Journal of Magnetic Resonance **149,** 251–257 (2001)
doi:10.1006/jmre.2000.2251, available online at http://www.idealibrary.com on IDEAL®

COMMUNICATIONS

Pitfalls in the Measurement of Metabolite Concentrations Using the One-Pulse Experiment in *in Vivo* NMR: Commentary on "On Neglecting Chemical Exchange Effects When Correcting *in Vivo* ^{31}P MRS Data for Partial Saturation"

Richard G. S. Spencer, Kenneth W. Fishbein, and Craig J. Galban

National Institutes of Health, National Institute on Aging, GRC 4D-08, 5600 Nathan Shock Drive, Baltimore, Maryland 21224

Received August 18, 2000; revised October 26, 2000

In an article in a previous issue of the *Journal of Magnetic Resonance*, Ouwerkerk and Bottomley (*J. Magn. Reson.* 148, pp. 425–435, 2001) show that even in the presence of chemical exchange, the dependence of saturation factors on repetition time in the one-pulse experiment is approximately monoexponential. They conclude from this fact that the effect of chemical exchange on the use of saturation factors when correcting for partial saturation is negligible. We take issue with this conclusion and demonstrate that because saturation factors in the presence of chemical exchange are strongly dependent upon all of the chemical parameters of the system, that is, upon all T_1's and M_0's of resonances in the exchange network and upon the reaction rates themselves, it is problematic to apply saturation factor corrections in situations in which any of these parameters may change. The error criterion we establish reflects actual errors in quantitation, rather than departures from monoexponentiality.

INTRODUCTION

Factors influencing signal-to-noise ratio (SNR) per unit time have been discussed extensively in the NMR literature. In terms of experimental technique, it is almost axiomatic that, following the work of Ernst and Anderson in 1966 (*1*), SNR is improved by pulsing with a repetition time TR which is too short to permit full relaxation of spins between pulses in a one-pulse experiment. Spectra obtained in this way require correction for incomplete relaxation, called partial saturation in this context, to achieve quantitative accuracy. If the saturation factor (SF) is defined as the ratio of the observed resonance magnetization to the equilibrium magnetization, then the correction procedure is to divide observed resonance amplitudes by a previously measured SF. Following Ernst and Anderson, such a procedure will accurately account for partial saturation provided only that the T_1 of the resonance in question does not change; this is because in their analysis the degree of partial saturation depends only upon the two externally selected pulse parameters, the interpulse delay, TR and flip angle, θ, and the T_1 of the resonance.

Assuming the usual case of $\mathrm{TR} \gg T_2$ (although this condition is not essential (*2, 3*)), Ernst and Anderson showed

$$\mathrm{SF}(T_1; \theta, \mathrm{TR}) = \frac{\left(1 - e^{-\mathrm{TR}/T_1}\right)\sin\theta}{\left(1 - e^{-\mathrm{TR}/T_1}\right)\cos\theta}. \qquad [1]$$

In particular, the SF of a particular resonance is independent of its equilibrium magnetization (M_0) and of the T_1's and M_0's of all other resonances in the spectrum. Chemical exchange is not incorporated into the formalism so that the SF is also *a priori* independent of chemical exchange rates.

Since the work of Ernst and Anderson, study of systems with chemical exchange, in particular *in vivo* systems, has become commonplace. Accordingly, we have extended the work of Ernst and Anderson to include the effects of chemical exchange on SFs (*2–5*). Our result for the general case of N mutually exchanging sites is (*5*)

$$\vec{\mathbf{SF}} = \vec{\mathbf{M}}_0^{-1}(\vec{\vec{\mathbf{I}}} - e^{\vec{\vec{\mathbf{A}}}\mathrm{TR}}\cos\theta)^{-1}(\vec{\vec{\mathbf{I}}} - e^{\vec{\vec{\mathbf{A}}}\mathrm{TR}})\vec{\mathbf{M}}_0 \sin\theta, \qquad [2]$$

where $\vec{\mathbf{M}}_0 = (M_{0S_1}, M_{0S_2}, \ldots, M_{0S_N})$, $\vec{\vec{\mathbf{I}}}$ is the $N \times N$ identity matrix, $\vec{\mathbf{M}}_0 = \vec{\vec{\mathbf{I}}}\vec{\mathbf{M}}_0$, and

$$\vec{\vec{\mathbf{A}}} = \begin{pmatrix} -\left(\frac{1}{T_{1S_1}} + \sum_{j\neq 1} k_{S_1S_j}\right) & k_{S_2S_1} & \cdots & k_{S_NS_1} \\ k_{S_1S_2} & -\left(\frac{1}{T_{1S_2}} + \sum_{j\neq 2} k_{S_2S_j}\right) & \cdots & k_{S_NS_2} \\ \cdots & \cdots & \cdots & \cdots \\ k_{S_1S_N} & k_{S_2S_N} & \cdots & -\left(\frac{1}{T_{1S_N}} + \sum_{j\neq N} k_{S_NS_j}\right) \end{pmatrix}.$$

This result demonstrates that in exchanging systems, the degree

1090-7807/01

of saturation of a resonance depends upon the M_0's and T_1's of all of the metabolites in the exchange network and upon all exchange rates, in addition to the usual parameters of θ and TR. A limited form of this result for the special case of $\theta = 90^\circ$, obtained by integrating the Bloch–McConnell equations, appears, for example, in Binzoni and Cerretelli (*6*).

