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Double Quantum Filtered ^{1}H NMR Spectroscopy Enables Quantification of Lactate in Muscle

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In this study we address the question of quantification of muscle lactate using double quantum filtered (DQF) ^{1}H NMR spectroscopy where dipolar and scalar coupled spectra are acquired. For this, lactate content in muscle samples was independently determined using a conventional enzymatic assay and DQF, ^{1}H NMR spectroscopy. NMR quantification of lactate relied on comparison of muscle spectra with similarly acquired spectra of standard lactate solutions. Transverse relaxation, T_2, and dipolar coupling effects were investigated at two different orientations of muscle fibers relative to B_0 and at various lactate concentrations. In all cases, we found a bi-exponential T_2 decay of the lactate methyl signal with a long T_2 of 142 ms ($\pm$8 ms, $n = 24$) and a short T_2 of 37 ms ($\pm$6 ms, $n = 24$). Lactate content of muscle determined by NMR spectroscopy agreed with the results obtained from enzymatic assays of the same samples provided that T_2 effects as well as the presence of both scalar and dipolar coupling interactions of lactate in muscle were taken into account.

Key Words: **double quantum; NMR spectroscopy; lactate; dipolar coupling; scalar coupling; transverse relaxation.**

INTRODUCTION

^{1}H NMR spectra of low molecular weight metabolites in skeletal muscle (e.g., creatine and carnosine) show anisotropic features due to residual dipolar coupling interactions (*1–3*). These interactions originate from an incomplete motional averaging of molecules that is not observed in aqueous solution where molecules rotate freely and isotropically. Recently, we showed that double quantum filtered (DQF), ^{1}H NMR spectra of lactate in postmortem muscle have a novel frequency splitting in addition to the well-known 7-Hz splitting caused by scalar coupling (*1*). This additional frequency splitting was independent of the static magnetic field, B_0, but varied with the orientation of muscle fibers relative to B_0 in accordance with the theory of dipolar coupling interactions (*1*).

The purpose of this study was to show that in order to edit and quantify lactate in muscle, one needs to account for the presence of both dipolar and scalar coupling interactions of lactate molecules in the tissue. Quantification of lactate during and following exercise is an important measure for muscle physiology since lactate is a key metabolite in active muscle metabolism (*4, 5*). Furthermore, an adequate quantification method for muscle would likely be useful for other ordered anisotropic tissues and organs such as the brain and heart.

In addition to dipolar coupling effects, one also has to take into account the T_2 decay of the signal during the preparation and refocusing times of spectra acquisition. In the particular case of postmortem muscle, there are two pools of lactate, one with anisotropic dipolar coupling interactions and the other without any detectable dipolar coupling effects (*1*). It is likely that the transverse relaxation rates of the two pools differ. The hypothesis tested here was that the raw, observed NMR signal intensities of DQF spectra from muscle lactate, when correctly adjusted for T_2 relaxation and dipolar coupling effects, agree with the signal intensity expected if the lactate were in a homogeneous aqueous solution. The need for this work was motivated by our desire for a rigorously defined spectroscopic method for observing and quantifying lactate during physiological manipulations of living muscle and by several studies which have concluded there is an NMR-invisible pool of lactate in muscle (see (*6*)).

METHODS

Experimental Design

The protocol consisted of four parts (i) DQF, ^{1}H NMR spectra were acquired on three different batches of muscle samples with and without added exogenous lactate. The methyl peak areas from the muscle spectra were converted to raw, observed lactate concentration in these samples using a calibration curve obtained from DQF, ^{1}H NMR spectra of solutions with known lactate concentrations. (ii) After spectra acquisition, all solution and muscle samples were assayed for true lactate content by enzymatic assay of perchloric acid extracts. (iii) T_2 measurements were made on muscle samples with endogenous lactate content and also on samples supplemented with exogenous lactate. (iv) Observed lactate content (expressed in μmol/g. tissue) as determined by DQF, ^{1}H NMR, before and after correcting for T_2 and

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dipolar coupling effects, was compared with the values obtained by the subsequent enzymatic assay on the same samples.

Sample Preparation

$L(+)$ lactate standard solutions (pH, 7) were prepared from sodium lactate salt obtained from Sigma (catalog L7022) diluted from a 5.05-M standard solution. Solutions were put in a glass tube with the same dimensions as muscle samples (18-mm diameter by 18-mm height). Bovine abdominal muscle, commonly referred to as flank steak, was obtained from a local meat market; its properties have been well defined (*1*). The fibers from this muscle are uniformly oriented and free of visible fat. Cylindrical samples were cut from the slab of meat in a region of uniform thickness to generate samples of reproducible size and shape. Five cylindrical samples were prepared from each slab. The protocol carried independent batches from three different slabs through the experimental design. One muscle sample in each batch was not treated before analysis; exogenous lactate was added to the other four samples in each batch. Those samples were injected with 15, 30, 45, or 60 μL of 5.05-M $L(+)$ lactate solution, respectively. The injection was done at different sites to enhance diffusion within the sample and to avoid surface liquid accumulation. Lactate was left to diffuse throughout the sample for 2 h.

