attempted by measuring the ratio of [products]/[reactants] whose binding constants for Mg^{2+} are very different, such as myokinase (EC 2.7.4.3) (10) or aconitase (EC 4.2.1.3) (9). In tissues containing mitochondria, where there is significant compartmentation of ADP (3), use of the metabolite ratio of the myokinase reaction is unsuitable, but changes induced by alteration of free [Mg^{2+}] in the equilibrium ratio of [citrate]/[isocitrate] agreed well with the estimation of free [Mg^{2+}] by other means (9). Supporting the role of intracellular citrate as a natural indicator of free [Mg^{2+}] was the observation that the chemical shift of the citrate resonance in ^{13}C NMR spectra of rat liver perfused with acetate, gave a free [Mg^{2+}] of 0.46 mM (14). However, citrate levels in most normal tissues are below the sensitivity of ^{13}C NMR, and enzymatic measurement of intracellular [citrate]/[isocitrate] is not suitable for use in humans and measurement of [isocitrate] is technically demanding. These difficulties have prompted the search for other suitable indicators of free intracellular [Mg^{2+}].

^{31}P (15) and combined ^1H, ^{31}P NMR (16, 17) studies of the effects of Mg^{2+} on ATP have shown that Mg^{2+} induces the greatest chemical shift of the β and the least on the α resonance at physiological pH. These findings have been interpreted as indicating that Mg^{2+} binds mainly to the β and γ phosphorus residues, but not the α residue (15, 17) (Fig. 1). Subsequently, measurement of the difference of the chemical shift of the β from the α resonance of ATP, $\delta_{\alpha\beta}$, became the dominant method for determination of intracellular free [Mg^{2+}] in red cell, muscle (18–20), liver (21), and kidney (22). The formalism applied in this method, however, contains an apparent binding con-

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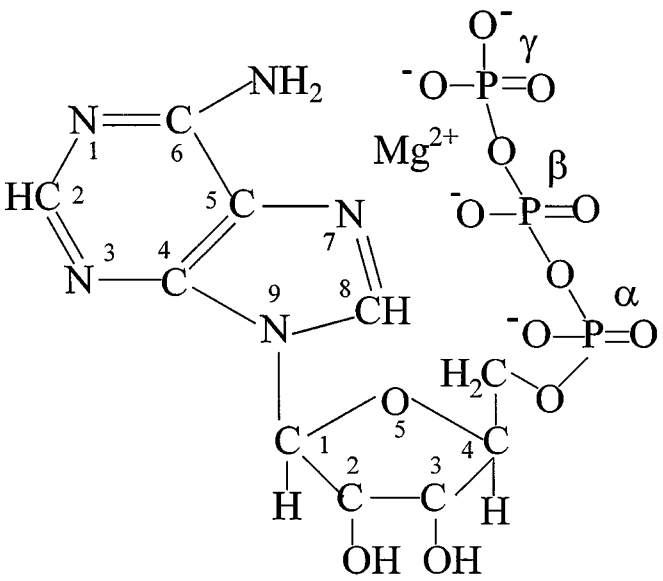

FIG. 1. **Structure of MgATP.** At pH between 7.0 and 7.3, raising the free [Mg^{2+}] from 0.25 to about 1.0 mM free [Mg^{2+}] increased the chemical shift of the α resonance of ATP by 0.06 ppm, β by 0.40 ppm and γ by 0.39 ppm. Such observations have led to the inference that Mg^{2+} forms ionic bonds with the β and γ phosphate of ATP, but not to the α.

stant and maximum chemical shifts for a mixture of ionic species of ATP for Mg^{2+} at a defined pH (23). While attempts have been made to correct mathematically this apparent binding constant for the effects of variations in intracellular pH (8, 24), no actual calibration of the characteristics of ^{31}P NMR spectra of defined ionic species of binding ligands under defined conditions of pH, free [Mg^{2+}], temperature of 38 °C, and ionic strength of 0.25 have been made. We have chosen to analyze the observed changes in peak height ratio and chemical shift difference in the ^{31}P NMR spectra of ATP in terms of its known acid and Mg^{2+} dissociation constants.

Because of the difficulty in determining free [Mg^{2+}] in tissue (25, 26) and the fundamental (6) and clinical importance of free [Mg^{2+}] (27–33), we have attempted to determine if ^{31}P spectral analysis can serve as an adequate indicator of intracellular free [Mg^{2+}] in intact tissue. We have measured the $\delta_{\alpha\beta}$ of ATP and δ_{P_i} *in vitro* under intracellular conditions in solutions containing 5 mM each of ATP, PCr, Glu-6-P, and P_i while at the same time observing other pertinent ^{31}P NMR spectral characteristics such as line width and peak heights as a function of changing pH and free [Mg^{2+}]. We have applied this analysis to ^{31}P NMR spectra of the working rat heart perfused with two different substrate combinations and compared the results with estimates of free [Mg^{2+}] determined using the [citrate]/[isocitrate] ratio.

EXPERIMENTAL PROCEDURES

Solutions—Solutions were prepared containing 5 mM each of K_2ATP, Na_2PCr, K_2Glc-6-P (Sigma), and KH_2PO_4 (British Drug House, Poole, UK) at 38 °C, $I = 0.25$. The pH of the solutions ranged from 5.75 to 8.5, and the total magnesium ranged from 0 to 12.2 mM. Ionic strength was adjusted to 0.25 by adding KCl and the pH was adjusted using KOH or HCl to give a set of 77 samples from pH 5.75 to 8.5 at each free [Mg^{2+}] concentration (Table I). Known acid dissociation constants, K_a, and Mg^{2+} binding constants for each particular ionic species, K_B, were adjusted to 38 °C and an ionic strength of 0.25 (6). Defining the ionic species of Mg^{2+} binding ligands, A^{z-}, present in each solution gave a statement from which the amount of total $MgCl_2$, [Mg_{Tot}], to be added to give the desired free [Mg^{2+}] could be calculated:

$$[Mg_{Tot}] = [Mg^{2+}]\left[1 + \sum_i \left(\frac{[A_{Tot}]_i \times K_{Bi}}{fA_i}\right)\right] \quad \text{(Eq. 1)}$$

where f is a ratio of the ionized ligand, A^{z-}, to the total ligand, $A_{Tot} \times fA_i = [A_{Tot_i}]/[A^{z-}]$. For ligands with multiple magnesium binding states such as ATP^{4-} and $HATP^{3-}$, the formalism is somewhat more complex than single binding ligands (6, 34). [Mg_{Tot}] to be added to give the desired free [Mg^{2+}] in the calibration solutions containing the four magnesium binding ligands, ATP, PCr, Glu-6-P, and P_i may be estimated by:

$$[Mg_{Tot}] = [Mg^{2+}] \times \left[1 + \frac{[P_{i_{Tot}}] \times K_{BP_i}}{fP_i} + \frac{[ATP_{Tot}] \times \left(K_{BATP^{4-}} + \frac{[H^+]}{K_{aHATP^{3-}}} - K_{BHATP^{3-}}\right)}{fATP} + \frac{[PCr_{Tot}] \times K_{BPCr}}{fPCr} + \frac{[G6P_{Tot}] \times K_{BG6P}}{fG6P}\right] \quad \text{(Eq. 2)}$$

Solution of this formalism may be achieved using any suitable program for the evaluation of simultaneous equations (35) or with commercially available software (*Mathematica*™, Wolfram Research, Champaign, IL) (36). By this method sufficient total magnesium was added to give solutions with free [Mg^{2+}] of 0, 0.25, 0.50, 0.75, 1.0, 2.5, and 5 mM.

^{31}P NMR Spectroscopy—^{31}P NMR spectra of the 77 different calibration solutions at the various pH and free [Mg^{2+}] at 38 °C and $I = 0.25$ were acquired using a 9.4-Tesla, Oxford Instrument 400 MHz wide-bore superconducting magnet interfaced with a Bruker Aspect 2000 spectrometer set at 161.9 MHz for phosphorus. The homogeneity of the magnetic field was optimized by shimming on the ^1H free induction decay providing line widths of 10 ± 3 Hz. For each sample, a 90° pulse was used and eight averaged transients were collected with an acquisition time of 0.32 s (8K data points), a spectral width of 12.8 kHz, an interpulse delay of 30 s, and no proton decoupling. A capillary containing phenyl phosphonic acid was used as an external reference standard. Prior to Fourier transformation, the time domain signals were zero filled to 16K data points, providing 1.5 Hz resolution per point and an exponential filter was applied that produced 10 Hz line broadening and in perfused heart spectra, 20 Hz line broadening was used. The ^{31}P NMR spectral resonances were fitted to a Lorentzian line shape using the NMR1 program (Tripos, UK). This program determined the best fit base line, following which the chemical shifts, line widths, areas, and peak heights were determined. The δ_{P_i} observed by the ^{31}P NMR spectral analysis of the standard samples at each known free [Mg^{2+}] were fitted to equations using the nonlinear fit package in *Mathematica* (Wolfram Research, Champaign, IL).

