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Quantification of the Contribution of Extracellular Sodium to ^{23}Na Multiple-Quantum-Filtered NMR Spectra of Suspensions of Human Red Blood Cells[1]

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^{23}Na double-quantum-filtered (DQF) NMR enables the detection of anisotropic motion of sodium ions due to their interaction with ordered structures in biological tissues. Using the technique, anisotropic motion was found for sodium ions in mammalian red blood cell suspensions (RBC) and the effect was shown to correlate with the integrity of membrane cytoskeleton. In the present study relative contributions to the DQF and triple-quantum-filtered (TQF) spectra of sodium bound to anisotropic and isotropic binding sites in the intra- and extracellular sodium pools (Na content being 15 and 150 mM, respectively) of human RBC were quantified for different hematocrits. DQF spectra were measured by a modified Jeener–Broekaert pulse sequence which enabled exclusive detection of anisotropically moving sodium ions. The relative contributions of the extracellular sodium to the TQF and DQF spectra decreased as the hematocrit increased, but their efficiency relative to the sodium content increased. The contribution of the extracellular sodium to the TQF signal was found to dominate the spectrum of the RBC suspension at all hematocrits studied. The contribution of the extracellular sodium to the DQF was significantly smaller than that to the TQF and was only 22% at a high hematocrit of about 90%.

Key Words: **^{23}Na NMR; multiple-quantum-filtered NMR; human red blood cells; intracellular sodium.**

INTRODUCTION

^{23}Na multiple-quantum-filtered NMR is being used to probe intracellular sodium ions in biological systems. However, it has been shown that extracellular sodium contributes to these spectra quite significantly (*1*). ^{23}Na double-quantum-filtered (DQF) NMR has been proven useful for detecting sodium ions that experience anisotropic motion due to their binding to ordered structures in biological tissues (*2*). Thus, anisotropic motion of sodium ions was found in human red blood cells (RBC) and a high correlation was found between the residual quadrupolar interaction and the conformation of the cytoskeleton (*3, 4*). In the present study the relative contributions to the DQF and triple-quantum-filtered (TQF) spectra of sodium bound to anisotropic and isotropic binding sites in the intra- and extracellular sodium pools of human RBC were quantified. DQF spectra were measured by a modified Jeener–Broekaert pulse sequence which enabled exclusive detection of anisotropically moving sodium ions (*5*). The relative contributions of the extracellular sodium to the TQF and DQF spectra decreased as the hematocrit increased, but their efficiency relative to the sodium content increased. The contribution of the extracellular sodium to the DQF was, however, significantly smaller, and was only 22% at a hematocrit of about 90%.

MATERIALS AND METHODS

The study was performed on human RBC from healthy volunteers. Venous blood was collected with EDTA (35 ml of blood with 1 ml of 0.2 M EDTA) and kept for 24 h at 4°C until the NMR experiments. The pellet of RBC was separated from plasma by centrifugation and then washed three times with a solution containing 145 mM NaCl, 10 mM Hepes at pH 7.4. The RBC pellet was divided into twelve 2-ml portions. The hematocrit of the RBC suspension was adjusted by adding an appropriate amount of solution to each sample. The added solution was composed of different proportions of isotonic 30 mM Na_3H_2TmDOTP 3 NaCl, shift reagent (SR) (*6*), and supernatant according to the final desired SR concentration. The hematocrit of each sample was determined by comparing the integrated intensity of the SQ spectrum of the supernatant to that of the extracellular sodium of the RBC suspension in the presence of the SR. The intra- and extracellular sodium concentrations were determined by comparing their integrated peak intensities to a known standard, taking the hematocrits into consideration, according to the method of Gupta and Gupta (*7*).

^{23}Na NMR spectra were recorded on a Bruker AMX-360-WB NMR spectrometer operating at 95.3 MHz. TQF spectra

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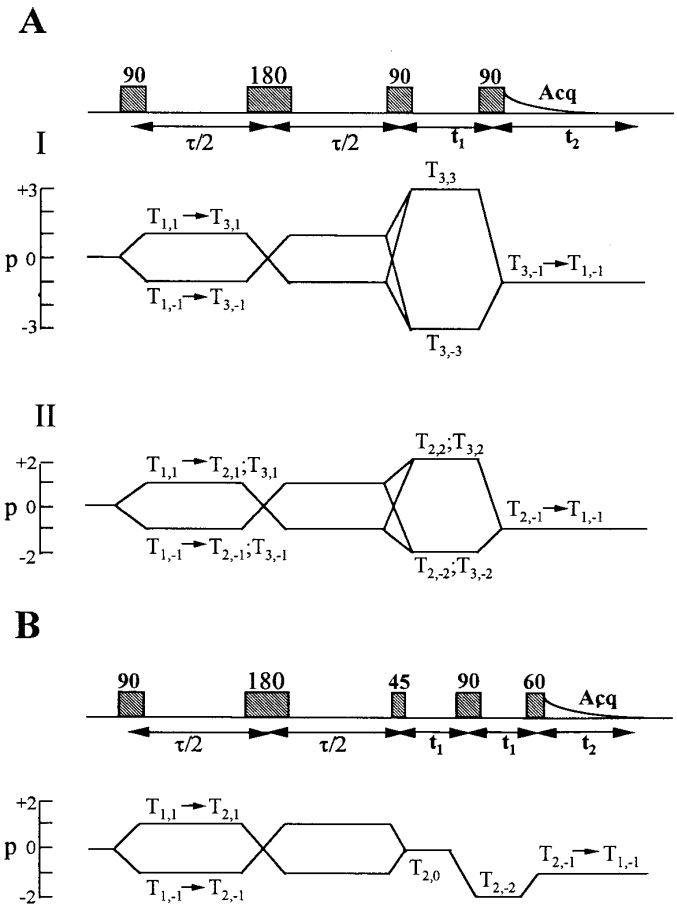