Enzymatic Lactate Assay

After spectra acquisitions, muscle samples were weighed, frozen in liquid nitrogen, wrapped with aluminum foil, and stored in a freezer (-70°C) for subsequent perchloric acid extraction and enzymatic assay. The enzymatic assay was done as described in (*1*). The calibration solutions for this assay were prepared from the same 5.05-M lactate standard used throughout this study.

NMR Spectroscopy

Spectra were acquired on a Bruker (GE Omega) 4.7-T (200.1 MHz) CSI spectrometer, with 10 G/cm maximum gradient strength. The coil was a single-turn solenoid (a copper band with 25-mm diameter and 12-mm height) mounted symmetrically around a glass cylinder of 18-mm height.

One-dimensional, DQ filtered spectra were acquired using the pulse sequence shown in Fig. 1. To remove any frequency dependent phase modulations due to chemical shift precession during DQ evolution time, τ', the pulse sequence was modified from the one originally used in (*1*) by adding a 180° pulse in the middle of τ'. After shimming, a DQF sequence with a pulse width of 16 μs at 1 kW for 160 acquisitions of 1024 points was used to selectively excite the coupled AX_3 spin system of lactate methyl–methine protons. All spectra were acquired with a recycle delay of 6 s and τ' of 6 ms. DQF spectra of lactate standard solutions were acquired using the same pulse parameters as for muscle samples (gain, r.f. power, sweep width, 90° pulse, and other instrument settings were identical).

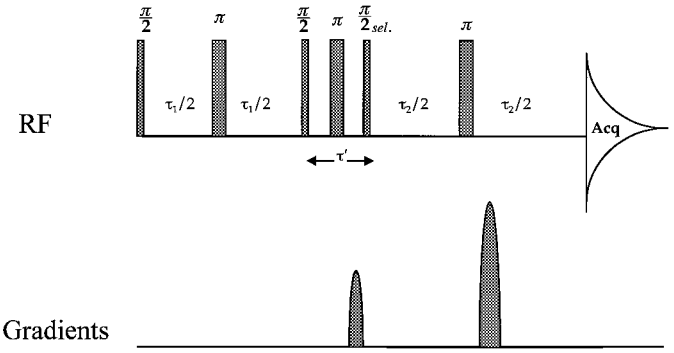

FIG. 1. Pulse sequence (DQF) used to select double quantum coherence between methyl and methine protons in lactate. The pulse phases were cycled according to well-defined schemes (*1*) to reduce unwanted coherences. To select for double quantum coherence between methyl and methine protons in lactate, the strength of the second gradient pulse was double that of the first gradient. In all cases τ' and the recycle time were set at 6 ms and 6 s, respectively.

For all samples, the 90° pulse for water was checked before each acquisition and was found not to vary more than 1 from 16 μsec. This procedure resulted in a stable absolute peak area of a standard lactate solution throughout the study. This result shows the performance of the instrument and the r.f. coil was stable throughout these experiments. In addition, the constancy of the 90° pulse for both muscle and solution samples suggests that all samples loaded the coil equivalently and that the B_1 field distribution in the samples was similar, although the latter is strictly not true because the field homogeneity of muscle and aqueous solutions was not identical. All acquisitions were done at ambient temperature, which varied between 17.5° and 18.5°C, as measured by a thermocouple in the magnet bore. The effects of temperature drift were shown not to be significant over the temperature range of 5 degrees.

For each muscle sample, spectra were acquired for both parallel and perpendicular orientations of muscle fibers with B_o. For each orientation, two sets of spectra were acquired. In one set, the echo times were $\tau_1 = \tau_2 = 1/(2J)$, where $J = 7$ Hz, while in the other set, τ_1 and τ_2 were fixed at $1/(2\Delta)$, where $\Delta = \Delta_{dc} + J$ with Δ_{dc} denoting the residual dipolar coupling. Note that the magnitude of Δ depends on the orientation of muscle fibers relative to B_o; its values are 24 and 2 Hz for parallel and perpendicular orientations, respectively (*1*).

Measurements of Relaxation Times

To cover the whole range of lactate concentrations in muscle samples studied, transverse relaxation time, T_2, of lactate was measured in endogenous samples ($n = 3$) as well as in samples supplemented with 60 μL of 5.05-M lactate solution ($n = 3$). For each sample, T_2 was determined by varying τ_1 for both parallel and perpendicular orientations of muscle fibers relative to B_o. For each orientation, spectra were acquired for two different values of τ_2 while varying τ_1 in a range from 10 to 350 ms; τ_2 was set to either 71.4 ms to refocus only the scalar

coupling or to 20.8 and 250 ms refocusing the sum of scalar and dipolar coupling for the parallel and perpendicular orientations, respectively. The same procedure was repeated by fixing τ_1 and varying τ_2.