Perfusion of Working Rat Hearts—Hearts from 450–500-g fed, male Wistar rats were perfused in the working mode using modified Krebs Henseleit buffer containing 10 mM glucose as described previously (37). Hearts were perfused in the Langendorff mode for 15 min, followed by a 15-min period of stabilization in the working mode and a 30 min period of perfusion at the end of which hearts were freeze clamped. The frozen tissue was extracted and assayed fluorometrically for contents of citrate and isocitrate (38). The intracellular free [Mg^{2+}] and pH was determined simultaneously from δ_{P_i} and the ^{31}P spectral properties of ATP as described below. Using the pH estimated from this result, intracellular free [Mg^{2+}] was also estimated independently by enzymatic measurements of the [citrate]/[isocitrate] ratios in freeze clamped perfused hearts as described previously (5, 6, 9). [creatine], [citrate] and [isocitrate] were determined enzymatically from freeze-clamped heart (37).

In a separate series, hearts were perfused as above in the ^{31}P NMR spectrometer, and the relevant spectral parameters were determined on the instrument described above using the same 20-mm broad band probe (Paul Morris Inc.). The temperature in the coil was maintained at 38 °C by a variable temperature unit controlled by the spectrometer. Fully relaxed spectra were obtained using a 90° (40 μs) pulse with a 15-s interpulse delay. There was a time resolution of 15 min as 64 free induction decays were collected to make a fully relaxed spectrum. [P_i], [PCr], and [ATP] were determined by ^{31}P NMR spectra (39). Cytosolic free [ΣADP] was calculated from the equilibrium constant, K_{CK}, and the measured components of the creatine kinase reaction (EC 2.7.3.2) [ΣADP] = [ΣATP][creatine]/K_{CK}[ΣPCr][H^+] (3). The value of K_{CK} was corrected for changes in free [Mg^{2+}] and pH as described previously (6).

RESULTS

Determination of Intracellular pH Using the P_i-PCr Chemical Shift Difference, δ_{P_i}—The measured δ_{P_i} showed the extrapo-

TABLE I
Concentrations of KCl and $MgCl_2$ added to calibrating solutions

The concentration of KCl and $MgCl_2$ in mM, which was added to the calibration standards containing 5 mM each of K_2ATP, Na_2PCr, K_2Glc-6-P, and KH_2PO_4 at 38°C to give $I = 0.25$ and free [Mg^{2+}] from 0 to 5 mM.

pH	[Mg^{2+}] (mM)													
	0		0.25		0.50		0.75		1.00		2.50		5.00	
	KCl	$MgCl_2$	KCl	$MgCl_2$	KCl	$MgCl_2$	KCl	$MgCl_2$	KCl	$MgCl_2$	KCl	$MgCl_2$	KCl	$MgCl_2$
5.75	188.8	0.0	190.9	1.7	191.8	2.7	192.1	3.5	192.1	4.2	189.8	6.9	183.5	10.3
6.00	185.4	0.0	189.1	2.1	190.4	3.3	190.8	4.1	190.8	4.7	188.3	7.3	181.9	10.7
6.25	181.5	0.0	187.2	2.6	188.9	3.8	189.4	4.6	189.4	5.2	186.8	7.7	180.3	11.0
6.50	177.3	0.0	185.5	3.0	187.4	4.2	187.9	4.9	188.0	5.5	185.3	7.9	178.8	11.3
6.75	173.3	0.0	183.8	3.4	185.9	4.5	186.5	5.1	186.6	5.7	183.9	8.1	177.5	11.6
7.00	169.8	0.0	183.4	3.6	184.7	4.6	185.3	5.3	185.3	5.8	182.7	8.3	176.4	11.8
7.15	168.1	0.0	181.8	3.7	184.1	4.7	184.7	5.4	184.7	5.9	182.1	8.4	175.9	11.9
7.30	166.7	0.0	181.2	3.7	183.5	4.8	184.2	5.4	184.2	5.9	181.7	8.4	175.5	12.0
7.50	165.3	0.0	180.6	3.8	183.0	4.8	183.6	5.5	183.7	6.0	181.2	8.5	175.1	12.1
8.00	163.5	0.0	179.8	3.9	182.3	4.9	182.9	5.5	183.0	6.0	180.6	8.6	174.6	12.2
8.50	162.8	0.0	179.6	3.9	182.0	4.9	182.7	5.5	182.8	6.1	180.4	8.6	174.4	12.2

lated limits for HPO_4^{2-} at pH > 8.5, HPO_4^{2-}, and $H_2PO_4^-$ at pH < 5.75, $\delta_{H_2PO_4^-}$ in magnesium-free standard solutions to be 5.64 and 3.30 ppm, respectively (Fig. 2*a*). The δ_{P_i} of the $MgHPO_4$ form, δ_{MgPO_4}, determined at pH 8.5 and free [Mg^{2+}] of 5 mM, was 5.59 ppm.

Intracellular pH using ^{31}P NMR spectroscopy has been determined by the δ_{P_i} and applying the formalism (40, 41):

$$\mathrm{pH} = pK_{aP_i} + \log \frac{\delta_{P_i} - \delta_{H_2PO_4^-}}{\delta_{HPO_4^{2-}} - \delta_{P_i}} \quad \text{(Eq. 3)}$$

However, it was previously predicted on theoretical grounds that pK_{aP_i} would decrease with increasing free [Mg^{2+}] (42) (Table II). Fitting the observed data (Fig. 2*a*) for each concentration of free [Mg^{2+}] to Equation 3 confirmed that the apparent pK_{aP_i} decreased from 6.78 at free [Mg^{2+}] of 0 to 6.66 at free [Mg^{2+}] of 5 mM. This uncertainty in the estimation of intracellular pH using δ_{P_i} induced by changes in intracellular free [Mg^{2+}] was eliminated by modifying Equation 3 with statements defining the ionic species present and relating the observed chemical shifts to those species:

$$[P_{i_{Tot}}] = [HPO_4^{2-}] + [H_2PO_4^-] + [MgHPO_4],$$

$$\delta_{P_i} = \frac{[HPO_4^{2-}]}{[P_{i_{Tot}}]}\delta_{HPO_4^{2-}} + \frac{[H_2PO_4^-]}{[P_{i_{Tot}}]}\delta_{H_2PO_4^-} + \frac{[MgHPO_4]}{[P_{i_{Tot}}]}\delta_{MgHPO_4}$$

$$\therefore \mathrm{pH} = pK_{aH_2PO_4^-} + \log \frac{(\delta_{P_i} - \delta_{H_2PO_4^-})}{(\delta_{HPO_4^{2-}} - \delta_{P_i}) + \frac{[Mg^{2+}]}{K_{DMgHPO_4}}(\delta_{MgHPO_4} - \delta_{P_i})} \quad \text{(Eq. 4)}$$

The values of $\delta_{H_2PO_4^-}$, $\delta_{HPO_4^{2-}}$, and δ_{MgHPO_4} were taken from the observed data (Fig. 2*a*, see above). The $pK_{aH_2PO_4^-}$ is the negative logarithm of the second acid dissociation constant of phosphoric acid, which was taken to be 6.77 from fitting the observed data (Fig. 2*a*) for each concentration of free [Mg^{2+}] to Equations 4 to 5 (Table II). The K_{DMgHPO_4} was taken to be 13.4 mM (6). Equation 4 is similar to previous predictions (43) except that the $MgH_2PO_4^+$ term is insignificant within the physiological range of pH and free [Mg^{2+}] and its inclusion is unnecessary. The theoretical surface predicted by Equation 4 (Fig. 2*b*) accounts well for the plot of the observed data (Fig. 2*a*).

Determination of Cytosolic Free [Mg^{2+}] Using the Chemical Shift Differences between the α-β Resonances of ATP, $\delta_{\alpha\beta}$—The measured chemical shifts for $HATP^{3-}$, $\delta_{HATP^{3-}}$, and ATP^{4-}, $\delta_{ATP^{4-}}$, were determined in magnesium free standard solutions and found to be 11.35 and 10.6 ppm at pH 5.75 and 8.5, respectively. The chemical shift of the $MgATP^{2-}$ form, $\delta_{MgATP^{2-}}$, determined at pH 8.5 and 5 mM free [Mg^{2+}], was 8.27 ppm. The plot of $\delta_{\alpha\beta}$ (Fig. 3*a*) showed little change within the physiological range of pH and free [Mg^{2+}]. Large chemical shift changes occurred below 0.5 mM free [Mg^{2+}] and at pH < 7.0. Within the physiological range of free [Mg^{2+}] from 0.25 to 1.5 mM and pH of 7.0, the actual observed chemical shift difference between the α and β peaks of ATP, $\delta_{\alpha\beta}$, decreased only 0.6 ppm or 7%.

