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Experimental demonstration of quantitation errors in MR spectroscopy resulting from saturation corrections under changing conditions

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Abstract

Metabolite concentration measurements in in vivo NMR are generally performed under partially saturated conditions, with correction for partial saturation performed after data collection using a measured saturation factor. Here, we present an experimental test of the hypothesis that quantitation errors can occur due to application of such saturation factor corrections in changing systems. Thus, this extends our previous theoretical work on quantitation errors due to varying saturation factors. We obtained results for two systems frequently studied by ^{31}P NMR, the ischemic rat heart and the electrically stimulated rat gastrocnemius muscle. The results are interpreted in light of previous theoretical work which defined the degree of saturation occurring in a one-pulse experiment for a system with given spin–lattice relaxation times, T_1s, equilibrium magnetizations, M_0s, and reaction rates. We found that (i) the assumption of constancy of saturation factors leads to quantitation errors on the order of 40% in inorganic phosphate; (ii) the dominant contributor to the quantitation errors in inorganic phosphate is most likely changes in T_1; (iii) T_1 and M_0 changes between control and intervention periods, and chemical exchange contribute to different extents to quantitation errors in phosphocreatine and γ-ATP; (iv) relatively small increases in interpulse delay substantially decreased quantitation errors for metabolites in ischemic rat hearts; (v) random error due to finite SNR led to approximately 4% error in quantitation, and hence was a substantially smaller contributor than were changes in saturation factors.
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1. Introduction

The one-pulse and related experiments are frequently used to quantify metabolite concentrations in in vivo NMR studies. These experiments are typically performed under partially saturated conditions in which the interpulse delay, TR, is less than the T_1s of the observed resonances. While rapid pulsing is beneficial in increasing signal-to-noise ratio per unit time (SNR), the resulting resonance amplitudes must be corrected for partial saturation [1], as follows.

Let M_0 denote the equilibrium magnetization and $M_{\text{obs}}(\theta, \text{TR})$ the observed magnetization in the steady state resulting from the one-pulse sequence with flip angle θ and interpulse delay TR. $M_{\text{obs}}(\theta, \text{TR}) = M_0$ whenever $\text{TR} \to \infty$ and $\theta = 90°$. Otherwise, the saturation factor, SF, is defined as the ratio of the observed magnetization to the equilibrium magnetization of the resonance:

$$\text{SF}(\theta, \text{TR}) = \frac{M_{\text{obs}}(\theta, \text{TR})}{M_0}. \tag{1}$$

It has been shown [1] that

$$\frac{M_{\text{obs}}(\theta, \text{TR})}{M_0} = \frac{(1 - \text{e}^{-\text{TR}/T_1}) \sin\theta}{(1 - \text{e}^{-\text{TR}/T_1} \cos\theta)} \tag{2}$$

for noninteracting resonances. Eq. (2) is not typically used explicitly in determining SFs. Rather, Eq. (1), based directly upon observed magnetizations, is used.

A common application of quantitation is the determination of resonance amplitudes following an intervention (Int). Define $M_0^{\text{Int,Apparent}}$ to be the value obtained according to the procedure outlined above, based on the measurement of a SF during a control period (Ctl):

$$M_0^{\text{Int,Apparent}} = \frac{M_{\text{obs}}^{\text{Int}}(\theta, \text{TR})}{\text{SF}^{\text{Ctl}}(\theta, \text{TR})}. \tag{3}$$

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The true value of the resonance amplitude during the intervention will be designated M_0^{Int}. The desired relation,

$$M_0^{\mathrm{Int,Apparent}} = M_0^{\mathrm{Int}} \tag{4}$$

holds when $\mathrm{SF}^{\mathrm{Ctl}}$ does not change between the time it is measured and the time $M_{\mathrm{obs}}^{\mathrm{Int}}(\theta, \mathrm{TR})$ is measured. According to Eq. (2), this requires only that the T_1 be unchanged. However, we have shown more recently that Eq. (2) is strictly valid only when the species under consideration is not in chemical exchange, CE [2–7]. Otherwise, the SFs depend also upon the T_1s and M_0s of all the other resonances in the exchange network, as well as on the rates of exchange between sites. Thus, these also must remain constant for Eq. (4) to hold strictly. The complete functional form of $\mathrm{SF}(T_{1,\mathrm{S_i}}, M_{0,\mathrm{S_i}}, k_{\mathrm{S_iS_j}})$, where the $\mathrm{S_i}$ are species labels, has been given previously [3].