Based upon calculations, simulations, and experiments (*2–5*), we have demonstrated that modeling exchanging systems by use of Eq. [1], that is, ignoring the effect of chemical exchange, can lead to quantification errors in M_0 and T_1 measurements. The magnitude of these errors depends upon all the system parameters. The potential importance of this is underscored by Ouwerkerk and Bottomley (*7*), who state, "Clearly, these concerns. . . could undermine metabolite quantification in virtually all human and animal ^{31}P MRS studies performed under partially saturated conditions."

Thus, Ouwerkerk and Bottomley and we agree upon the potential importance of chemical exchange effects. In addition, Ouwerkerk and Bottomley have not questioned the mathematical analysis leading to Eq. [2]. However, we come to opposite conclusions as to whether the effects on saturation factors due to chemical exchange are of a significant or even measurable magnitude in metabolite quantitation.

As has been previously stated, our concerns relate to the use of SFs for quantitation in experiments with potential variation in M_0's, T_1's, and reaction rates (*4, 5*). Such variation can come about through an intervention, as in a dynamic NMR experiment, or when samples are changed, as in the case in which SFs obtained from one set of samples are applied to another set of samples (*8*). It is in these cases that the M_0's, T_1's, and exchange rates of the system from which SFs are measured will in general differ from the M_0's, T_1's, and exchange rates of the system to which they are applied in an effort to correct for partial saturation, leading to incorrect quantitation.

METABOLITE QUANTITATION IN THE ONE-PULSE EXPERIMENT

We now describe a typical, empirical way in which SFs are applied to correct for partial saturation in the one-pulse experiment and define the error associated with that procedure. For concreteness we describe a dynamic NMR experiment.

Consider an experiment with a control period (Ctl) followed by an intervention period (Int). A common example is a perfused heart preparation in which, after stabilization and measurement of metabolite concentrations, the heart is rendered ischemic. The usual correction for the saturation of a given resonance resulting from pulsing with a short delay time TR^S with a fixed θ is as follows. $M_{obs}(\theta, TR)$ will denote the observed magnetization using a fixed θ and repetition time TR.

During Ctl two spectra are acquired, one with a short repetition time, TR^S, and $\theta < 90^\circ$ selected for high SNR per unit time during the subsequent Int period, and one with a long repetition time, TR^L, permitting complete relaxation; for practical purposes, essentially complete relaxation is generally assumed to have occurred for $TR^L \gtrsim 5 \times T_1$. For optimal SNR, the experiment with TR^L is assumed to be performed with $\theta = 90^\circ$; this is not essential to the argument. It is important to point out that (*5*)

$$M_{obs}^{Ctl}(TR^L) = M_0^{Ctl} \quad [3]$$

and

$$M_{obs}^{Int}(TR^L) = M_0^{Int}, \quad [4]$$

whether or not chemical exchange is present. The spectrum with TR^L is required in order to calculate the saturation factor:

$$SF^{Ctl} = M_{obs}^{Ctl}(\theta, TR^S)/M_{obs}^{Ctl}(TR^L). \quad [5]$$

During Int, one wishes to obtain a value for M_0^{Int} from spectra acquired with θ and TR^S. For quantitation, the non-measured fully relaxed resonance amplitude during Int is taken to be

$$M_0^{Int,Apparent} = M_{obs}^{Int}(\theta, TR^S)/SF^{Ctl}. \quad [6]$$

This is entirely valid provided that $SF^{Ctl} = SF^{Int}$; in the Ernst and Anderson formalism (*1*), this requires only that the T_1 of the resonance under consideration remains unchanged between Ctl and Int. In the formalism incorporating chemical exchange (*5*), as appropriate for *in vivo* systems, this equality generally requires that the T_1's and M_0's of all resonances in the exchange network with the resonance under consideration, as well as all of the rate constants, be equal in the Ctl and Int periods. This is manifestly not assured in intervention experiments.