A formalism relating changes in free [Mg^{2+}] to $\delta_{\alpha\beta}$, described previously (18–23), was:

$$\delta_{\alpha\beta} = \frac{\delta_{ATP} + \frac{[Mg^{2+}]}{K_{D\Sigma ATP_{app}}} \times \delta_{MgATP}}{1 + \frac{[Mg^{2+}]}{K_{D\Sigma ATP_{app}}}} \quad \text{(Eq. 5)}$$

where δ_{MgATP} was 8.27 ppm, δ_{ATP} was 10.81 ppm, and $K_{D\Sigma ATP_{app}}$ was 0.05 mM at pH 7.3, 37 °C (22). Equation 5 was modified in a manner similar to the derivation of Equation 4 from Equation 3 to yield a statement (Equation 6 and Fig. 3*c*) relating the chemical shift of the α and β peak of ATP to both free [Mg^{2+}] and pH:

$$[ATP_{Tot}] = [ATP^{4-}] + [HATP^{3-}] + [MgATP^{2-}]$$

$$\delta_{\alpha\beta} = \frac{[ATP^{4-}]}{[ATP_{Tot}]}\delta_{ATP^{3-}} + \frac{[HATP^{3-}]}{[ATP_{Tot}]}\delta_{HATP^{3-}} + \frac{[MgATP^{2-}]}{[ATP_{Tot}]}\delta_{MgATP^{2-}}$$

$$\therefore \delta_{\alpha\beta} = \frac{\delta_{ATP^{4-}} + \delta_{HATP^{3-}} \times 10^{(pK_{a_{HATP^{3-}}} - \mathrm{pH})} + \delta_{MgATP^{2-}}\frac{[Mg^{2+}]}{K_{DMgATP^{2-}}}}{1 + 10^{(pK_{a_{HATP^{3-}}} - \mathrm{pH})} + \frac{[Mg^{2+}]}{K_{DMgATP^{2-}}}} \quad \text{(Eq. 6)}$$

where $\delta_{MgATP^{2-}}$, $\delta_{ATP^{4-}}$, and $\delta_{HATP^{3-}}$ and were taken from the observed data (see above). $pK_{aHATP^{3-}}$ was taken to be 6.50 at 38 °C, $I = 0.25$, and 0 mM free [Mg^{2+}] (6), and $K_{DMgATP^{2-}}$ is K_D of $MgATP^{2-}$ at $I = 0.25$, 38 °C, which was taken to be 0.106 mM (6, 44). The theoretical surface (Fig. 3*c*) described by Equation 6 incorporates a free [Mg^{2+}] term, a pH term, and the Mg^{2+} dissociation constant for a defined ionic species of ATP. This curve is very slightly higher than the empirically measured data (Fig. 3*a*), but can be made superimposable by multiplying $K_{DMgATP^{2-}}$ by 0.6. The $MgHATP^-$ and Mg_2ATP terms were ignored because of their low concentrations under these conditions.

Determination of Cytosolic Free [Mg^{2+}] with Change in the Peak Height Ratio of the β/α Resonances of ATP, $h_{\beta/\alpha}$—It was observed that the peak heights of both the β and γ ^{31}P NMR resonances of ATP changed as free [Mg^{2+}] was varied while the peak height of the α resonance remained constant (Fig. 4). In the area encompassed by pH 7.0–7.3 and free [Mg^{2+}] of 0.25–1.5 mM, the ratio of the peak heights of the β/α resonances

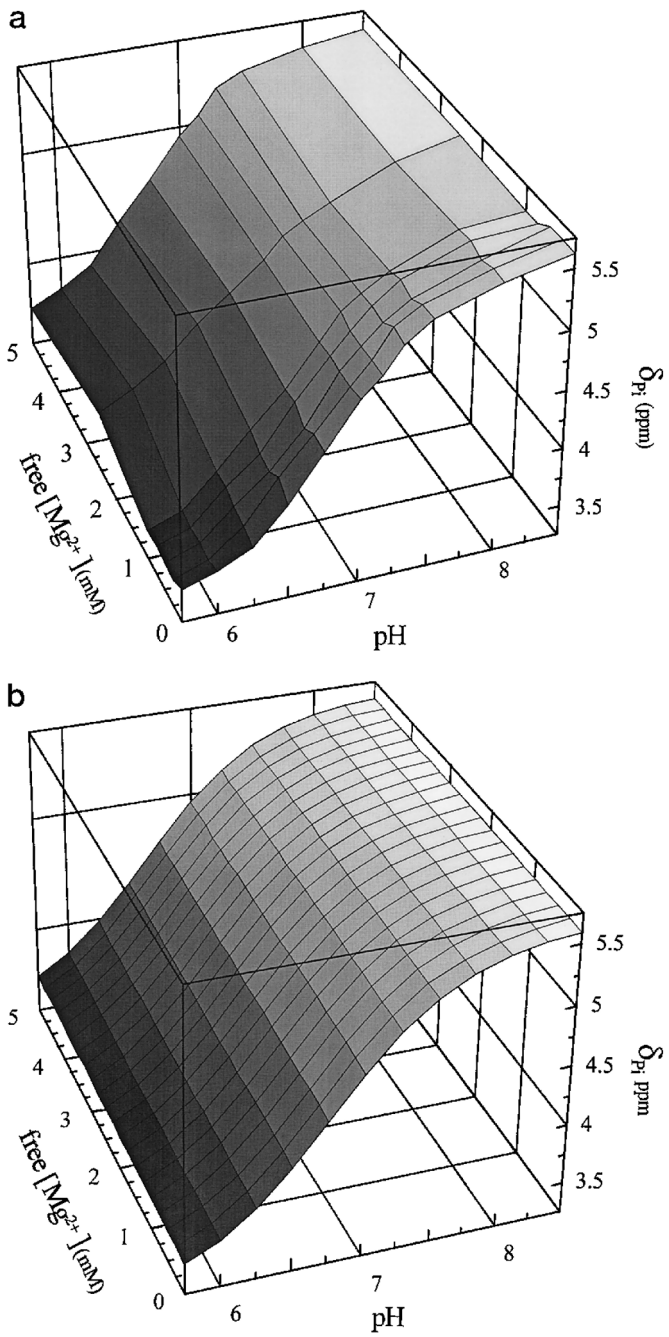

FIG. 2. **Plot of the ^{31}P NMR spectral experimental (*a*) chemical shift difference of P_i from phosphocreatine, δ_{P_i}, and the theoretical (*b*) surface relating δ_{P_i} to both pH and free [Mg^{2+}].** The ^{31}P NMR spectral measurements of the chemical shift difference of P_i from phosphocreatine as a function of alterations in pH from 5.75 to 8.5 and in free [Mg^{2+}] from 0 to 5 mM shows a typical sigmoidal curve with changing pH (*a*). At free [Mg^{2+}] of 0, the apparent pK_a of $H_2PO_4^-$ was 6.83 and at 0.25 mM free [Mg^{2+}] and above around 6.75. A new formalism (Equation 4) relating the pK_a, K_D for Mg^{2+} to pH, free [Mg^{2+}] and δ_{P_i} generates a surface (*b*) similar to the observed changes shown in (*a*). The δ_{P_i} reflects changes in pH and also changes in free [Mg^{2+}] as [Mg^{2+}] approaches the K_D of $MgHPO_4$.

varied 2.5-fold from 0.30 to about 0.75 (Fig. 5*a*). Two maxima, $h_{\beta/\alpha max}$, were observed, about 0.9, at the extremes of free [Mg^{2+}] examined and minima were observed, 0.1 to 0.4, at 0.25 mM free [Mg^{2+}], the experimental point closest to $K_{D\mathrm{MgATP}^{2-}}$.

The plot of the surface of the observed $h_{\beta/\alpha}$ (Fig. 5*a*) at free [Mg^{2+}] at or above 0.25 mM and all pH values from 5.75 to 8.5 could be simulated (Fig. 5*c*) by the formalism derived from the acid dissociation constants and the Mg^{2+} binding constants for ATP:

$$h_{\beta/\alpha} = h_{\beta/\alpha\mathrm{Max}} \times \frac{(1 + 10^{pK_{a4}-\mathrm{pH}})}{\left(1 + 2.5 \times \frac{K_{D\mathrm{MgATP}^{2-}}}{[Mg^{2+}]} \times (1 + 10^{pK_{a4}-\mathrm{pH}})\right)} \quad \text{(Eq. 7a)}$$

At free [Mg^{2+}] < 0.25 mM

$$h_{\beta/\alpha} = h_{\beta/\alpha\mathrm{Max}} \times \frac{(1 + 10^{pK_{a4}-\mathrm{pH}})}{\left(1 + 2.5 \times \frac{[Mg^{2+}]}{K_{D\mathrm{MgATP}^{2-}}} \times (1 + 10^{pK_{a4}-\mathrm{pH}})\right)} \quad \text{(Eq. 7b)}$$

The pK_a of the α phosphate was taken to be 3.75, pK_{a3} of H_2ATP^{2-} (6). The apparent acid dissociation constant of $HATP^{3-}$, pK_{a4}, determined by taking the first derivative of the line describing the change in the chemical shift differences from PCr to βATP with pH at 0 free [Mg^{2+}] gave a pK_{a4} of 6.71 from the β phosphorus of $HATP^{3-}$. This value, while somewhat higher, is within experimental error of the pK_{a4} of $HATP^{3-}$ of 6.50 obtained titrametrically and corrected to the desired temperature and ionic strength (6). The factor of 2.5 was estimated from the measured γ/β peak height ratio, $h_{\gamma/\beta}$, at 0.25 mM free [Mg^{2+}] and represents the greater chemical shift difference and hence broadening of the β relative to the α or γ phosphorus resonances.