For determining T_2 of lactate in muscle, data were initially fit to a monoexponential function. The correlation coefficients, r^2, of the monoexponential fit on data collected on endogenous samples varied from 0.12 to 0.17. The data were better fit by a biexponential function described in Eq. [1]. This nonlinear regression fit (Fig. P for Windows, Biosoft, Cambridge, UK) increased r^2 to values ranging from 0.87 to 0.93.

$$A = A_o\{a((\sin(\pi J\tau_1)\sin(\pi J\tau_2)(1+\cos^2(\pi J\tau_1)) \times \exp(-\tau_1/T_{2l})\exp(-\tau_2/T_{2l})) + b(\sin(\pi\Delta\tau_1)\sin(\pi\Delta\tau_2) \times (1+\cos^2(\pi\Delta\tau_1))\exp(-\tau_1/T_{2s})\exp(-\tau_2/T_{2s}))\}, \quad [1]$$

where A is the measured NMR methyl lactate signal from each spectrum. The fitting procedure yielded values for the following parameters: A_o which denotes the theoretical maximum signal at $\tau_1 = 0$ and $\tau_2 = 0$ in the absence of modulation; the preexponential parameters, a and b, which denote the fraction of lactate in each pool; and the relaxation times, T_{2l} and T_{2s}. This equation is derived from the spin evolution for an AX_3 homonuclear coupled spin system by means of product operator formalism (*7*) assuming that only A–X interactions are present. In our prior study (*1*), triple quantum coherence studies and decoupling experiments showed that even though intramethyl dipolar coupling is present, the observed orientation-dependent frequency splitting is due entirely to the methyl–methine interactions. However, this result does not necessarily exclude intramethyl dipolar couplings from affecting the signal modulation during acquisition. To estimate the possible magnitude of these effects, full numerical simulations were performed which showed that when the signal is averaged over a range of intramethyl couplings spanning as little as 20 Hz and as large as 400 Hz, the effect of these interactions on signal modulation averages to zero. These empirical findings validate the use of Eq. [1] for data analysis. The fitted values for T_{2s} and T_{2l} and a and b are given as means ±SD.

Transverse relaxation time, T_2, was also determined for lactate in aqueous solution. For this, spectra were acquired from a 0.2-M lactate solution using the same procedure as described above. In this case, τ_1 was varied from 10 to 850 ms, while τ_2, due to the absence of dipolar coupling interactions in solution, was set at 71.4 ms. To determine T_2, data were fit to a monoexponential function. The fitted T_2 value is given as mean ±SD.

To set the recycle time to avoid saturation due to T_1 decay of the signal, a set of spectra were acquired on an endogenous muscle sample using the same DQF pulse sequence with recycle time varying from 0.2 to 15 s. The methyl peak was integrated for all the spectra. Since no change in signal was observed for recycle times longer than 3 s, the recycle time for all acquisitions was set at 6 s.

Data Analysis

Prior to Fourier transformation, free induction decays from all one-dimensional spectra were first baseline corrected to remove any DC offset between the real and imaginary components and then apodized with a matched exponential function based on the linewidth of the spectra. For all muscle and standard samples, the integration of the methyl peaks was done on phased DQF spectra using a numerical integration method (MatLab) in the region from 0.35 ± 0.10 to 1.72 ± 0.15 ppm. This integration was done in triplicate on independent days and the results were averaged. Despite the operator intervention the integration random error was small; the maximum range in any set of integrations was 4% of mean.

The peak areas were converted to raw, observed NMR lactate content in muscle samples using the calibration curve yielded by standard solutions. This calibration curve was obtained by plotting the methyl peak area of DQF spectra from lactate solutions versus their known lactate concentrations.

The raw, observed NMR lactate content in muscle samples was corrected to yield the true values which account for T_2 signal decay and dipolar coupling effects. For this, Eq. [1] was rearranged to

$$\text{True NMR Lactate Concentration} = \frac{\text{Observed NMR Lactate Concentration}}{a((\sin(\pi J\tau_1)\sin(\pi J\tau_2)(1+\cos^2(\pi J\tau_1))\exp(-\tau_1/T_{2l})\exp(-\tau_2/T_{2l})) + b(\sin(\pi\Delta\tau_1)\sin(\pi\Delta\tau_2)(1+\cos^2(\pi\Delta\tau_1))\exp(-\tau_1/T_{2s})\exp(-\tau_2/T_{2s}))}$$

in which the T_2 values and constants a and b are the averaged values obtained from T_2 experiments. This is a straightforward algebraic calculation which adjusts the observed NMR lactate concentration to that fully corrected for T_2 and orientational dipolar effects.