It is clear from the continuity of the dependence of SF on system parameters that small variations in these parameters will lead to near-validity of Eq. (4) [4,8]. The question however remains whether substantial errors can result from use of Eq. (3) under realistic circumstances. While this has been discussed theoretically [3,4,7–9], the present manuscript experimentally tests the specific hypothesis that departures from Eq. (3) are observable under normal conditions.

A widely studied exchange network in in vivo NMR is the three-site system of phosphocreatine (PCr), γ-ATP, and inorganic phosphate ($\mathrm{P_i}$):

$$\mathrm{PCr} \underset{k_{\gamma\text{-ATP}\to\mathrm{PCr}}}{\overset{k_{\mathrm{PCr}\to\gamma\text{-ATP}}}{\rightleftarrows}} \gamma\text{-ATP} \underset{k_{\mathrm{P_i}\to\gamma\text{-ATP}}}{\overset{k_{\gamma\text{-ATP}\to\mathrm{P_i}}}{\rightleftarrows}} \mathrm{P_i} \tag{5}$$

where the forward and reverse creatine kinase (CK) reaction rates are denoted by $k_{\mathrm{PCr}\to\gamma\text{-ATP}}$ and $k_{\gamma\text{-ATP}\to\mathrm{PCr}}$, respectively, and the ATP synthesis and hydrolysis rates are given by $k_{\mathrm{P_i}\to\gamma\text{-ATP}}$ and $k_{\gamma\text{-ATP}\to\mathrm{P_i}}$. Accordingly, we test the above hypothesis using ^{31}P NMR. We present experimental results for two systems, the isolated perfused rat heart undergoing ischemia and the stimulated rat gastrocnemius muscle in vivo. Simulation results are then presented to confirm that our experimental results may be explained within the framework of the dependence of SFs on system parameters [3]. Negative controls with high and low SNR were used to demonstrate both the validity of the usual correction for partial saturation for nonchanging systems, and that random error contributes substantially less to quantitation error than does departure from Eq. (4) in our experiments.

2. Methods

2.1. Perfused rat heart spectroscopy

Hearts from male Wistar rats weighing 375–400 g were perfused retrograde through the aorta with phosphate-free Krebs–Henseleit buffer gased with a 95% O_2–5% CO_2 mixture (pH 7.4) at 37 °C. Buffer flow was held constant at 21 ml/min during control conditions and reduced to 5 ml/min during ischemia. Left ventricular pressures were monitored via a fluid-filled catheter connected to a water-filled latex balloon inserted into the left ventricle. Initial left ventricular end-diastolic pressure (LVEDP) was set to 6 mmHg in all hearts. Hearts were paced at 240 beats per minute. Cardiac function was monitored throughout in order to assess stability of the preparation; unstable hearts were discarded.

The perfused hearts were placed into a 20 mm NMR sample tube and commercial probe, and inserted into the bore of a 9.4 T Bruker DMX NMR spectrometer (Bruker Medizintechnik GmbH, Ettlingen, Germany). Experiments were performed on two groups of hearts for which the expected errors differed substantially. In the first group ($n = 8$), spectra were recorded with $\mathrm{TR} = 15\,\mathrm{s}$, $\theta = 90°$ (56 acquisitions) and $\mathrm{TR} = 1\,\mathrm{s}$, $\theta = 90°$ (400 acquisitions) both during the control period and after stability was attained during the ischemic period. In the second group of hearts ($n = 8$), the pulse parameters were $\mathrm{TR} = 15\,\mathrm{s}$, $\theta = 60°$ (56 acquisitions) and $\mathrm{TR} = 2\,\mathrm{s}$, $\theta = 60°$ (200 acquisitions). The spectral data acquisition time, and hence time resolution, during the ischemic period was the same for the short TR spectra in these two groups of hearts. The order of the spectra acquired with short TR and with a TR of 15 s, for both Ctl and Int periods, was randomized. Eight dummy pulses were used for all spectra acquired at a short TR. Spectra were quantified by using Lorentzian fits to resonance lines. Intensities were normalized by the number of acquisitions.