Because changes in free [Mg^{2+}] can affect the observed δ_{P_i} used to estimate intracellular pH, the estimation of either pH or free [Mg^{2+}] requires that Equations 4 and 7 be solved simultaneously. Since pK_{a3} is about 3 orders of magnitude below intracellular pH, the term $(1 + 10^{pK_{a3}-\mathrm{pH}})$ in Equation 7 can be taken to be 1, and Equation 7a simplified to:

$$[Mg^{2+}] = \frac{2.5 \times K_{D\mathrm{MgATP}^{2-}} \times (1 + 10^{pK_{a4}-\mathrm{pH}}) \times h_{\beta/\alpha}}{h_{\beta/\alpha\mathrm{Max}} - h_{\beta/\alpha}} \quad \text{(Eq. 7c)}$$

It should be noted however, that when the observed $h_{\beta/\alpha}$ approaches 0.8, the free [Mg^{2+}] becomes indeterminate other than to say it is > 1.5 mM and cannot be estimated from this measurement (see Fig. 5*c*).

While changes in the β/α peak height ratio appear to be greater within the physiologically normal range of free [Mg^{2+}] from 0.25 to 1.5 mM, the γ/α peak height ratio, $h_{\gamma/\alpha}$, also described changes in free [Mg^{2+}], but only within an acidotic pH range of 5.75–7.0 and a range of free [Mg^{2+}] from 0.25–0.75 mM where the $h_{\gamma/\alpha}$ decreased precipitously from 0.96 to 0.2 (Fig. 6*a*). These levels of pH and free [Mg^{2+}] are generally below those encountered in normal tissue. Nevertheless, the behavior of the γ/α peak height ratio with changes in pH and free [Mg^{2+}] can be described by an equation derived from acid and Mg dissociation constants of the same form as Equation 8

$$h_{\gamma/\alpha} = h_{\gamma/\alpha\mathrm{Max}} \times \frac{(1 + 10^{pK_{a4}-\mathrm{pH}})}{\left(1 + \frac{K_{D\mathrm{MgATP}^{2-}}}{[Mg^{2+}]} \times (1 + 10^{pK_{a4}-\mathrm{pH}})\right)} \quad \text{(Eq. 8)}$$

Where pK_{a4} = 6.65: the minimum of the first differential of the curve of the chemical shift differences from PCr to γATP *versus* pH at 0 free [Mg^{2+}]; $h_{\gamma/\alpha\,\mathrm{Max}}$ = 0.96: the average ratio at 0 and 5 mM [Mg^{2+}] and all pH values. The similarity between the $h_{\beta/\alpha}$ or the $h_{\gamma/\alpha}$ predicted by Equations 7b or 8 (Figs. 5*c* or 6*b*) and the values observed in ^{31}P NMR spectroscopic measurements (Figs. 5*a* or 6*a*) suggests that an equation derived from simple physicochemical constants can account for the observed spectral changes in peak heights, at least in *in vitro* solutions.

Comparison of the Estimation of Free [Mg^{2+}] in Tissue by Three Different Methods—Estimation of intracellular free [Mg^{2+}] and pH in the working rat heart perfused with 10 mM glucose, obtained by the simultaneous solution of Equation 4

TABLE II

Variation in pK_a by ^{31}P NMR spectroscopy in solutions of I = 0.25, 38°C

The values for $pK_{a\mathrm{H_2PO_4^-}}$ were assigned by fitting the experimental data to Equation 6 for each known free [Mg^{2+}] condition. The apparent changes in the value of $pK_{a\mathrm{P_iapp}}$ were assigned by fitting the experimental data to Equation 5 which contains no free [Mg^{2+}] term.

	Free [Mg^{2+}] (mM)							
	0	0.25	0.5	0.75	1	2.5	5	Mean ± S.E.
$pK_{a\mathrm{H_2PO_4^-}}$	6.78	6.77	6.81	6.78	6.78	6.69	6.79	6.77 ± 0.015
$pK_{a\mathrm{P_iapp}}$	6.78	6.76	6.79	6.76	6.75	6.62	6.66	
Difference	0	0.01	0.02	0.02	0.03	0.07	0.13	

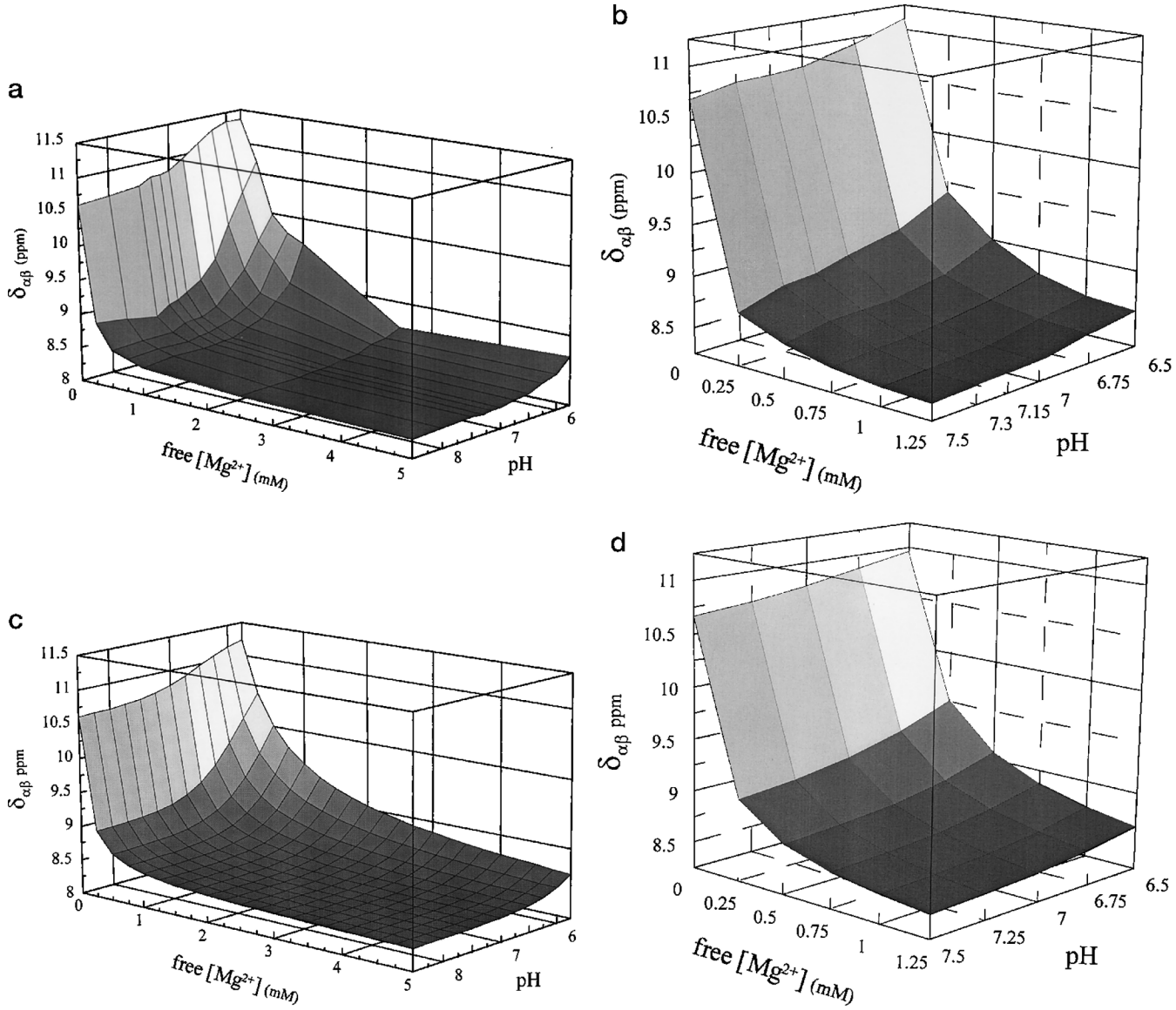

FIG. 3. **Plot of ^{31}P NMR the experimental (*a*) spectral chemical shift difference of the β from the α resonance of ATP, $\delta_{\alpha\beta}$, and the theoretical (*c*) surface relating $\delta_{\alpha\beta}$ to free [Mg^{2+}] and pH.** The plot of the ^{31}P NMR spectra of the chemical shift difference of the β from the α resonances of ATP, $\delta_{\alpha\beta}$, show that nearly all of the change in $\delta_{\alpha\beta}$ occurs at free [Mg^{2+}] < 0.5 mM and pH < 7.0 (*a*) both of which are below the normal physiological range. The theoretical surface (*c*) generated by a new formalism (Equation 6) relating changes in $\delta_{\alpha\beta}$ to a known ionic K_D for $MgATP^{2-}$, pH, and free [Mg^{2+}] duplicated the observed $\delta_{\alpha\beta}$ (*a*) quite well. Within the physiological range of free [Mg^{2+}] from 0.25 to 1 mM at pH 7, the measured $\delta_{\alpha\beta}$ changed by only 0.6 ppm making this parameter relatively insensitive (*b*, experimental; *d*, theoretical).

using δ_{P_i} and Equation 7c using $h_{\beta/\alpha}$, was 1.0 ± 0.1 mM and 7.03 ± 0.01 (Table III). In contrast, the free [Mg^{2+}] estimated from the measured $\delta_{\alpha\beta}$ using Equation 5 was 0.51 ± 0.06 mM, 2-fold lower than that obtained by $h_{\beta/\alpha}$. For comparison, the free [Mg^{2+}] estimated from the measured [citrate]/[isocitrate] ratio (6, 9) was 1.2 ± 0.03 mM (n = 6) which did not differ significantly from the free [Mg^{2+}] estimated by $h_{\beta/\alpha}$.