Errors in quantitation during Int due to neglect of chemical exchange are given by the proportional difference between the apparent corrected magnetization and the true magnetization:

$$\text{Error}\left(M_0^{Int}\right) = \left(M_0^{Int,Apparent} - M_0^{Int}\right)/M_0^{Int}. \quad [7]$$

EXAMPLE OF QUANTITATION ERRORS DUE TO NEGLECT OF CHEMICAL EXCHANGE

We now consider a specific example with values taken from the perfused heart literature. Data from several papers are required in order to obtain realistic specific values for the simulation. We take $T_1(PCr) = 2.78$ s, $T_1(\gamma\text{-ATP}) = 0.64$ s, $T_1(Pi) = 2.4$ s; these values were obtained from well-oxygenated hearts at a moderate workload (*9*) and are used in the following for both preischemic and postischemic values. Note that this assumption of constancy of T_1's generally leads to smaller quantitation errors than if the T_1's vary. We take preischemic [PCr] = 6.9 μmol/g ww, [γ-ATP] = 4.3 μmol/g ww, [Pi] = 1.6 μmol/g ww; postischemic [PCr] = 0.345 μmol/g ww, [γ-ATP] = 0.215 μmol/g ww, [Pi] = 19.2 μmol/g ww (*10*); preischemic $k_{PCr\rightarrow\gamma\text{-ATP}} = 0.7$ s^{-1}, postischemic $k_{PCr\rightarrow\gamma\text{-ATP}} = 0.2$ s^{-1} (*11*), preischemic $k_{Pi\rightarrow\gamma\text{-ATP}} = 0.37$ s^{-1} (*11*); and

TABLE 1

Magnitude of the Errors in Metabolite Quantitation during Ischemia Due to Neglect of Chemical Exchange for the System Described in the Example

TR	θ (°)	Errors in PCr and in PCr/β-ATP (%)	Errors in Pi and in Pi/β-ATP (%)	Error in PCr/Pi (%)
2 s	60	15	9	6
1 s	60	20	12	9
1 s	90	25	14	12

Note. β-ATP is not involved in chemical exchange in the usual formulation of high-energy phosphate metabolism, which we have followed here, so that the errors in PCr and Pi are identical to those in the ratios PCr/β-ATP and Pi/β-ATP, respectively.

postischemic $k_{\mathrm{Pi}\rightarrow\gamma\text{-ATP}} = 0.1\ \mathrm{s}^{-1}$. Lacking a literature value, this last value was obtained by multiplying the known preischemic value by the ratio of the post- and preischemic values for $k_{\mathrm{PCr}\rightarrow\gamma\text{-ATP}}$. Quantitation errors due to chemical exchange for magnetizations during Int, as defined by Eq. [7], are shown in Table 1 for three choices of TR and θ. There is no error due to chemical exchange for β-ATP, as it does not participate in a significant amount of chemical exchange under normal circumstances. The largest error, obtained when TR = 1 s and $\theta = 90°$ (*12*), is found to be fully 25% for the ratio PCr/β-ATP. Of course, in many applications, errors of the magnitude shown in Table 1 may be acceptable, while in others they will not be.

The above example is typical of only one type of *in vivo* NMR experiment, albeit a rather common one. Many other experimental paradigms are also of interest, such as comparisons between healthy subjects and those with a particular pathology (*8*). A SF obtained from one set of subjects may be applied to another set in an effort to correct for partial saturation. However, this approach is in general valid only if the M_0's, T_1's, and exchange rates are the same for the two groups of subjects. Clearly, this is not guaranteed.

An alternative approach to correcting for partial saturation is in principle possible (*7*). This involves performing an explicit measurement of each resonance's apparent T_1, T_1^{obs}, and then using these values to correct for partial saturation by application of the formula given by Ernst and Anderson, using T_1^{obs},

$$\mathrm{SF}\left(T_1^{\mathrm{obs}}; \theta, \mathrm{TR}\right) = \frac{\left(1 - e^{-\mathrm{TR}/T_1^{\mathrm{obs}}}\right)\sin\theta}{\left(1 - e^{-\mathrm{TR}/T_1^{\mathrm{obs}}}\cos\theta\right)}. \qquad [8]$$

If these T_1^{obs} values are obtained from a partial saturation based method such as progressive saturation or the dual angle method (*13*), they will in general be significantly different from the true T_1 values of the metabolites (*2, 3, 5*). By true T_1 values, we mean the T_1's which appear in, e.g., the Bloch–McConnell equations (*14*) and which Ouwerkerk and Bottomley refer to as the "so-called 'intrinsic' T_1's." These T_1^{obs} will also have a strong dependence upon TR and flip angle (*2, 3, 5*), which are obviously not intrinsic chemical properties of the system. This fact holds regardless of whether two or three (*2, 3*) parameters are used in the nonlinear fit to the data to obtain T_1^{obs}, in the case of progressive saturation, and regardless of the choice of TR, α, and β in the case of the dual-angle method. However, note that the T_1's which appear in the Bloch–McConnell equations can in fact be obtained even in the presence of chemical exchange by means of saturation transfer, inversion recovery, and certain other experiments.