2.2. In vivo rat gastrocnemius muscle spectroscopy

Male Wistar rats ($n = 9$) weighing 375–400 g were anesthetized with isofluorane and placed on a temperature controlled heating mat within a homebuilt animal holder. The right gastrocnemius muscle was fastened to a home-built two-turn elliptical surface coil (1.2×2.0 cm). Two platinum electrodes were placed percutaneously into the Achilles tendon and into the proximal portion of the lateral head of the gastrocnemius muscle, respectively. Supramaximal electrical stimulation was performed with a pair of rectangular pulses each of duration 5 ms, with an interval between the pulses of 200 ms and a 2 s interval between the pulse pairs. Contraction force was measured using a force transducer attached to the foot of the leg under study. Data from unstable preparations were discarded.

Spectra were obtained using a 1.9 T, 31 cm Bruker ABX Biospec (Bruker Medizintechnik GmbH, Ettlingen, Germany). An adiabatic half passage sin/cos pulse

[10] of 0.512 ms duration was applied to achieve uniform 90° excitation of spins. Spectra were recorded with TR = 10 s, $\theta = 90°$ (56 acquisitions) and TR = 1 s, $\theta = 90°$ (400 acquisitions) both during the control period and after stability was attained during stimulation. The order of the spectra acquired with a TR of 1 s and with a TR of 10 s was randomized for both Ctl and Int periods. A sufficient number of dummy pulses was used for spectra acquired at the short TR. Spectra were quantified using the same procedure as in the heart experiments.

2.3. Methylenediphosphonic acid spectroscopy

An external standard of methylenediphosphonic acid (MDP), contained in a sealed capillary adjacent to the heart during all heart experiments, was incorporated as a negative control with high SNR (≈14) which undergoes no CE and had a constant T_1 throughout the experiment. Spectra were quantified using the same procedure as in the heart experiments. The SNR was defined as the maximal signal divided by 2× the noise amplitude.

2.4. Phosphate-buffered saline spectroscopy

^{31}P NMR was performed at 9.4 T using a Bruker DMX NMR spectrometer on a sample of phosphate-buffered saline (PBS) within a 20 mm NMR sample tube. Twelve spectra were acquired using a TR = 1 s and $\theta = 90^{\circ}$ (300 acquisitions, 4 dummy scans), and another 12 were acquired using a TR = 15 s and $\theta = 90°$ (3 acquisitions). These pulse parameters were chosen to approximately match the SNR of these experiments to that of P_i in the isolated perfused heart during control conditions, which ranges from 2 to 5. Spectra were quantified using the same procedure as in the heart experiments. The SNR was calculated as described for MDP.

2.5. Definition of quantitation errors

SF(θ,TR) was determined in accordance with Eq. (1), where all values refer to the control period. These SF(θ,TR) were then used for correction of the short TR spectra obtained during the intervention (ischemia for hearts, stimulation for rat gastrocnemius). $M_0^{\text{Int,Apparent}}$ was then compared with M_0^{Int} [4,7], with percent error defined as

$$\%\ \text{error in}\ M_0^{\text{Int,Apparent}} = \frac{\left[M_0^{\text{Int,Apparent}} - M_0^{\text{Int}}\right]}{M_0^{\text{Int}}} 100\%. \tag{6}$$

Results of metabolite quantitation are reported as mean (SEM). Statistical analysis was performed using the Student's t test, where two-tailed values of $p < 0.05$ were considered significant.