Addition of 4 mM D-β-hydroxybutyrate and 1 mM acetoacetate to the perfusion media significantly decreased free cytosolic [Mg^{2+}] to 0.61 ± 0.025 as estimated by $h_{\beta/\alpha}$ while cytosolic pH was 7.03 ± 0.01. The decrease in free [Mg^{2+}] inferred from $h_{\beta/\alpha}$ was confirmed by a decrease in free [Mg^{2+}] estimated by the [citrate]/[isocitrate] ratio to 0.74 ± 0.10. In contrast, the free [Mg^{2+}] estimated from $\delta_{\alpha\beta}$ after addition of ketones was not

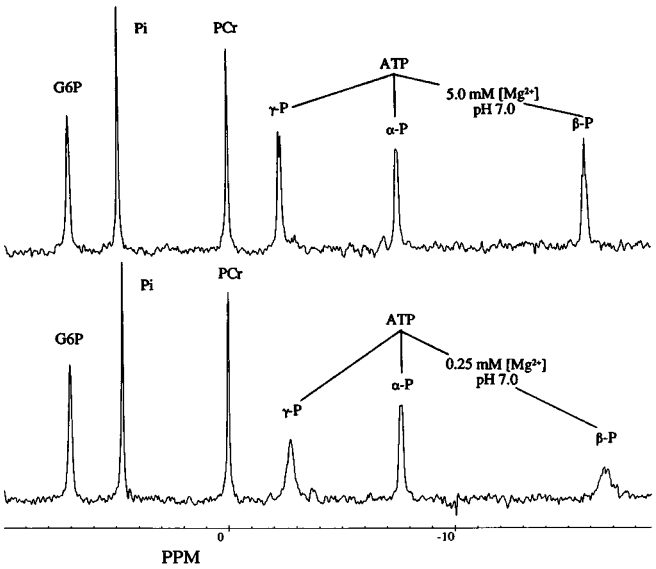

FIG. 4. **^{31}P NMR spectra of ATP at I = 0.25, 38 °C, pH 7.0, and 0.25 and 5 mM free [Mg^{2+}].** The β and γ resonance of ATP show broadening and a decrease of peak height at free [Mg^{2+}] of 0.25 mM reflecting the multiple ionic forms, whereas at 5 mM free [Mg^{2+}] these peaks were not broadened. In contrast, the α resonance shows little change in peak height. These changes in the relative peak heights of the β *versus* the α resonance, allow the β/α peak height ratio, $h_{\beta/\alpha}$ to be used to estimate the free [Mg^{2+}].

significantly changed from hearts perfused with glucose alone at 0.46 ± 0.04 mM (Table III).

In the glucose perfused hearts, K_{CK} (Table III) was 1.65 × 10^9 M when free [Mg^{2+}] was estimated by $h_{\beta/\alpha}$, but significantly decreased to 1.27 × 10^9 M when free [Mg^{2+}] was estimated by $\delta_{\alpha\beta}$. As a result of this variation, the free cytosolic [ΣADP] was 0.172 ± 0.012 when calculated using the $h_{\beta/\alpha}$ estimate of [Mg^{2+}], but was significantly elevated to 0.223 ± 0.015 mM when estimated using the $\delta_{\alpha\beta}$ result. In hearts perfused with glucose and ketones, variation in estimated free [Mg^{2+}] also significantly altered K_{CK}.

DISCUSSION

^{31}P NMR spectroscopy provides a method for determination of intracellular pH in intact tissues. Small changes in pH are thought to result from the action of a number of important hormones (45) and a number of pathological states. Precise determination of cytosolic pH and free [Mg^{2+}] (6) is required to evaluate the pH gradient and electrical potential between mitochondria and cytosol and the free energy of hydrolysis of ATP (39). Alterations in tissue magnesium content is thought to occur in a majority of patients in intensive care units (32) and to play an etiological role in hypertension, arteriosclerosis, certain severe cardiac arrhythmias (31), malnutrition, and alcohol withdrawal syndrome (27). Alterations in free [Mg^{2+}] have been shown experimentally to have widespread effects on the reactions of energy metabolism and on the kinetics of ion channels (7). However, evaluation of intracellular [Mg^{2+}] has been difficult. Here we present evidence that measurement of the β/α peak height ratio of ATP in ^{31}P NMR can give an accurate estimate of the free [Mg^{2+}] in tissue in a noninvasive manner.

The measured $\delta_{HPO_4^{2-}} - \delta_{H_2PO_4^-}$ (Fig. 2*a*) of 2.34 ppm is similar to that reported by Bailey *et al.* (41) of 2.42 ppm. Previous attempts to measure pH from δ_{P_i} using a pK_a apparent for inorganic phosphate (Equation 3) agreed within 0.1 pH units with estimates of intracellular pH using measurements of tissue contents of bicarbonate and CO_2 (46). Solving simultaneously the new formalism for cytosolic pH (Equations 4 and 7a), using a $pK_{aH_2PO_4^-}$ of 6.77, the δ_{P_i} and the observed $h_{\beta/\alpha}$ (Equation 7a) gave cytosolic pH values of 7.03 in glucose-perfused hearts. While the true $pK_{aH_2PO_4^-}$ remained constant, the apparent pK_{aP_i} (Equation 3) varied with changing [Mg^{2+}], with its maximum deviation observed at the highest [Mg^{2+}] examined. Previous estimates of $pK_{aH_2PO_4^-}$ at 25 °C and various ionic strengths ranged from 6.78 at I = 0.2 (34, 47) to 7.206 at I = 0 and 0 free [Mg^{2+}] (48). At physiological ionic strength and temperature the value has been estimated to be 6.71 (6) or 6.75 (42). From the curve fitting routine using Equation 3 in the absence of free [Mg^{2+}], the point of maximum value of $d\delta_{P_i}/d$pH identifies the pH where the ratio of [$H_2PO_4^-$]/[HPO_4^{2-}] is 1. The pH at that point is equivalent to the negative log of the acid dissociation constant. When free [Mg^{2+}] is present in the solution, the maximum value of $d\delta_{P_i}/d$pH no longer identifies the pH where [$H_2PO_4^-$]/[HPO_4^{2-}] is 1, and therefore does not identify the true acid dissociation constant. We set the value of Equation 4 to that obtained with δ_{P_i} and solved for $pK_{aH_2PO_4^-}$ as the unknown. We obtained an essentially invariant acid dissociation constant in the range of free [Mg^{2+}] from 0 to 5 mM. On the other hand when we applied this method to Equation 3 which does not have free [Mg^{2+}] term, we obtained variable constants depending on the free [Mg^{2+}] because this number is not a true acid dissociation constant. It follows that a precise determination of intracellular pH from δ_{P_i} requires knowledge of the free [Mg^{2+}]. Use of Equation 3 to determine cytosolic pH yielded pH values which were 0.02–0.04 pH units higher than the values obtained by the simultaneous solution of Equations 4 and 7.

$\delta_{\alpha\beta}$ has previously been used for determination of free [Mg^{2+}] (18–23). However, Equation 5 does not account for chemical shift changes with pH of the α and β peaks of the complexed, δ_{MgATP}, and uncomplexed δ_{ATP}, forms of ATP, or in the apparent binding constant for all the ionic species of ATP,ΣATP. In our study the $\delta_{\alpha\beta}$ at pH 7.3 and [Mg^{2+}] = 0 was 10.77, similar to 10.81 previously reported (22). These values result from contributions of both ATP^{4-} and $HATP^{3-}$ and therefore may not be used as the chemical shift limit for ATP^{4-}. The proper formalism relating $\delta_{\alpha\beta}$ to free [Mg^{2+}] (Equation 6) uses defined acid and magnesium dissociation constants for the ionic species involved. However, Fig. 3, *a* and *b*, shows that the changes observed in $\delta_{\alpha\beta}$ were very small within the normal intracellular range of free [Mg^{2+}] and pH, and occur mainly below 0.5 mM free [Mg^{2+}].