The fact that $T_1^{\mathrm{obs}} \neq T_1$ is of obvious significance for T_1 measurements, but one can ask the mathematical question of whether use of T_1^{obs} in place of T_1 in the Ernst formula [1] leads to nearly correct saturation factors. The answer, as shown by Ouwerkerk and Bottomley in (*7*), is yes; for any fixed values of θ and TR, and fixed M_0's, T_1's, and rate constants, and hence fixed T_1^{obs}, the dependence of SF on TR is, qualitatively, nearly monoexponential.

This mathematical fact, that use of Eq. [8] with a T_1^{obs} yields virtually identical SFs to Eq. [2], is of limited practical value for two reasons. First, in a typical NMR experiment involving an intervention, as in the example above, the M_0's, T_1's, and reaction rates change, or at least have the potential to change—otherwise there's no point in doing the experiment. As a result, in general $T_1^{\mathrm{obs,Int}} \neq T_1^{\mathrm{obs,Ctl}}$. Therefore, use of a SF derived during a control period to correct for partial saturation during an intervention period is incorrect and can lead to precisely the errors discussed above unless it is known *a priori* that T_1^{obs} is unchanged by this intervention. Similar considerations hold for a SF derived from one set of subjects and applied to another, unless the subjects are known to be identical (*15*) or to have the same T_1^{obs}. Second, it is likely to be impractical and time consuming to perform an accurate T_1^{obs} measurement during an intervention, when SNR per unit experimental time is likely to be of paramount importance. It is for this reason that experiments are performed using empirical SFs, rather than SFs derived from specific measurement of T_1^{obs} in virtually all cases. This point will be further addressed below.

DISCUSSION

We believe that Eq. [2], rather than Eq. [1], forms the correct basis for further analysis of the one-pulse and related experiments in *in vivo* NMR and in other chemical systems demonstrating chemical exchange. Equation [2] follows directly from the Bloch–McConnell equations (*14*), which have been extensively applied to the analysis of specialized pulse sequences such as saturation transfer and inversion recovery. The reason for the lack of incorporation of chemical exchange in the analysis of arguably the most commonly performed *in vivo* NMR experiment, the one-pulse experiment, prior to our first discussion of this topic in 1989 (*4*), is unclear to us.

The work of Ouwerkerk and Bottomley explicitly demonstrates the near-monoexponentiality of the function SF(TR) for systems with exchange. Thus, knowing {SF(TR_1), SF(TR_2), . . . , SF(TR_n)} for some set of TR values, one could predict the value of SF at a different value of TR, SF(TR_{n+1}).

Similar comments apply to the ability to predict $SF(\theta_{n+1})$ from measurements of $\{SF(\theta_1), SF(\theta_2), \ldots, SF(\theta_n)\}$ (*7*). However, such a process of deriving a $SF(TR_{n+1})$ or a $SF(\theta_{n+1})$ does not address the use of saturation factors in correcting for partial saturation when any of the chemical parameters change as in the above example or in other *in vivo* NMR experiments.

Although a SF can often be approximated by a monoexponential form

$$SF(TR) = f_1(T_{1i}, M_{0i}, k_{ij}) \exp(-f_2(T_{1i}, M_{0i}, k_{ij}) \cdot TR),$$

It will nevertheless vary with the system parameters. What is significant is the fact that *each SF is dependent upon all of the system's M_0's, T_1's, and k's, and these parameters may change appreciably during the course of a dynamic experiment or between subject groups*. As a result, monoexponential dependence of SF on TR in no way implies that chemical exchange effects can be neglected in metabolite quantitation. In a similar fashion, even if the dependence of SFs on θ in the presence of chemical exchange is well-described by Eq. [1], these SFs are still dependent on all system parameters.

The dependence of SFs on chemical parameters is given by Eq. [2]. However, it is clearly impractical to measure all of these parameters and, from that data, use Eq. [2] to calculate SFs. Rather, an empirical approach must be taken for saturation correction in a way that does not neglect chemical exchange.

Our published concerns regarding metabolite quantitation (*4, 5*) have centered upon experiments in which the SF measured during one period, typically a control period, is used to correct for partial saturation subsequent to an intervention. We state (*5*) that "the [usual] correction scheme . . . may lead to large errors unless all of the system's M_0's and k's, [and] T_1's, are unchanged. That is, the naïve correction scheme is valid only when nothing happens to the sample." Alternatively, the correction scheme is accurate if T_1^{obs} is somehow known to be unchanged. An analogous situation obtains when a SF measured on one set of samples or subjects is applied to another set.