2.6. Simulations

Simulations were performed in order to evaluate the potential basis for the experimental results. The simulations were based on the fundamental equation describing partial saturation in the presence of CE as derived previously [3]:

$$\mathbf{SF} = \mathbf{M}_0^{-1}(\mathbf{I} - e^{\mathbf{A}\,TR}\cos\theta)^{-1}(\mathbf{I} - e^{\mathbf{A}\,TR})\mathbf{M}_0 \sin\theta, \tag{7}$$

where $\mathbf{M}_0 = (M_{0S_1}, M_{0S_2}, \ldots, M_{0S_N})$, with S_i labeling species, $\mathbf{I}$ is the $N \times N$ identity matrix, $\mathbf{M}_0 = \mathbf{I}\mathbf{M}_0$, and $\mathbf{A}$ is a matrix depending upon all of the T_1s of the metabolites within an exchange network and upon all of the reaction rates between these metabolites.

Simulations in the Mathematica (Wolfram Research, Champaign, IL) programming language were performed for the three-site reaction network described in Eq. (5). Simulation input values are given in Table 1. Equilibrium magnetizations for the control and intervention periods were experimentally measured using long TRs

Table 1
Physiological parameters used for the model simulations

	T_1(PCr)	T_1(γ-ATP)	T_1(P_i)	M_0(PCr)	M_0(γ-ATP)	M_0(P_i)	$k_{PCr\to\gamma\text{-ATP}}$	$k_{Pi\to\gamma\text{-ATP}}$
Heart								
(1 s, 90°)								
Control	2.78 [11]	0.64 [11]	2.4 [11]	0.158 (0.008)	0.140 (0.008)	0.039 (0.007)	0.7 [12]	0.37 [11]
Ischemia	**1.76**	**0.7**	**4.79**	0.102 (0.009)	0.111 (0.009)	0.121 (0.019)	**0.10**	**0.13**
(2 s, 60°)								
Control	2.78 [11]	0.64 [11]	2.4 [11]	0.215 (0.011)	0.149 (0.007)	0.034 (0.007)	0.7 [12]	0.37 [11]
Ischemia	**2.16**	**0.66**	**3.81**	0.143 (0.014)	0.145 (0.010)	0.088 (0.006)	**0.52**	**0.12**
Skeletal								
Control	6.3 [6]	2.5 [6]	4.0 [6]	6.13 (0.269)	1.15 (0.094)	0.249 (0.043)	0.21 [6]	**0.23**
Stimulation	**7.01**	**2.0**	**8.0**	2.59 (0.298)	0.874 (0.087)	2.39 (0.309)	0.32 [6]	**0.21**

Referenced T_1s and rate constants were obtained from the literature. Bolded values are estimates calculated using referenced T_1s and rate constants and fitting Eqs. (6) and (7) to the experimental data. M_0s were determined experimentally and are given as the mean (SEM).

as described above. T_1s and rate constants were obtained insofar as possible from literature reports of experiments comparable to those performed here [6,11,12]. Bolded values for the rat hearts were calculated using literature T_1s and reaction rates for comparable control experiments, as referenced in Table 1. Bolded values for the gastrocnemius muscle were calculated using literature T_1s for control experiments and forward CK rates for control and intervention experiments, as referenced in Table 1. These literature values, along with our experimentally observed M_0s, were used as input parameters to a global optimization program written in the Mathematica programming language and based upon Eqs. (6) and (7). The resulting best-fit values (bolded) are comparable to literature results [6,11,12]. Simulations were first performed with both CE and T_1 changes. It is straightforward to identify many sets of input parameters which generate results which match the experiments. Our approach was to use plausible values to assess the likely dominant contributors to the observed errors. In particular, the error due to CE alone was estimated by calculating $M_0^{\text{Int,Apparent}}$ with all T_1s set to control values. The error due to T_1 changes alone was estimated by calculating $M_0^{\text{Int,Apparent}}$ with all reaction rates set to zero.