In this study, we show the ability of changes in free [Mg^{2+}] to alter both the chemical shift differences and the peak heights the β and γ, but not the α resonances of ATP (Figs. 3 and 5). At pH between 7.0 and 7.3, raising the free [Mg^{2+}] from 0.25 to about 1.0 mM increased the chemical shift differences between PCr and the α resonance of ATP by 0.06 ppm, β by 0.40 ppm, and γ by 0.39 ppm. ^{31}P NMR chemical shifts are generally neither sensitive nor specific enough to specify binding sites of a metal ion (16) with certainty. There remains uncertainty about the meaning to the greater chemical shift in the β resonance of ATP with [Mg^{2+}] (16). However, our ability to duplicate the changes in the ^{31}P NMR spectra of ATP using simple binding and acid dissociation constants leads us to confirm, that within usual physiological concentrations of ATP, free Mg^{2+} and pH, binding of Mg^{2+} occurs predominantly at the β and γ phosphorus resonances of ATP but not to the α resonance (15, 17) (Fig. 1).

Under the physiological conditions examined, ATP_{Tot} consisted principally of the species ATP^{4-}, $MgATP^{2-}$, and $HATP^{3-}$. At 0 free [Mg^{2+}] and pH 5.75, $HATP^{3-}$ is the predominant species and the chemical shift of the ^{31}P NMR spec-

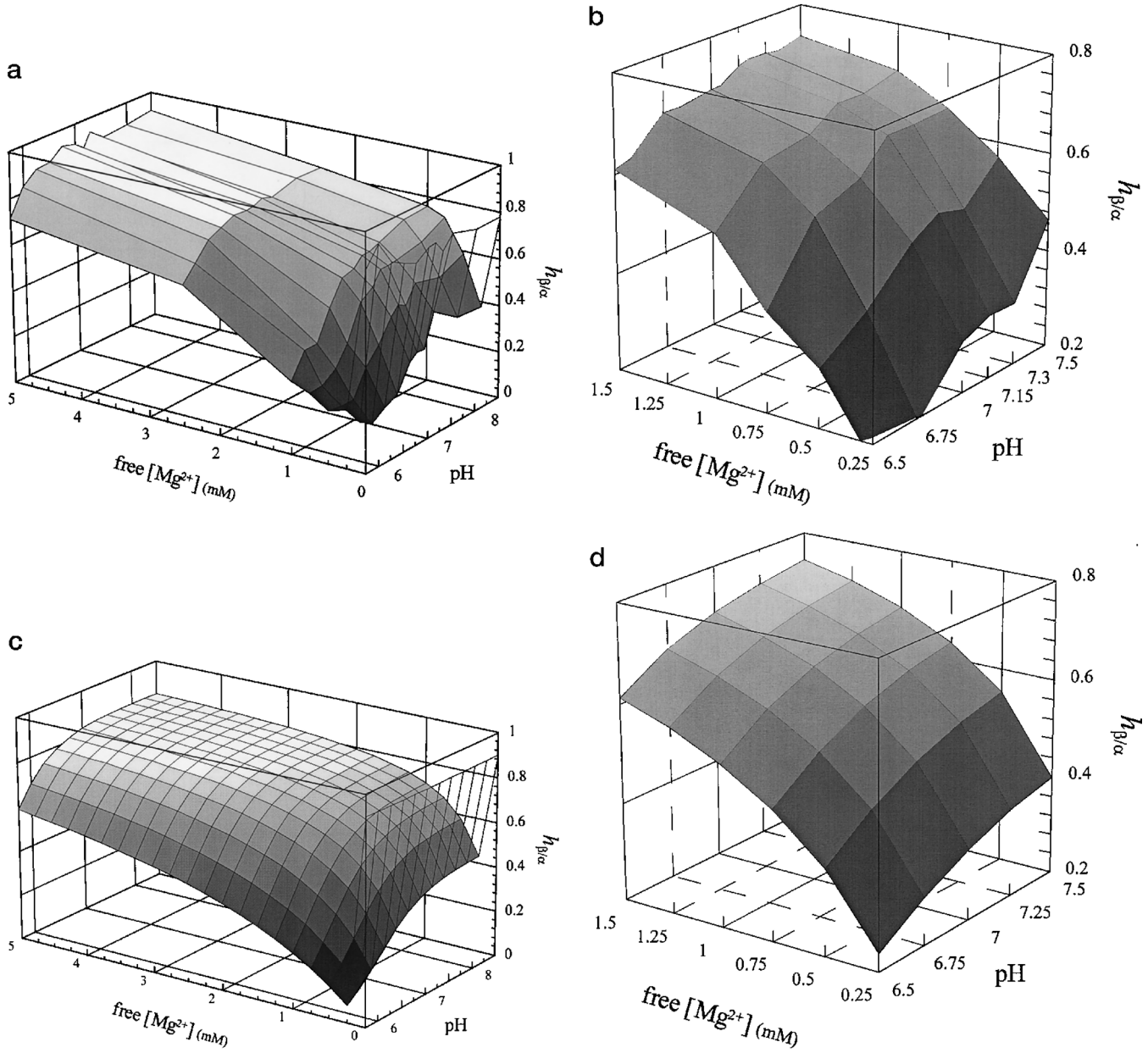

FIG. 5. **Plot of ^{31}P NMR spectra of the experimental (*a*) β/α peak height resonances of ATP, $h_{\beta/\alpha}$, and the theoretical (*c*) surface relating $h_{\beta/\alpha}$ to free [Mg^{2+}] and pH.** The plot of the experimental (*a*) ^{31}P NMR spectral measurements of $h_{\beta/\alpha}$ which show two maxima, $h_{\beta/\alpha\ \mathrm{Max}}$, of about 0.9 at 0 and 5 mM free [Mg^{2+}] and a minima near to K_D of $MgATP^{2-}$ of 0.1 mM. The formalism (Equation 7) describing the relationship between free [Mg^{2+}], pH, and $h_{\beta/\alpha}$ (*c*) duplicates the observed changes in this measured parameter. Within the physiological range of free [Mg^{2+}] from 0.25 to 1.5 mM, the $h_{\beta/\alpha}$ varies over 2-fold (*b*, experimental; *d*, theoretical) making this measurement sensitive to changes in free cytosolic [Mg^{2+}] within living cells.

tra between α and either β or γ is maximum. At free [Mg^{2+}] of 5 mM and pH 8.5, $MgATP^{2-}$ is the predominant species of ATP, and the chemical shift differences are minimal. At all conditions of pH and free [Mg^{2+}] between these extremes, increasing numbers of molecular species which undergo intermediate exchange are formed. This yields β or γ peaks at intermediate positions. Since α, β, and γ resonances are all part of the same ATP molecule, the area under all of the peaks must be equal with consequent broadening of these resonances so that the peak height of these resonances decrease relative to that of the α resonance which remains in the unprotonated uncomplexed form. Advantage may be taken of the multiple ionic species formed with Mg^{2+} in the broadening of the β and γ resonance peaks, the heights of which are determined by the predominant species, to estimate free [Mg^{2+}] so long as pH is also known (Equations 4 and 7c).

In the range of 0.25 to 5 mM free [Mg^{2+}], the ratio of β or γ to α peak height represents the ratio of the number of magnesium-bound β or γ phosphorus to the number of the nonprotonated α phosphorus residues of ATP. In the range of 0–0.25 of free [Mg^{2+}], this ratio represents the number of the nonprotonated and non-magnesium-bound β or γ phosphorus to the nonprotonated α phosphorus nuclei of ATP. Therefore when [Mg^{2+}] is above K_D:

$$h_{\beta/\alpha} = h_{\beta/\alpha\mathrm{Max}} \times \frac{\mathrm{MgP}_\beta}{\mathrm{P}_\alpha^-} \qquad \text{(Eq. 9)}$$

where P_α^- is the unprotonated α phosphate of ATP and MgP_β is the magnesium complex of the β phosphorus of ATP. The

$h_{\beta/\alpha\,\mathrm{Max}}$ term is the maximum β/α peak height ratio of the two phosphorus nuclei within the same ATP molecule observed at 0 and saturating free [Mg^{2+}]. Within the range of [Mg^{2+}] tested, the number of total ATP, ATP_{Tot}, may be represented by:

$$ATP_{Tot} = P^-_\alpha + HP_\alpha = P^-_\beta + HP_\beta + MgP_\beta \qquad \text{(Eq. 10)}$$

where HP_α is the protonated α phosphate of ATP, and P^-_β and HP_β are the unprotonated and protonated β phosphate of ATP.