The error criteria established by Ouwerkerk and Bottomley assess the quality of the fit of a monoexponential function to SF(TR) data during an experiment in which no system parameters change. The quality of this fit is unrelated to the quantitation errors which can result from the use of SFs to correct for partial saturation when chemical parameters change, whether through a dynamic process or from changing samples. As shown in the example above, these quantitation errors can be quite substantial, on the order of 20% or more. The demonstration that deviations from monoexponential dependence of SF on TR decrease with increasing TR and smaller θ (*7*) does not contradict our previous assertion that deviations of T_1^{obs} from the true T_1 are minimized by employing shorter TR and larger θ (*5*). Similarly, the fact that SFs are well-approximated by a monoexponential over a wide range of fixed rate constants, as shown in Fig. 4 of Ref. (*7*), does not imply that SFs are weakly dependent on rates.

Ouwerkerk and Bottomley cite the work of Binzoni and Cerretelli on muscle metabolism (*6*) as indicating that in a typical system with chemical exchange, the dependence of SF on reaction rates is small. Using Monte Carlo methods, Binzoni and Cerretelli demonstrated that the standard deviation of the function $SF(k_{PCr\rightarrow\gamma\text{-ATP}}, k_{Pi\rightarrow\gamma\text{-ATP}})$ for each metabolite over a random set of pairs $(k_{PCr\rightarrow\gamma\text{-ATP}}, k_{Pi\rightarrow\gamma\text{-ATP}})$ is small compared with its mean value. However, $k_{PCr\rightarrow\gamma\text{-ATP}}$ and $k_{Pi\rightarrow\gamma\text{-ATP}}$ are not random variables, and analysis of $SF(k_{PCr\rightarrow\gamma\text{-ATP}}, k_{Pi\rightarrow\gamma\text{-ATP}})$ as a random function is not pertinent to the analysis of any specific system, for which $k_{PCr\rightarrow\gamma\text{-ATP}}$ and $k_{Pi\rightarrow\gamma\text{-ATP}}$, as well as the other independent variables in Eq. [2], take specific values.

Figure 1 shows a plot of $SF_{PCr}(k_{PCr\rightarrow\gamma\text{-ATP}})$ and $SF_{Pi}(k_{PCr\rightarrow\gamma\text{-ATP}})$ with the other parameter values as in the control period of the example in the text, including $k_{Pi\rightarrow\gamma\text{-ATP}} = 0.37\ s^{-1}$. In Fig. 1, TR = 2 s and $\theta = 60°$; similar results are obtained for other pulse parameters. The difference between the value $SF_{PCr} = 0.59$ when $k_{PCr\rightarrow\gamma\text{-ATP}} = 0$, and the asymptotic value $SF_{PCr} = 0.76$ for large $k_{PCr\rightarrow\gamma\text{-ATP}}$, is fully 29%. Thus, the dependence of SF_{PCr} on $k_{PCr\rightarrow\gamma\text{-ATP}}$ is in fact substantial. Similar comments pertain to SF_{Pi}.

Following Binzoni and Cerretelli, we can calculate the mean and standard deviation of all of the values of SF_{PCr} used to generate Fig. 1. These are 0.74 ± 0.036. This mean value is fully 25% different from the $k_{PCr\rightarrow\gamma\text{-ATP}} = 0$ value. The fact that the standard deviation is small merely reflects the fact that the values of SF_{PCr} cluster near the asymptotic value over most of the domain of $k_{PCr\rightarrow\gamma\text{-ATP}}$ as in the Monte Carlo simulation of Binzoni and Cerretelli (*6*). Thus, characterizing the function $SF(k)$ by its mean and standard deviation with respect to variation in k gives the misleading impression that $SF(k)$ is weakly dependent on k.

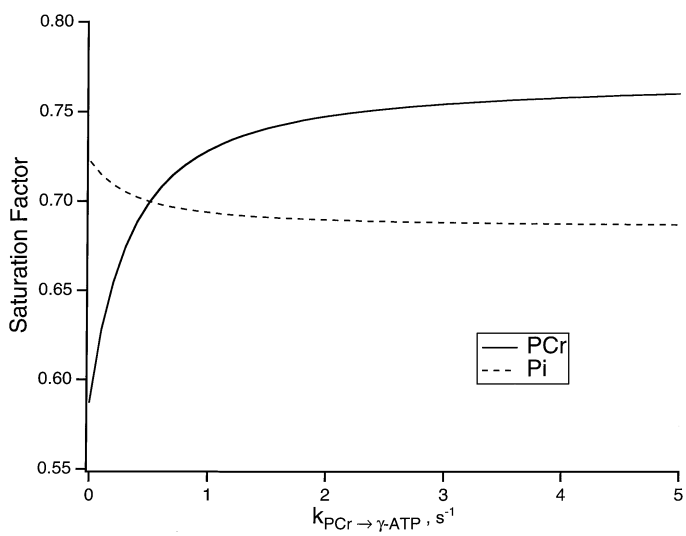

FIG. 1. Plot of SF(PCr) (solid line) and SF(Pi) (dashed line) as a function of $k_{PCr\rightarrow\gamma\text{-ATP}}$ for a one-pulse experiment with $\theta = 60°$ and TR = 2 s. The values of $k_{Pi\rightarrow\gamma\text{-ATP}}$ and the other system parameters are as given in the example in the text for the well-oxygenated state.