3. Experimental results

3.1. Heart experiments, $TR = 1$ s and $\theta = 90°$

Table 2 shows the comparison between the true equilibrium magnetizations, as measured directly using a long TR acquisition, and apparent equilibrium magnetizations as determined by Eq. (3). The error in P_i, −44.3%, was large, with $M_0^{\text{Int,Apparent}}$ nearly a factor of two smaller than M_0^{Int} ($p < 0.05$). Smaller errors were seen for PCr and γ-ATP.

3.2. Heart experiments, $TR = 2$ s and $\theta = 60°$

Again, a significant difference between $M_0^{\text{Int,Apparent}}$ and M_0^{Int} was seen for P_i (−18.7%, $p < 0.05$), with smaller errors in PCr and γ-ATP (Table 2).

The overall reduction in errors, as compared with the TR = 1 s, $\theta = 90°$ dataset, is attributable primarily to the increase in TR [3,7,14].

3.3. Gastrocnemius experiments

Experiments were performed with TR = 1 s and $\theta = 90°$. Again, a significant difference between $M_0^{\text{Int,Apparent}}$ and M_0^{Int} was seen for P_i (−39.8%, $p < 0.05$). Small differences between M_0^{Int} and $M_0^{\text{Int,Apparent}}$ were seen for PCr and γ-ATP (Table 2).

3.4. Methylenediphosphonic acid experiments

Experiments were performed with TR = 1 s and $\theta = 90°$, and TR = 2 s and $\theta = 60°$ to demonstrate the validity of Eq. (4) when CE and T_1 changes are not present for a sample with a large SNR. As expected, $M_0^{\text{Int,Apparent}}(\text{MDP}) \approx M_0^{\text{Int}}(\text{MDP})$ in all experiments, with errors <1% (Table 2).

3.5. Phosphate-buffered saline experiments

Experiments were performed with TR = 1 s and $\theta = 90°$ to determine the contribution of random error to the difference between M_0^{Int} and $M_0^{\text{Int,Apparent}}$ for metabolites with low SNR. This contribution, approximately 4%, was much smaller than the error

Table 2
Experimental values of $M_0^{\text{Int,Apparent}}$, M_0^{Int}, and % error of $M_0^{\text{Int,Apparent}}$ (bolded) for heart and skeletal muscle

	MDP	P_i	PCr	γ-ATP
Heart				
(1 s, 90°)				
$\mathbf{M_0^{Int,Apparent}}$	0.270 (0.017)	0.067 (0.015)	0.106 (0.015)	0.117 (0.006)
$\mathbf{M_0^{Int}}$	0.272 (0.021)	0.121 (0.019)	0.102 (0.009)	0.111 (0.009)
% **Error in** $\mathbf{M_0^{Int,Apparent}}$	**−0.793**	**−44.3***	**3.55**	**5.59**
(2 s, 60°)				
$\mathbf{M_0^{Int,Apparent}}$	0.233 (0.023)	0.071 (0.004)	0.146 (0.011)	0.148 (0.012)
$\mathbf{M_0^{Int}}$	0.234 (0.011)	0.088 (0.006)	0.143 (0.014)	0.145 (0.010)
% **Error in** $\mathbf{M_0^{Int,Apparent}}$	**−0.639**	**−18.7***	**2.46**	**2.23**
Skeletal				
$\mathbf{M_0^{Int,Apparent}}$		1.44 (0.198)	2.57 (0.295)	0.929 (0.118)
$\mathbf{M_0^{Int}}$		2.39 (0.309)	2.59 (0.298)	0.874 (0.087)
% **Error in** $\mathbf{M_0^{Int,Apparent}}$		**−39.8***	**−0.676**	**6.35**

Interpulse delay and flip angle are given for each set of data and are presented as (TR,θ). M_0s were determined experimentally and are given as the mean (SEM).

* Denotes statistical significance using the Student's t test ($p < 0.05$).