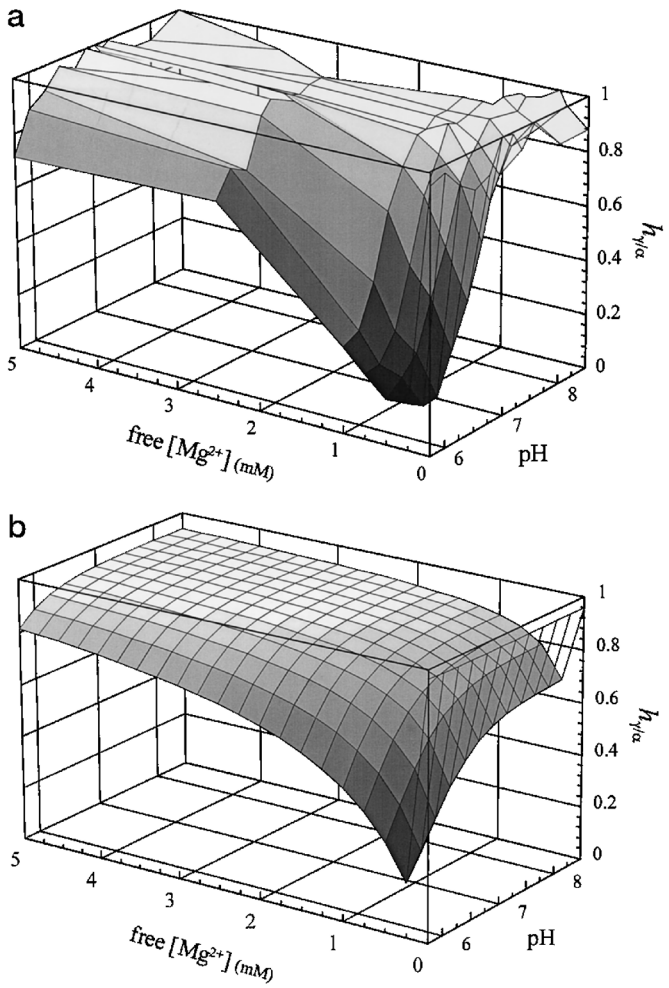

FIG. 6. **Plot of ^{31}P NMR experimental (*a*) data for the γ/α peak height resonances of ATP, $h_{\gamma/\alpha}$, and the theoretical (*b*) surface relating $h_{\gamma/\alpha}$ to free [Mg^{2+}] and pH.** The plot of the $h_{\gamma/\alpha}$ of ATP (*a*) as $h_{\gamma/\alpha}$ varies with free [Mg^{2+}] and pH. The measured $h_{\gamma/\alpha}$ has two maxima at 0 and 5 mM free [Mg^{2+}] at about 0.96 and sharply decreases from near maximum at free [Mg^{2+}] of 0.75 mM to a mimimum near a free [Mg^{2+}] of 0.1 mM and at pH values below 7.0, making the measurement of $h_{\gamma/\alpha}$ less sensitive for estimation of cytosolic free [Mg^{2+}] under normal intracellular conditions. The observed changes in $h_{\gamma/\alpha}$ with pH and free [Mg^{2+}] are duplicated (b) by an equation of the same form (Equation 8) as that describing changes in $h_{\beta/\alpha}$ which is derived from known acid and magnesium dissociation constants and a statement of the ionic forms present in the solution.

From Equation 10, using the K_α values of ATP and the K_D of $MgATP^{2-}$, we may write:

$$P^-_\alpha + HP_\alpha = P^-_\alpha \times (1 + 10^{pK_{\alpha 4} - \mathrm{pH}})$$

$$P^-_\beta + HP_\beta + MgP_\beta = MgP_\beta \times \left(1 + \frac{K_{D\mathrm{MgATP}^{2-}}}{[Mg^{2+}]} \times (1 + 10^{pK_{\alpha 4} - \mathrm{pH}})\right)$$

$$\therefore \frac{MgP_\beta}{P^-_\alpha} = \frac{(1 + 10^{pK_{\alpha 4} - \mathrm{pH}})}{\left(1 + \frac{K_{D\mathrm{MgATP}^{2-}}}{[Mg^{2+}]} \times (1 + 10^{pK_{\alpha 4} - \mathrm{pH}})\right)} \qquad \text{(Eq. 11)}$$

Equation 7a was obtained from Equation 11 by multiplying $K_{D\mathrm{MgATP}^{2-}}/[Mg^{2+}]$ by 2.5, the observed ratio of the γ/β peak heights of ATP near $K_{D\mathrm{MgATP}^{2-}}$ and pH 8.5. This factor appears to be related to the greater chemical shift difference of the β peak of 2.76 ppm relative to that of α and γ, which were 0.43 and 0.59 ppm, respectively. Because the chemical shift differences with free [Mg^{2+}] of α or the γ resonances of unprotonated ATP were quite similar, the sensitivity of $h_{\beta/\alpha}$ was taken to be similar to that observed in $h_{\gamma/\beta}$. For the same reasons, inclusion of such a factor is not required in the derivation of the formalism which describes the changes in the $h_{\gamma/\alpha}$ with free [Mg^{2+}] and pH (Equation 8), because their shift differences are roughly similar. The $h_{\beta/\alpha}$ was however more sensitive within the physiological range because the $\delta_{\alpha\beta}$ was three times that of $\delta_{\alpha\gamma}$ enhancing the broadening of the β peak and hence causing a greater diminution of its height. Under conditions where [Mg^{2+}] is less than 0.25 mM, the concentration closest to K_D tested in this study, the ATP^{4-} species was used for both phosphorus nuclei (Equation 1b). Since the β/α ratio approached 1, this indicated that protonation of ATP^{4-} to $HATP^{3-}$ had little effect on the $h_{\beta/\alpha}$. In contrast, addition of Mg^{2+} at any pH had a large effect on $h_{\beta/\alpha}$ (Fig. 4). We incorporated this observation into Equation 7b in the statement

$$HP_\beta = P^-_\beta \times \frac{[Mg^{2+}]}{K_{D\mathrm{MgATP}^{2-}}}[HATP^{3-}] \qquad \text{(Eq. 12)}$$

The peak height of the β resonance of ATP was maximal at 0 and saturating free [Mg^{2+}] and decreased at intermediate free [Mg^{2+}], while the α resonance of ATP was essentially invariant, as had been observed previously (18). What was not previously known was the formalism relating this decrease in peak height to free [Mg^{2+}] and that this variation in β/α peak height would occur within the range of the physiological intracellular free [Mg^{2+}] of 0.25 to 1.5 mM (9). In attempting to use the measured $h_{\beta/\alpha}$ to estimate free [Mg^{2+}] it is essential that care be taken in determining the position of the base line under the β and α peaks of ATP in order to obtain an accurate measurement of peak height. When the measured β/α peak height ratio approaches the $h_{\beta/\alpha\mathrm{Max}}$ of about 0.90, the calculation of free [Mg^{2+}] using Equation 8 becomes indeterminate except to conclude that it is >1.5 mM (Fig. 3*a*). Likewise, when the concentration of free [Mg^{2+}] falls below 0.25 mM, approaching the K_D of $MgATP^{2-}$ of 0.1 mM, the estimation of free [Mg^{2+}] by this method would become indeterminate.

TABLE III

Variation in perfused heart free cytosolic [Mg^{2+}] using chemical shift or peak height ratios of ATP

Cytosolic free [Mg^{2+}] in working rat hearts was evaluated using two methods: measurement of $h_{\beta/\alpha}$ and $\delta_{\alpha\beta}$, and given as means ± standard error of the mean with the number observations given in (). *indicates a significant difference of the means of the $h_{\beta/\alpha}$ and $\delta_{\alpha\beta}$ groups at $p < 0.05$ as judged by the Mann-Whitney *U* test. K_{CK} is the apparent equilibrium constant of the creatine kinase reaction (EC 2.7.3.2) at the pH and free [Mg^{2+}] specified.

	Glucose (6)		Glucose + ketones (4)	
	$h_{\beta/\alpha}$	$\delta_{\alpha\beta}$	$h_{\beta/\alpha}$	$\delta_{\alpha\beta}$
$[Mg^{2+}]_{cyto}$	1.0 ± 0.1	0.51 ± 0.06*	0.61 ± 0.02	0.46 ± 0.04*
$K_{CK} \times 10^{-9}$ M	1.65 ± 0.00	1.27 ± 0.00*	1.37 ± 0.00	1.21 ± 0.00*
$[\Sigma ADP]_{cyto}$	0.172 ± 0.012	0.223 ± 0.015*	0.083 ± 0.005	0.094 ± 0.005

The value of the apparent equilibrium constants of a number of important intracellular reactions change with both pH and free [Mg^{2+}]. In particular free cytosolic [ΣADP] has been estimated from the measured components of the creatine kinase reaction (3, 4, 49, 50), particularly in studies attempting to determine the factors controlling O_2 consumption in intact tissues (39) or to explain variations in [ΣPCr]/[ΣATP] ratios observed by ^{31}PNMR spectroscopy of human subjects (4) in various physiological states. The apparent equilibrium constant of the creatine kinase reaction:

$$K_{CK} = \frac{[\Sigma ATP][Creatine]}{[\Sigma ADP][\Sigma PCr]} = K_{ionic}[H^+]^{-(-1)} \times \frac{f ATP}{f ADP \times f PCr},$$

where

$$K_{ionic} = \frac{[ATP^{4-}][Creatine^{\pm}]}{[ADP^{3-}][PCr^{2-}][H^+]} = 3.88 \times 10^8 M^{-1} \qquad \text{(Eq. 13)}$$

and the value of K'_{CK} at pH 7.0, free [Mg^{2+}] = 1 mM, I = 0.25 and T = 38 °C is 166 (1). This value has been confirmed to within about 5% (36, 51). It is not widely appreciated, however, that without accurate assessment of both cytosolic pH and free [Mg^{2+}], the estimation of cytosolic [ΣADP] may be significantly in error (Table III). Determination of both pH and free [Mg^{2+}] from δ_{P_i} and $h_{\beta/\alpha}$ by the simultaneous solution of Equations 5 and 8 not only improves the estimation of both cytosolic free [Mg^{2+}] and pH, but also the estimation of cytosolic free [ΣADP].