RESULTS

NMR Spectroscopy

Figure 2A shows a DQF ^{1}H spectrum from an endogenous muscle sample with fibers oriented perpendicular to B_o. In this case, to account for both dipolar and scalar coupling, τ_1 and τ_2 were set at $1/2\Delta$. In these experiments, we are concerned only with the lactate methyl resonances, which are those on the right at ~1.2 ppm. The middle peaks were not specifically assigned

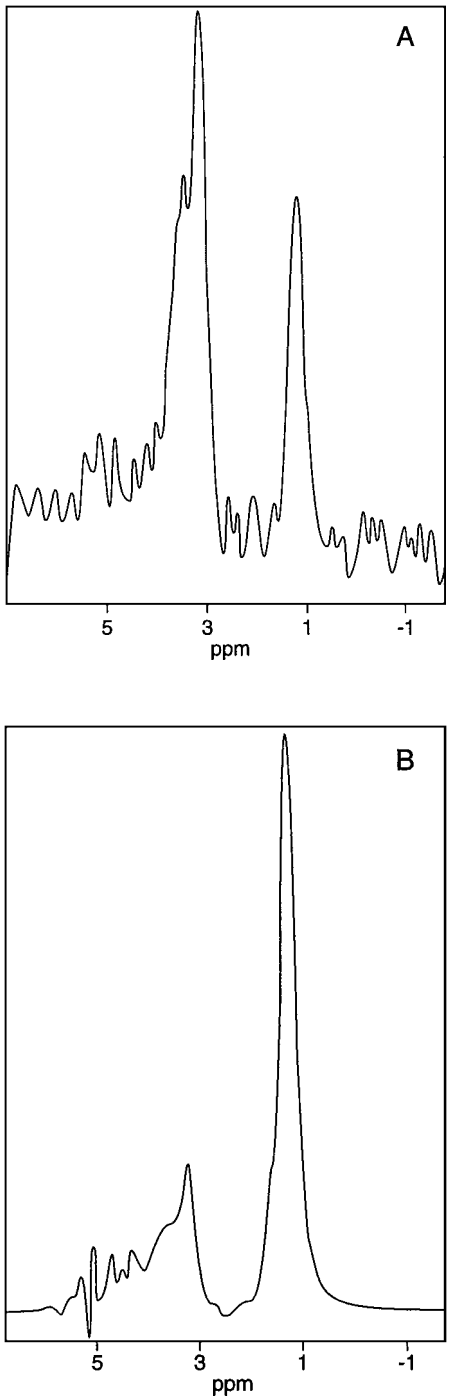

FIG. 2. DQF ^{1}H NMR spectra from (A) endogenous muscle sample with fibers oriented perpendicular to B_o with τ_1 and τ_2 set at 250 ms and (B) muscle sample supplemented with exogenous lactate with fibers parallel to B_o with τ_1 and τ_2 set at 20.8 ms. Spectra were baseline corrected and apodized with a matched exponential function prior to Fourier transformation. Note that (A) represents the spectrum with the lowest signal-to-noise ratio while (B) is representative of a spectrum with a high signal-to-noise ratio. The lactate methyl resonance is centered at 1.2 ppm; the other resonances were not studied in detail or assigned (see text).

and likely represent major contributions from methyl protons of trimethylamines (*2*, *3*). The leftmost peaks represent the residual water. Other spectra, with shorter τ_1 and τ_2 values, yielded higher signal-to-noise ratios (range from 23 to 1100). For comparison, Fig. 2B displays a typical high signal-to-noise DQF, ^{1}H spectrum acquired from a supplemented muscle sample with fibers oriented parallel to B_o. Shimming on water solution standards and muscle samples gave an average linewidth of $\sim 15 \pm 2$ and $\sim 39 \pm 8$ Hz, respectively.

To check the quality of the filter and the nature of the residual signals at 3.2 ppm, a nonDQF spectrum with the same echo times was acquired on an endogenous sample with fibers parallel with B_o. As predicted, the spectrum showed only a large water peak (at 4.7 ppm) and a small lipid peak (at 1.2 ppm). No signal was detected at 3.2 ppm (data not shown).

TABLE 1

Mean Values of Long, T_{2l}, and Short, T_{2s}, Relaxation Times and Preexponential Constant, *a* and *b*, Obtained from Triplicate Measurements on All Samples

Sample description	T_{2l} [ms]	T_{2s} [ms]	*a*, preexponential constant for T_{2l} in %	*b*, preexponential constant for T_{2s} in %
Endogenous muscle samples with fibers oriented parallel to B_o (echo times refocusing only *J*-coupling)	142	37	19	82
Endogenous muscle samples with fibers oriented parallel to B_o (echo times refocusing both couplings)	149	45	18	85
Endogenous muscle samples with fibers oriented perpendicular to B_o (echo times refocusing only *J*-coupling)	141	34	19	83
Endogenous muscle samples with fibers oriented perpendicular to B_o (echo times refocusing both couplings)	140	37	17	83
Supplemented muscle samples with fibers oriented parallel to B_o (echo times refocusing only *J*-coupling)	142	32	19	80
Supplemented muscle samples with fibers oriented parallel to B_o (echo times refocusing both couplings)	147	46	16	83
Supplemented muscle samples with fibers oriented perpendicular to B_o (echo times refocusing only *J*-coupling)	133	36	21	81
Supplemented muscle samples with fibers oriented perpendicular to B_o (echo times refocusing both couplings)	138	31	20	79

T_2 Measurements

For the orientation-independent case of lactate in solution the best fit yielded a single T_2 value of 1900 ms (± 75, $n = 4$).