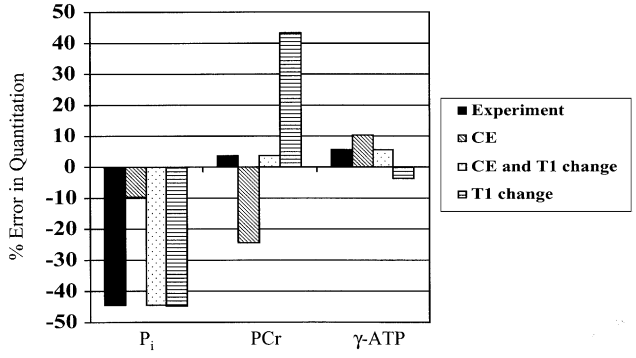

Fig. 1. Simulated errors compared to experimental errors for P_i, PCr, and γ-ATP for the ischemic heart ($n = 8$). The pulse parameters used were TR = 1 s and $\theta = 90°$. Errors resulting from chemical exchange (CE) and T_1 changes are identical to experimental errors. The contribution of CE alone and T_1 changes alone are shown.

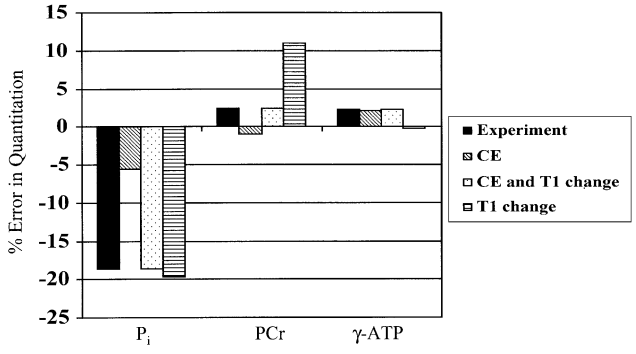

Fig. 2. Similar to Fig. 1, except with pulse parameters of TR = 2 s and $\theta = 60°$ ($n = 8$).

experimentally determined for P_i in both the isolated perfused heart and gastrocnemius experiments.

4. Simulation results

4.1. Heart simulations, TR = 1 s, θ = 90°

A comparison of the experimental results presented in Table 2 and model simulations is shown in Fig. 1. Simulation results for the case of CE and changes in T_1 are identical to the experimental errors, since the bolded values in Table 1 were acquired by fitting these parameters to the experimental data. Examination of the figure suggests that relative contributions of CE and T_1 changes to errors differ between metabolites.

The simulated error in P_i for the case of CE alone was small relative to the experimental error. T_1 changes alone produced an error almost identical to the experimental error for P_i. These results show a strong dependence of error on changes in T_1. Errors in PCr due to CE and T_1 were nearly equal and opposite, resulting in a small net error and indicating the importance of both effects on total error. Similarly, for γ-ATP, the two

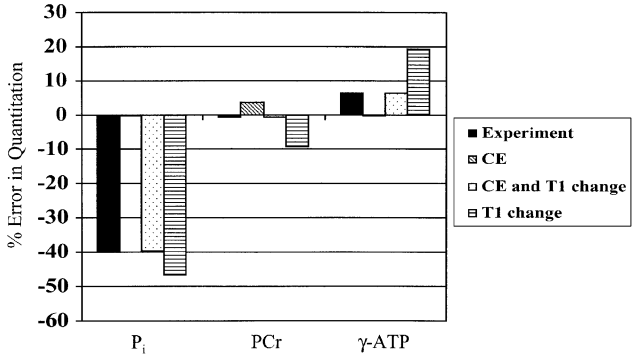

Fig. 3. Similar to Fig. 1, except for gastrocnemius muscle ($n = 9$). Pulse parameters were TR = 1 s and $\theta = 90°$.

sources of error partially offset each other with CE making a somewhat greater contribution.