The use of $h_{\beta/\alpha}$ to determine intracellular free [Mg^{2+}] has advantages over the measurement of the [citrate]/[isocitrate] ratio or the measurement of $\delta_{\alpha\beta}$. First, use of metabolite ratios requires tissue destruction and the enzymatic measurement of isocitrate is technically difficult. Secondly estimation of the [citrate]/[isocitrate] ratio can give spurious estimates of the cytosolic free [Mg^{2+}] because of intraorganelle compartmentation of metabolite or of Mg^{2+} or because of a failure of aconitase to achieve near-equilibrium. Measurement of $\delta_{\alpha\beta}$ for the determination of intracellular free [Mg^{2+}] is limited primarily by its lack of sensitivity in the physiological range, and by its analysis with an inaccurate formalism. The alternative use of $h_{\beta/\alpha}$ takes advantage of tissue analysis by ^{31}P NMR spectroscopy which is non destructive and therefore capable of use in living subjects. Additionally, the $h_{\beta/\alpha}$ measures only cytosolic ATP and requires neither an enzyme catalyzed equilibrium nor the administration of an exogenous agent of lesser known tissue distribution and allergenic potential. While peak heights may be altered by a number of other factors, such as excess amounts of paramagnetic substances or other factors severely altering the magnetic environment, this new method for estimation of free [Mg^{2+}] in tissue may provide further insights into the physiological and therapeutic role of [Mg^{2+}] in a number of disease states (32). Hitherto a deficiency of free intracellular [Mg^{2+}] could only be inferred from measurement of magnesium retention after intravenous administration (30).

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REFERENCES

1. Lawson, J. W. R., and Veech, R. L. (1979) *J. Biol. Chem.* **254,** 6528–6537
2. Cornell, N. W., Leadbetter, M., and Veech, R. L. (1979) *J. Biol. Chem.* **254,** 6522–6527
3. Veech, R. L., Lawson, J. W. R., Cornell, N. W., and Krebs, H. A. (1979) *J. Biol. Chem.* **254,** 6538–6547
4. Hochachka, P. W., Clark, C. M., Holden, J. E., Stanley, C., Ugurbil, K., and Menon, R. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 1215–1220
5. Kwack, H., and Veech, R. L. (1992) in *From Metabolite, to Metabolism to Metabolon* (Stadtman, E. R., and Chock, P. B., eds) pp. 185–207, Academic Press, San Diego
6. Veech, R. L., Gates, D. N., Crutchfield, C. W., Gitomer, W. L., Kashiwaya, Y., King, M. T., and Wondergem, R. (1994) *Alcohol. Clin. Exp. Res.* **18,** 1040–1056
7. Hille, B. *Ionic Channels of Excitable Membranes*, Sinauer, Sunderland, MA
8. London, R. E. (1991) *Annu. Rev. Physiol.* **53,** 241–258
9. Veloso, D., Guynn, R. W., Oskarsson, M., and Veech, R. L. (1973) *J. Biol. Chem.* **248,** 4811–4819
10. Rose, I. A. (1968) *Proc. Natl. Acad. Sci. U. S. A.* **61,** 1079–1086
11. Bunn, H. F., Ransil, B. J., and Chao, A. (1971) *J Biol. Chem.* **246,** 5273–5279
12. Walser, M. (1967) *Ergeb. Physiol.* **59,** 185–296
13. Masuda, T., Dobson, G. P., and Veech, R. L. (1990) *J. Biol. Chem.* **265,** 20321–20334
14. Cohen, S. M. (1983) *J. Biol. Chem.* **258,** 14294–14308
15. Cohn, M., and Hughes, T. R. (1962) *J. Biol. Chem.* **237,** 176–181
16. Bock, J. L. (1980) *J. Inorg. Biochem.* **12,** 119–130
17. Pecoraro, V. L., Hermes, J. D., and Cleland, W. W. (1984) *Biochemistry* **23,** 5262–5271
18. Gupta, R. K., Gupta, P., Yushok, W. D., and Rose, Z. B. (1983) *Physiol. Chem. Phys. Med. NMR* **15,** 265–280
19. Gupta, R. K., and Moore, R. D. (1980) *J. Biol. Chem.* **255,** 3987–3993
20. Gupta, R. K., Benovic, J. L., and Rose, Z. B. (1978) *J. Biol. Chem.* **253,** 6172–6176
21. Malloy, C. R., Cunningham, C. C., and Radda, G. K. (1986) *Biochim. Biophys. Acta* **885,** 1–11
22. Dowd, T. L., and Gupta, R. K. (1993) *J Biol. Chem.* **268,** 991–996
23. Gupta, R. K., Gupta, P., Yushok, W. D., and Rose, Z. B. (1983) *Biochem. Biophys. Res. Commun.* **117,** 210–216
24. Mosher, T. J., Williams, G. D., Doumen, C., LaNoue, K. F., and Smith, M. B. (1992) *Magn. Reson. Med.* **24,** 163–169
25. Gullestad, L., Dolva, L. O., Waage, A., Falch, D., Fagerthun, H., and Kjekshus, J. (1992) *Scand J. Clin. Lab. Invest.* **52,** 245–253
26. Murphy, E. (1993) *Miner. Electrolyte Metab.* **19,** 250–258
27. Jones, J. E., Shane, S. R., Jacobs, W. H., and Flink, E. B. (1969) *Ann. N. Y. Acad. Sci.* **162,** 934–946
28. Altura, B. M., Altura, B. T., Gebrewold, A., Ising, H., and Gunther, T. (1984) *Science* **223,** 1315–1317
29. Meema, H. E., Oreopoulos, D. G., and Rapoport, A. (1987) *Kidney Int.* **32,** 388–394
30. Rasmussen, H. S., McNair, P., Goransson, L., Balslov, S., Larsen, O. G., and Aurup, P. (1988) *Arch. Intern. Med* **148,** 329–332
31. Weiss, M., and Lasserre, B. (1994) *Magnes. Res.* **7,** 135–144
32. McLean, R. M. (1994) *Am. J. Med.* **96,** 63–76
33. Ruat, M., Snowman, A. M., Hester, L. D., and Snyder, S. H. (1996) *J. Biol. Chem.* **271,** 5972–5975
34. Alberty, R. A. (1969) *J. Biol. Chem.* **244,** 3290–3302
35. Lawson, J. W. R., Guynn, R. W., Cornell, N. W., and Veech, R. L. (1976) in *Gluconeogenesis, Its Regulation in Mammalian Species* (Hanson, R. W., and Mehlman, M. A., eds) pp. 481–514, John Wiley & Sons, New York
36. Teague, W. E., Jr., and Dobson, G. P. (1992) *J Biol. Chem.* **267,** 14084–14093
37. Kashiwaya, Y., Sato, K., Tsuchiya, N., Thomas, S., Fell, D. A., Veech, R. L., and Passonneau, J. V. (1994) *J. Biol. Chem.* **269,** 25502–25514
38. Passonneau, J. V., and Lowry, O. H. (1993) *Enzymatic Analysis: A Practical Guide*, pp. 128–130, 183–185, Humana Press, Totowa, NJ
39. Sato, K., Kashiwaya, Y., Keon, C. A., Tsuchiya, N., King, M. T., Radda, G. K., Chance, B., Clarke, K., and Veech, R. L. (1995) *FASEB J.* **9,** 651–658
40. Moon, R. B., and Richards, J. H. (1973) *J. Biol. Chem.* **248,** 7276–7278
41. Bailey, I. A., Williams, S. R., Radda, G. K., and Gadian, D. G. (1981) *Biochem. J.* **196,** 171–178
42. Roberts, J. K., Wade-Jardetzky, N., and Jardetzky, O. (1981) *Biochemistry* **20,** 5389–5394
43. Kost, G. J. (1990) *Magn. Reson. Med* **14,** 496–506
44. Goldberg, R. N., and Tewari, Y. B. (1991) *Biophys. Chem.* **241,** 241–261
45. Roos, A., and Boron, W. F. (1981) *Physiol. Rev.* **61,** 296–434
46. Dobson, G. P., Veech, R. L., Passonneau, J. V., Kobayashi, K., Inubushi, T., Wehrli, S., Nioka, S., and Chance, B. (1992) *NMR Biomed.* **5,** 20–28
47. Smith, R. M., and Alberty, R. A. (1956) *J. Am. Chem. Soc.* **78,** 2376–2380
48. Wagman, D. D., Evans, W. H., Parker, V. B., Schumm, W. H., Halow, I., Bailey, S. M., Churney, K. L., and Nuttall, R. L. (1982) *J. Phys. Chem. Ref. Data* **11,** Suppl. 2, 73
49. Balaban, R. S., Kantor, H. L., Katz, L. A., and Briggs, R. W. (1986) *Science* **232,** 1121–1123
50. Chance, B., Leigh, J. S. J., Kent, J., McCully, K., Nioka, S., Clark, B. J., Maris, J. M., and Graham, T. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 9458–9462
51. LoPresti, P., and Cohn, M. (1989) *Biochim. Biophys. Acta* **998,** 317–320