In postmortem muscle, to rule out any variation of T_2 over the entire range of lactate content in the batches of samples assayed, T_2 was measured on endogenous samples as well as on samples supplemented with exogenous $L(+)$ lactate (see Methods).

For each muscle sample, T_2 was determined for both parallel and perpendicular orientation of muscle fibers relative to B_o. Examples showing variation of the peak areas of the methyl signal as a function of preparation time τ_1 are presented in Figs. 3A–3D. In these figures, the solid line represents the bi-exponential function as described in Eq. [1] while the filled circles represent the integrated methyl peaks. In Figs. 3A and 3B, muscle fibers were oriented parallel with B_o, while in Fig. 3C and 3D, fibers were perpendicular to B_o. In Fig. 3A and 3C, τ_2 was fixed at $1/2J$ (refocusing only the scalar coupling) while in Fig. 3B and 3D, τ_2 was fixed at $1/2\Delta$ (refocusing the sum of both couplings in each orientation). Prior experiments showed that similar graphs are obtained when τ_2 is varied while τ_1 is fixed with and without accounting for dipolar coupling.

Table 1 gives the mean values of long, T_{2l}, and short, T_{2s}, relaxation times as well as preexponential constants, a and b, obtained from the triplicate, independent measurements for both endogenous and supplemented samples. The range for each measurement did not exceed 11% of the mean value. The results given in Table 1 show that relaxation times and preexponential constants do not depend on the orientation of muscle fibers relative to B_o (at 95% confidence level, a two-sample t-statistic significance test yielded P-values for T_{2l}, T_{2s} and a and b of $P = 0.88$, $P = 0.67$, $P = 0.85$, and $P = 0.71$, respectively). Also, there was no significant difference between relaxation times and