4.2. Heart simulations, TR = 2 s, θ = 60°

A comparison of the experimental results presented in Table 2 and model simulations is shown in Fig. 2. Again, T_1 changes dominate the error in P_i. Both CE and T_1 changes contribute to the error in PCr and CE is the dominant source of error for γ-ATP.

4.3. Gastrocnemius simulations, TR = 1 s, θ = 90°

A comparison of the experimental results presented in Table 2 and model simulations is shown in Fig. 3.

The error in P_i was dominated by T_1 changes; the error for CE alone was close to zero, which can be attributed to small changes in rate constants between Ctl and Int. The contribution to quantitation error in PCr was large in magnitude for both CE and T_1 changes, but these effects largely offset each other, leading to a small net error. The simulated error in γ-ATP for the case of CE alone was negligible, whereas for T_1 changes alone, it was much larger than the experimental error. These results suggest that both CE and T_1 changes contribute to the experimental error.

5. Discussion

It is almost axiomatic in in vivo NMR that metabolite concentrations obtained under partially saturated conditions may be corrected by use of a predetermined saturation factor. This procedure relies upon the implicit assumption that SF of the metabolite under consideration does not change between the time the saturation factor data is collected and the time of acquisition of the partially saturated data. To our knowledge, however, this hypothesis has never been subjected to experimental scrutiny; accordingly, we performed experiments directly testing the validity of saturation factor corrections

under typical experimental conditions. We find that correction for metabolite concentrations by use of saturation factors can in fact lead to substantial errors.

According to the Ernst and Anderson analysis of the one-pulse experiment [1], the accuracy of saturation corrections requires only that the T_1 of the metabolite under consideration remain unchanged. More recently, we have shown theoretically [2–4] that saturation corrections may be problematic even when the T_1 of the metabolite in question remains unchanged. This holds true since saturation factors depend upon all of the T_1s and M_0s of all the metabolites in the relevant exchange network, as well as upon all of the associated reaction rates. M_0s in particular are often directly observed to undergo large changes in ^{31}P NMR experiments in which a metabolic stressor is applied.

In the present work, the magnitude of these errors was determined experimentally in an isolated perfused rat heart preparation undergoing mild ischemia and in the gastrocnemius muscle of a living rat undergoing mild electrical stimulation. The results confirm our central hypothesis, that quantitation errors can occur from use of saturation factor corrections in changing systems. Simulation results indicate that both T_1 changes and CE contribute to quantitation errors.

Recently, Newcomer and Boska [13] also investigated the effects of chemical exchange in in vivo measurements, evaluating T_1 changes with exercise in the human gastrocnemius muscle. T_1 was derived from two progressive saturation datasets. One of these was comprised of data corresponding to nine values of TR, while the other used data corresponding to only two relatively small TR values. This latter method was chosen to minimize contributions from chemical exchange [5,6]. They found that T_1(PCr) decreased by 20%, $T_1(P_i)$ increased by 58%, and $T_1(\gamma$-ATP) remained unchanged compared to control values under muscle activation. They conclude that chemical exchange is a significant factor in T_1 measurements using progressive saturation in exercising human gastrocnemius, as has also been shown in electrically stimulated rat gastrocnemius [6].

It is clear that the magnitude of metabolite quantitation errors, and the relative contributions to these errors of changes in T_1s, M_0s, and rate constants, will depend upon the specifics of the experimental protocol and chemical parameters, which in general, remain unknown. It is important to note that in the present experiments, no attempt was made to maximize the contribution of any particular source of error or the size of the overall error. In addition, the range of possible experiments and interventions is unlimited. Therefore, the quantitative results presented here are representative rather than comprehensive.

As has been previously emphasized [4,14], the significance of the quantitation errors resulting from changing parameters which we have described here depends upon the magnitude of other sources of error and the overall requirement for measurement accuracy in a given application. Further, identical considerations to the ones discussed in this work apply to the use of SFs obtained from one set of experimental subjects to correct for partial saturation in experiments performed on another set of subjects which will, in general, have different chemical parameters.

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