Ouwerkerk and Bottomley present a simulation in which M_0's do change, as could come about by an intervention. They find, as do we, that T_1^{obs} can change substantially in such cases, demonstrating the error in applying corrections based on $T_1^{obs,Ctl}$ to measurements made during an intervention period. Their results suggest that the use of two dual-angle-method-based measurements, one to obtain an initial T_1^{obs} and one to obtain a final T_1^{obs} after an intervention, may yield the information required to correct for partial saturation in the presence of chemical exchange during this intervention. This may be called the double-dual-angle method. A proposal to perform future studies in this way, which would represent a distinct departure from the manner in which studies using the one-pulse and related experiments have been and currently are performed, merits further study. The potential advantage of the double-dual-angle method is that it may lead to overall greater SNR per unit time than long TR experiments; this may permit higher temporal resolution during an intervention study. However, if metabolite concentrations do not vary linearly with time, the linear interpolation procedure suggested by Ouwerkerk and Bottomley is likely to be problematic; the correct interpolating function will be unknown in the usual case. Further, there are a number of situations in which the second part of the double-dual-angle measurement protocol may be impossible, including instability of the experimental preparation or when making measurements on a subject exercising until exhaustion. Moreover, it is not clear whether the double-dual-angle method would give accurate metabolite quantitation if T_1's or reaction rates change. We note also that a dual angle measurement of T_1^{obs} may be required for each intervention period, as in a control, ischemia, reperfusion, or graded exercise protocol, or even at each time point.

An alternative to this might be to make use of the fact pointed out by Ouwerkerk and Bottomley that SF(TR) is generally closely fit by a monoexponential. This demonstrates that one could extrapolate from two or more short TR measurements of observed magnetization made at a given time point during an intervention to obtain a value for the fully relaxed magnetization at that time point. While this progressive saturation method avoids the use of a long TR, low SNR acquisition, it would require measurements at more than one TR. In addition, each of these will have a finite SNR which will propagate into errors in the derived M_0 value. The selection of appropriate TRs is also not obvious; they must be sufficiently different from each other that the data set has adequate dynamic range for a meaningful fit, but none of the TR values can be so large that any SNR advantage or increase in temporal resolution achieved by avoiding a single long TR experiment is nullified. Again, whether this progressive saturation method would be an efficient quantitation method in terms of accuracy and SNR would require explicit study.

A third approach would be to abandon the short TR experiment entirely and acquire data throughout the experiment with a sufficiently long TR that saturation corrections are negligible, as proposed in our previous work (*5*), and also noted by Ouwerkerk and Bottomley (*7*) to eliminate errors due to chemical exchange. This long TR method is certainly the simplest and most reliable approach, requiring minimal knowledge of the full set of chemical parameters and requiring only one measurement. Furthermore, as previously pointed out (Ouwerkerk, private communication), one salutary effect of chemical exchange is to increase the effective rate of recovery of longitudinal magnetization for resonances with long T_1's which are in chemical exchange with species having shorter T_1's, so that the TRs required for accurate measurements may be somewhat shorter, and incur a smaller SNR penalty, than otherwise expected. For the example presented above, Table 2 shows the TRs required to achieve specified degrees of accuracy. Comparison of the SNR consequences of such TR selection with the SNR consequences of the double-dual-angle method, and the progressive saturation method applied at each time point, remains to be fully explored.

TABLE 2

Minimum Repetition Times Resulting in the Specified Upper Bound on Quantitation Errors in PCr/Pi, PCr/β-ATP (or PCr), and Pi/β-ATP (or Pi) Resulting from Chemical Exchange Using the One-Pulse Experiment for the System Described in the Example

Flip angle	TR_{min} (s)	Error bound (%)
60°	5.2	5
60°	3.2	10
90°	7.0	5
90°	5.0	10

It is clear that implementation of a version of any of the above procedures for accurate quantitation will not serve to correct erroneous metabolite quantification in previous human and animal ^{31}P NMR studies performed under partially saturated conditions.

We do not claim that errors in the use of saturation factors are always or even usually the dominant source of error in ^{31}P NMR studies performed under partially saturated conditions, but rather that they can indeed in realistic circumstances be comparable to or larger than other sources of error. In each case, an attempt can be made to check this using plausible parameter values.