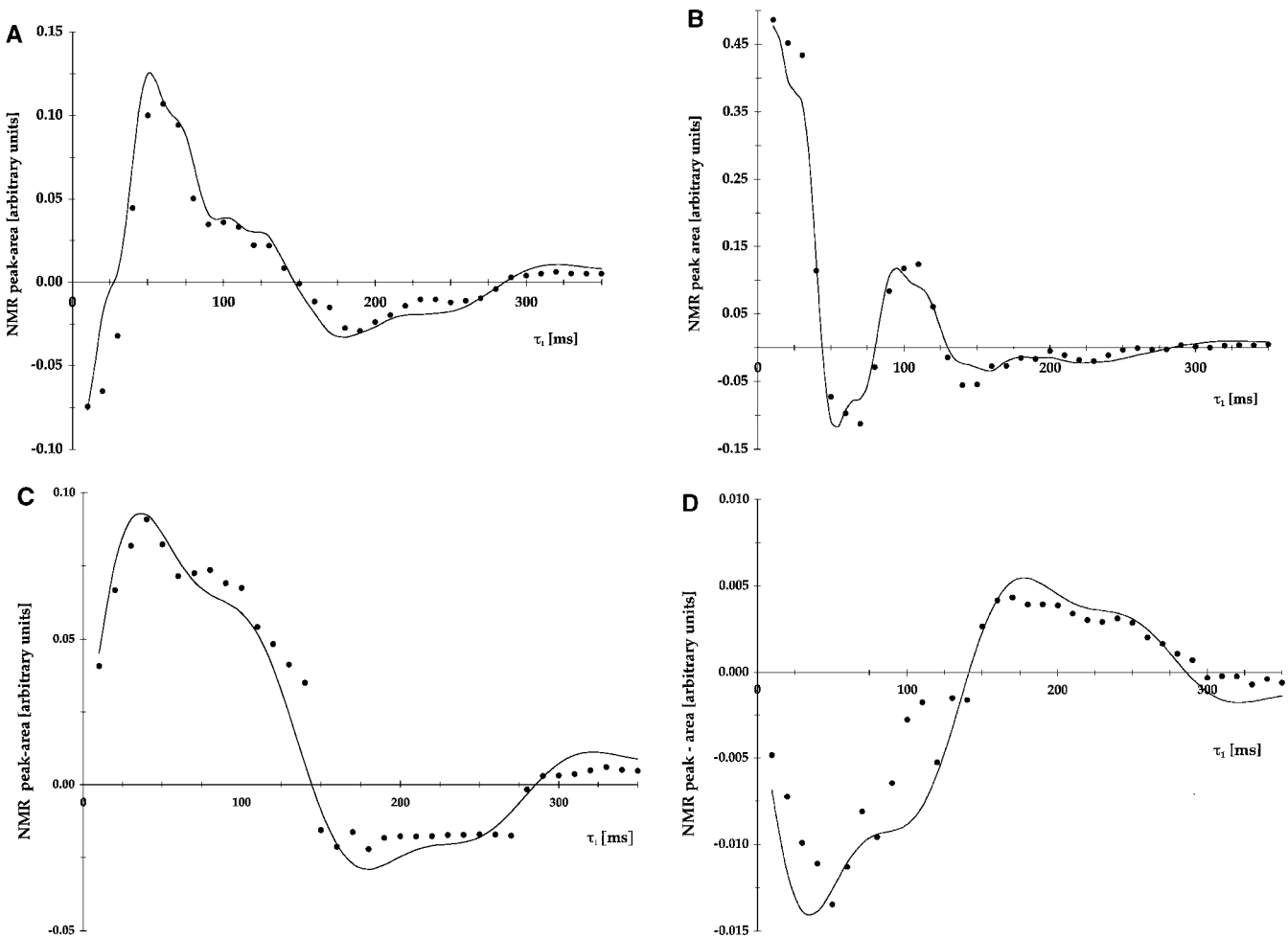

FIG. 3. Transverse relaxation as a function of sample orientation and τ_2. The variation of the methyl peak area is plotted as a function of τ_1. In (A) and (B) muscle fibers were oriented parallel to B_o with τ_2 set at 71.4 and 20.8 ms, respectively. In (C) and (D) the fibers were oriented perpendicular to B_o with τ_2 set at 71.4 and 250 ms, respectively. The solid line represents the theoretical response as described in Eq. [1] (see text). The filled circles represent the experimental data. The ordinate scale is in arbitrary units.

preexponential constants obtained from endogenous samples compared to those acquired from supplemented ones ($P = 0.85$, $P = 0.72$, $P = 0.65$, and $P = 0.74$ for T_{2l}, T_{2s}, a, and b, respectively). Thus, all the results from Table 1 were averaged to yield a long relaxation time, $T_{2l} = 142 \pm 8$ ms ($n = 24$) and a short relaxation time, $T_{2s} = 37 \pm 6$ ms ($n = 24$). The average values for the scaling factors a and b were $19 \pm 3\%$ ($n = 24$) and $82 \pm 2\%$ ($n = 24$), respectively. These factors represent the proportions of lactate pools that show distinct T_2's and orientation properties.

Calibration of NMR Spectra with Known Lactate Solution Standards

Methyl peak areas of spectra acquired on lactate solutions with known concentrations were used to calibrate the methyl peak areas of all the spectra acquired on muscle samples. The peak areas for the solutions yielded a regression line: $A = (3.8\,10^{12})N$, ($r^2 = 0.988$; SD = 0.011), where N is the number of lactate moles present in the standard sample and A is the area of lactate methyl peak from DQF ^{1}H NMR spectra in arbitrary units. This calibration was repeated at the beginning, middle, and end of the study. No tendency of the values to change over time was detected so the calibrations were averaged. This procedure allowed calculation of raw, observed NMR lactate concentration in muscle samples to be further corrected for T_2 and dipolar coupling effects.

Biochemical Assay

The endogenous lactate content of the muscle measured in perchloric acid extracts was found to be high as previously

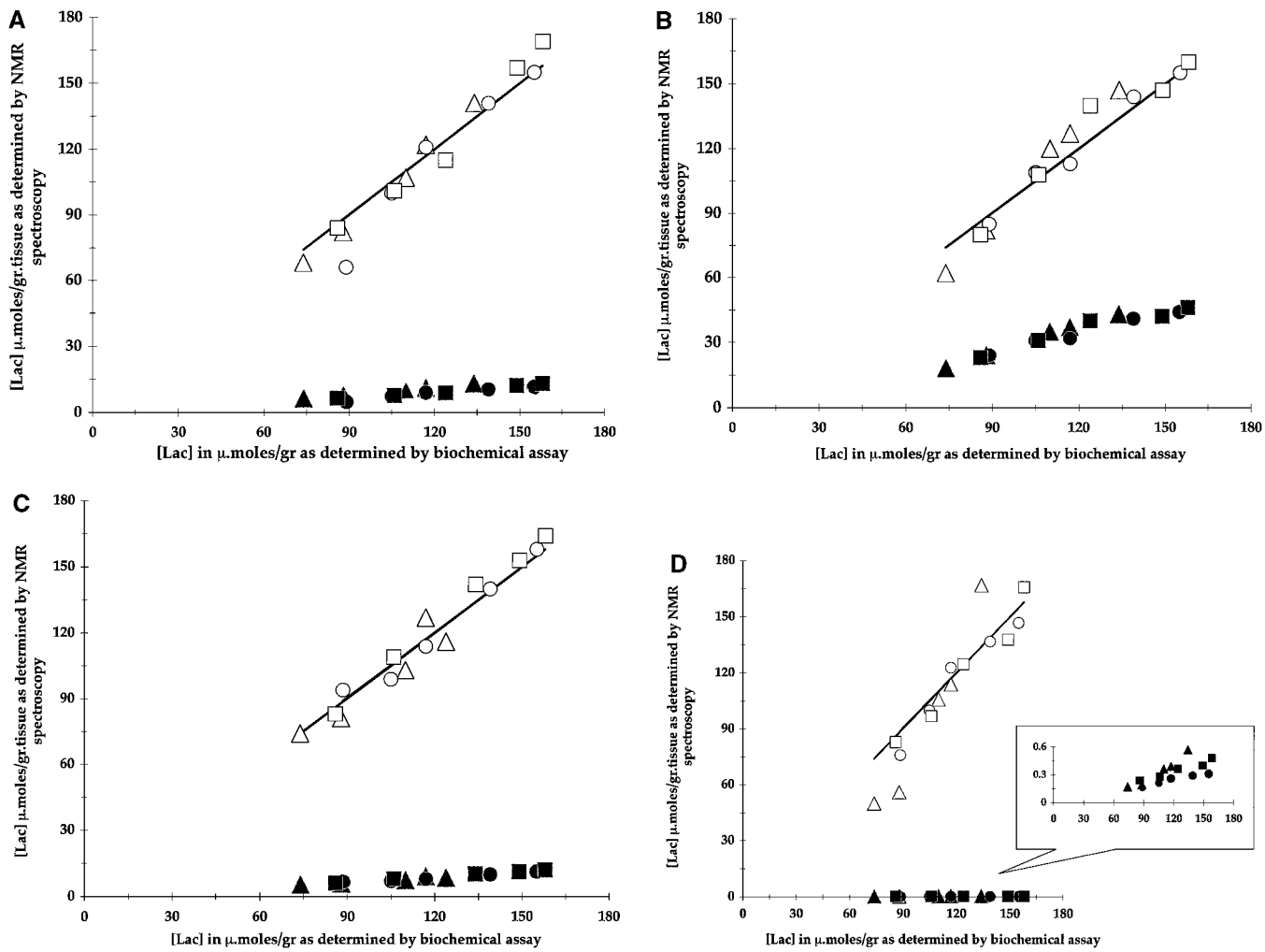

FIG. 4. Comparison of raw and adjusted intensity of lactate methyl resonance in muscle and in lactate standard solutions. The abscissa is the lactate concentration (μmol/g tissue) determined by enzymatic assay. The ordinate is the lactate concentration (μmol/g tissue) from the NMR areas scaled by the standard curve for lactate solutions. The solid line represents the regression line from standard lactate solutions. Each symbol (circle, triangle, and square) represents a separate batch of muscle samples. Filled symbols display results from raw, observed muscle spectra. Open symbols display results of these data adjusted for T_2-relaxation and dipolar coupling effects. In both (A) and (B) muscle fibers were oriented parallel with B_o. In (A) τ_1 and τ_2 were set at 71.4 ms and in (B) τ_1 and τ_2 were set at 20.8 ms. In (C) and (D) fibers were oriented perpendicular to B_o while τ_1 and τ_2 were set at 71.4 and 250 ms, respectively.

reported (*1*), 74 to 89 μmol/g wet weight of tissue. This procedure gave the absolute concentrations of lactate in each muscle sample which is compared in the following section to the lactate concentration determined through NMR spectroscopy.

Comparison of Lactate Content in Muscle as Determined by Biochemical Assay and NMR Spectroscopy

As mentioned above, the raw, observed NMR spectral area of methyl lactate peak from muscle samples were converted to lactate content in these samples using the calibration curve obtained from the standard solutions. These results are shown in Figs. 4A–4D; each graph shows data of a particular muscle fiber orientation before (filled symbols) and after (open symbols) correcting for T_2 and dipolar coupling effects. The solid line in each graph represents the linear regression line of NMR versus enzymatic assay data obtained from standard solutions. Each batch of samples is represented by a different symbol. In Figs. 4A and 4B, NMR spectra were acquired with muscle fibers oriented parallel to B_o with echo times set to refocus J-coupling and the sum of both couplings, respectively. The same procedure was followed in Figs. 4C and 4D, where samples were rotated with fibers perpendicular to B_o. Before any correction for T_2 relaxation and dipolar coupling (see Methods), lactate content in muscle samples determined from NMR spectra lay well below the data from biochemical assay (filled symbols in Figs. 4A–4D). However, a good match between NMR and biochemical results was achieved when raw, observed NMR data were adjusted to account for orientation-dependent dipolar coupling effects and for a biexponential T_2 decay of the signal (open symbols in Figs. 4A–4D).