As pointed out previously (*5*, *7*), there are clear SNR disadvantages to making measurements in a way which decreases the errors due to the neglect of chemical exchange. Thus, for qualitative results with high temporal resolution, accepting the errors due to chemical exchange may be the most reasonable approach. This is especially true when overall SNR is poor. On the other hand, these errors may not be acceptable for detailed measurements of, for example, intracellular energy charge, aerobic threshold, and phosphorylation potential, particularly in cases in which the SNR is relatively high.

Ouwerkerk and Bottomley conclude their paper by writing that "Reasonable accuracy is even possible in dynamic experiments in which the equilibrium magnetizations of the exchanging species vary, if measurements are interlaced with dual angle T_1 measurements." This is entirely consistent with the main conclusion of our work: accurate metabolite quantitation in *in vivo* NMR requires a significant departure from current practice.

It is unclear whether the double-dual-angle method (*7*), a dual-angle (*13*), progressive saturation, or long TR (*5*) experiment at each time point during an intervention or for each set of samples, or another as-yet-to-be-determined method will optimally address the problems of quantitation in human and animal ^{31}P MRS studies performed under partially saturated conditions. Nevertheless, it is clear that a modification of the current implementation of the one-pulse and related experiments is required in order that systematic errors related to chemical exchange be avoided. It is probable that this modification should be use of a long TR. The situation is not resolved by the goodness of the fit of SF(TR) to monoexponential functions; the problem resides in the dependence of SF on all of the system parameters, not in the functional form of SF(TR). This applies whether SFs are empirically measured, as is the usual practice, or are calculated from separate measurements of T_1^{obs}.

We stand by the conclusions of our previous work, which may be summarized by stating that implicit or explicit use of Eq. [1], rather than Eq. [2], in the analysis of one-pulse and related *in vivo* NMR spectroscopy experiments performed under conditions of partial saturation for exchanging systems can lead to significant errors in quantitation and that these errors cannot *a priori* be regarded as negligible. Further, as random errors in NMR spectroscopy continue to be reduced by improvements in experimental techniques and hardware, the importance of the systematic error introduced by ignoring exchange in the analysis of the one-pulse experiment will be correspondingly greater.

The note added in proof below addresses issues raised by Ouwerkerk and Bottomley in Ref. (*16*).

Note added in proof (*Received February 7, 2001*). We have reported (*4, 5, 17*, present paper) that substantial variations in SFs can occur when chemical parameters (M_0's and k's) change over plausible ranges. In contrast, Ouwerkerk and Bottomley conclude (*16*) that SFs depend only modestly on these parameters. These authors approximate the nonlinear function, SF, by initial terms in its Taylor expansion and consider relatively small deviations (25%) in M_0's and k's. However, our analysis ((*4*, Figs. 1, 2); Fig. 1 above) shows that local derivatives do not describe the change in SFs when parameter values change significantly. Further, availability of a simple closed form expression for SF (M_{0i}, T_{1i}, k_{sisj}) (*5*) obviates the need to analyze the functional dependencies of SFs through linear approximations.

We have not recommended the operating conditions of small TR, large θ for saturation factor correction. We noted (*2, 3, 5*) that these conditions minimize errors in T_1 measurements, but are inferior to long TR, small θ for magnetization corrections ((5, Figs. 8, 9)).

We stress again, with Ouwerkerk and Bottomley now apparently in agreement (*16*), that none of the salient arguments regarding the use of SFs to correct for partial saturation are based upon the monoexponentiality of SF(TR) (*7*).

We reiterate that our analysis does not derive from that of Binzoni and Cerretelli (*6*), valid only for $\theta = 90^\circ$. Our earliest contribution on this topic (*4*) predates their work (*6*) and includes θ variation. Our extension (*5*) to arbitrary exchange networks (Eq. [2] above) has formed the basis for the analyses of (θ, TR) selection by Ouwerkerk and Bottomley and by us. The formalism required for this was not presented in Binzoni and Cerretelli (*6*).

Figure 1 above demonstrates the flaw in the use of the standard deviation to assess variation of SF(k) (*6, 7*). We are not suggesting that k will change from a finite value to zero within a given pair of measurements.

It appears to us that all parties may now agree with our central point (*4, 5, 17*, present paper), that quantitation problems can occur when SFs are used to correct for partial saturation in situations in which chemical parameters vary substantially.

Ouwerkerk and Bottomley's analysis (*16*, Table 2) constructively furthers the goal of determining appropriate pulse parameters for one-pulse and related experiments in exchanging systems. That analysis supports our conclusion, stated explicitly in Ref. *5* (pp. 133–134), that long TR experiments, permitting full relaxation between pulses and hence eliminating the need for measuring SFs, circumvent the problems of exchange with significantly less of a SNR penalty than for equally accurate rapid pulsing (very short TR) experiments. See also Tables 1 and 2 above, demonstrating decreased quantitation errors as TR increases. Of course, in actual experimental situations pulse parameter selection will be complicated due to limited a priori knowledge of system parameters and the tradeoff between quantitation accuracy, temporal resolution, and SNR in TR selection.

We consider long TR experiments to be a "significant departure from current practice" because such experiments are infrequently done, due to loss of SNR as compared with short TR experiments (*1*).

REFERENCES

1. R. R. Ernst and W. A. Anderson, Application of Fourier transform spectroscopy to magnetic resonance. *Rev. Sci. Instrum.* **37,** 93–102 (1966).
2. A. Horská, J. Horsky, and R. G. S. Spencer, Measurement of spin–lattice relaxation times in systems undergoing chemical exchange. *J. Magn. Reson. A* **110,** 82–89 (1994).
3. A. Horská and R. G. S. Spencer, Measurement of spin–lattice relaxation times and kinetic rate constants in rat muscle using progressive partial saturation and steady-state saturation transfer. *Magn. Reson. Med.* **36,** 232–240 (1996).
4. R. G. S. Spencer, J. A. Ferretti, and G. H. Weiss, NMR saturation factors in the presence of chemical exchange. *J. Magn. Reson.* **84,** 223–235 (1989).
5. R. G. S. Spencer and K. W. Fishbein, Measurement of spin–lattice relaxation times and concentrations in systems with chemical exchange using the one-pulse sequence: Breakdown of the Ernst model for partial saturation in nuclear magnetic resonance spectroscopy. *J. Magn. Reson.* **142**(1), 120–135 (2000).
6. T. Binzoni and P. Cerretelli, Muscle ^{31}P-NMR in humans: Estimate of bias and qualitative assessment of ATPase activity. *J. Appl. Physiol.* **71**(5), 1700–1704 (1991).
7. R. Ouwerkerk and P. A. Bottomley, On neglecting chemical exchange effects when correcting *in vivo* ^{31}P MRS data for partial saturation. *J. Magn. Reson.* **148,** 425–435 (2001).
8. P. A. Bottomley, MR spectroscopy of the human heart: The status and the challenges. *Radiology* **191**(3), 593–612 (1994).
9. R. G. Spencer, J. A. Balschi, J. S. Leigh, Jr., and J. S. Ingwall, ATP synthesis and degradation rates in the perfused rat heart. ^{31} P-Nuclear magnetic resonance double saturation transfer measurements [published erratum appears in *Biophys. J.* **54**(6), following 1186 (1988)], *Biophys. J.* **54**(5), 921–929 (1988).
10. R. Kalil-Filho, G. Gerstenblith, R. G. Hansford, V. P. Chacko, K. Vandegaer, and R. G. Weiss, Regulation of myocardial glycogenolysis during post-ischemic reperfusion. *J. Mol. Cell. Cardiol.* **23**(12), 1467–1479 (1991).
11. S. Neubauer, B. L. Hamman, S. B. Perry, J. A. Bittl, and J. S. Ingwall, Velocity of the creatine kinase reaction decreases in postischemic myocardium: A ^{31}P-NMR magnetization transfer study of the isolated ferret heart. *Circ. Res.* **63**(1), 1–15 (1988).
12. J. H. Cieslar and G. P. Dobson, Free [ADP] and aerobic muscle work follow at least second order kinetics in rat gastrocnemius in vivo. *J. Biol. Chem.* **275**(9), 6129–6134 (2000).

13. P. A. Bottomley and R. Ouwerkerk, The dual-angle method for fast, sensitive T_1 measurements *in vivo* with low-angle adiabatic pulses. *J. Magn. Reson. B* **104,** 159–167 (1994).
14. H. M. McConnell, Reaction rates by nuclear magnetic resonance. *J. Chem. Phys.* **28**(3), 430–431 (1958).
15. K. H. Campbell, J. McWhir, W. A. Ritchie, and I. Wilmut, Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380**(6569), 64–66 (1996).
16. R. Ouwerkerk and P. A. Bottomley, On neglecting chemical exchange effects when correcting *in vivo* ^{31}P MRS data for partial saturation: Commentary on "Pitfalls in the measurement of metabolite concentrations using the one-pulse experiment in *in vivo* NMR." *J. Magn. Reson.* **148,** 425–435 (2001).
17. C. Galban and R. G. S. Spencer, Analysis of quantitation errors due to neglect of chemical exchange and T_1 variations when correcting for partial saturation in magnetic resonance spectroscopy: Theoretical and experimental. In *Book of Abstracts,* 9*th Annual Congress of the International Society for Magnetic Resonance in Medicine, Glasgow, Scotland, April, 2001,* to appear.