Dependent on the orientation of the muscle fibers relative to B_o, adjustment of the raw NMR data using a monoexponential fit ($T_2 = 153 \pm 21$ ms, $n = 24$) accounted only for between 15 (parallel) and 68% (perpendicular) of lactate concentration values as determined by biochemical assay (data not shown).

DISCUSSION AND CONCLUSIONS

This study demonstrates that for an adequate quantification of muscle lactate using DQF, ^{1}H NMR spectroscopy both scalar and residual dipolar coupling interactions need to be taken into account along with a biexponential T_2 decay. This biexponential T_2 decay of lactate signal suggests that there are two pools of lactate present in these muscle samples. Lactate in one pool has a longer T_2 ($\sim$142 ms) while lactate in the other pool has a much shorter T_2 ($\sim$37 ms). This conclusion is in agreement with our prior study (*1*), where we showed that DQ signal from lactate methyl protons has two sets of frequency splittings: a 7-Hz splitting, due to scalar coupling, as well as an orientation-dependent splitting which had contributions from residual dipolar coupling interactions.

Lactate molecules in one pool rotate freely and isotropically as they would in solution, consistent with a longer T_2 decay, while lactate molecules in the other pool have a somewhat restricted motion which would yield a shorter T_2 decay. However, it is important to emphasize that the biexponential model was used as the simplest one to give a statistically significant fit to the data. This model implies the presence of only two pools of lactate in muscle when in fact these compartments could be further subdivided hence requiring a multiexponential fit for a higher accuracy in determining the T_2 values of lactate in muscle. We have no independent verification of the identity of these pools. Based on the microscopic structure of this postmortem muscle, the shorter T_2 likely represents the lactate pool within the myofibrillar space.

A biexponential decay of lactate in muscle has been observed before (*8*) for excised rat leg muscle. In that paper a two-pool model was used to indicate a compartmentation of lactate between muscle intra- and extracellular spaces. Our results are in agreement with such a finding if one thinks of the extracellular space as a solution-like environment for lactate, while lactate in the myofibrillar space would tend to have a more restricted motion resulting in the observation of dipolar coupling interactions. These results were independent of lactate content in muscle. The simplest explanation for this finding is that the volume of the two pools is different, and lactate has an equal probability of diffusing into each pool. Thus added lactate appears in each pool in proportion to the volume available to that pool. The values for a and b as determined by the best fit of the data to Eq. [1] suggest that $\sim$20 % of the signal comes from the pool where lactate behaves as in solution while $\sim$80 % comes from the pool where residual dipolar coupling interactions are present. Our muscle samples are postmortem and have degraded membranes, which means a larger myofibrillar volume and less interstitial spaces as seen in the histologic sections (*1*).

T_2 relaxation anisotropy has been observed for other tissues such as tendon (*9*). An orientation-dependent T_2 mechanism, such as the dipolar coupling, would be expected for an anisotropic motion. We found that T_2 values were not dependent on the orientation of muscle fibers relative to B_o. This could be explained by the fact that the small splitting observed (*1*) indicates that considerable isotropic motion is present in both pools, with the anisotropic component of T_2 being too small to be observed.

These results were obtained under optimal experimental conditions. The muscle sample is metabolically stable because it is postmortem. The lactate content is also very high. Both factors simplified experimental design and enabled high quality data with very high signal-to-noise ratios. Both of these properties allowed long acquisition times and multiple measurements of the same preparation. Without these optimal conditions, the findings of this work could not have been obtained. These conditions will not be present in most dynamic physiological conditions of living muscle, such as exercise and recovery of skeletal muscle in which the lactate concentrations are much lower and change rapidly.

The results and conclusions are relevant to the interpretation of in vivo ^{1}H NMR spectroscopy as well as a better understanding of the mechanism behind the dipolar coupling effects in muscle and metabolite compartments. Since orientational dependency of ^{1}H NMR spectra has been shown for several muscle metabolites (*1–3*), an adequate quantification of these metabolites requires that this anisotropy is taken into account when the pulse sequence is applied as well as when the relaxation times are determined. This information could also be used to distinguish different compartments of these metabolites based on very distinct (generally much shorter) T_2 values which may accompany the observation of dipolar coupling.

The principles of dipolar coupling identified (*1*) and shown to be quantitatively accounted for in this paper apply for lactate, creatine, taurine, and likely other metabolites in structurally anisotropic cells and tissues (*1–3*) and should be used to guide future experimental design. Furthermore, many other metabolites present in muscle and other organs also show residual anisotropic dipolar coupling interactions (*1–3*). This means that adequate quantification in such circumstances requires that the factors identified in our work need to be taken into account. In practical experiments with living tissues, the magnitude of the dipolar coupling and T_2 effects will be known with less precision. Nonetheless, the success we demonstrated under optimal conditions provides a goal toward which future physiological work can be directed.

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