FIG. 1. (A) TQF (I) and DQF (II) pulse sequences and coherence pathways for $I = 3/2$ nuclei. (B) The modified Jeener–Broekaert pulse sequence, DQJB.

were measured using the traditional pulse sequence with 48-step phase cycling (Fig. 1A) (*8, 9*). DQF spectra were measured using a modified Jeener–Broekaert experiment, DQJB (Fig. 1B) (*5*). For MQF spectra the repetition time was 250 ms which is in the range 5–10 T_1 necessary for these measurements. The number of accumulations for SQ spectra was 16, for DQJB, 1152, and for TQF, 480.

THEORETICAL BACKGROUND

The applied pulse sequences and their coherence pathway diagrams are given in Fig. 1. Pulse sequence A enables the detection of either TQF or DQF depending on the phase cycling. The coherence pathways denoting only those spherical tensors which contribute to the TQF and DQF spectra are given in I and II, respectively. In isotropic solution, the $T_{1,\pm1}$ tensors formed by the first 90° pulse are converted by quadrupolar relaxation into the third-rank tensors, $T_{3,\pm1}$. In anisotropic systems the residual quadrupolar interaction allows the conversion of $T_{1,\pm1}$ into the second-rank tensors, $T_{2,\pm1}$, as well. The third pulse at the end of the creation time, τ, transfers $T_{3,\pm1}$ and $T_{2,\pm1}$ into $T_{3,\pm3}$, $T_{3,\pm2}$, and $T_{2,\pm2}$, respectively. The multiple-quantum evolution time, t_1, is kept short (20 μs) in order to avoid the effects of the triple- and double-quantum relaxation, respectively. The last 90° pulse transfers the magnetization into the SQ coherences, $T_{3,-1}$ and $T_{2,-1}$, which evolve into the observable $T_{1,-1}$ during the acquisition time. Thus, for TQF (I), the phase cycling that selects the triple-quantum coherences retains only the contribution of the $T_{3,\pm3}$ tensors. In the case of the DQF measurement (II) the contributions of both $T_{3,\pm2}$ and $T_{2,\pm2}$ tensors are retained. This effect is the reason for differences between the DQF and the TQF spectra for anisotropically moving $I = 3/2$ nuclei. It has been shown that only the contributions of $T_{2,\pm1}$ are observed in DQF spectra when the last two 90° pulses are substituted by two $\theta = 54.7°$ pulses (*2, 8*). However, the ability to measure the contribution of $T_{2,\pm1}$ exclusively depends on the accuracy of the flip angle 54.7° and, thus, the homogeneity of B_1. This problem can be overcome by using the Jeener–Broekaert experiment (*10*), as has been suggested by Kemp-Harper and Wimperis (*11*). We have recently proposed a modification of the Jeener–Broekaert experiment and have demonstrated its efficiency for the exclusive detection of the signal of the anisotropically moving sodium ions (*5*). Figure 1B illustrates the pulse sequence, the coherence pathway diagram, and only those spherical tensors which contribute to the resultant DQJB signal. As in pulse sequence A, the third- and the second-rank tensors, $T_{3,\pm1}$, and $T_{2,\pm1}$, are formed during the creation time, τ. The 45° pulse (due to its 90° phase shift with respect to the first two pulses) transfers only the $T_{2,\pm1}$ tensors into the zero-quantum coherence, $T_{2,0}$. The following 90° pulse transfers $T_{2,0}$ into the double-quantum coherence, $T_{2,-2}$, and the 60° pulse transfers $T_{2,-2}$ into the SQ coherence, $T_{2,-1}$, which evolves into the observable $T_{1,-1}$ during the acquisition time. Pulse sequence B employs an additional

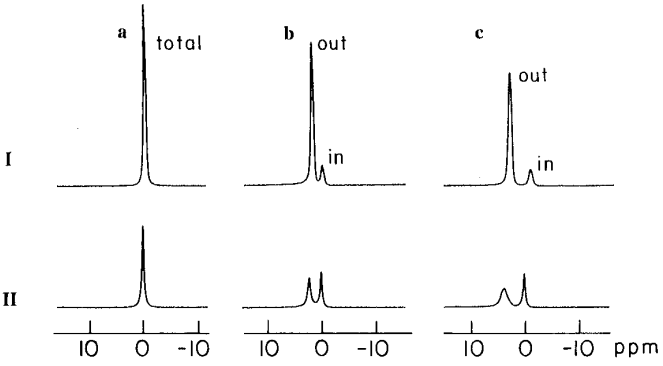

FIG. 2. ^{23}Na single-pulse NMR spectra of suspensions of human RBC (a) without SR, (b) in the presence of 1.15 mM SR, and (c) in the presence of 2.3 mM SR. Spectra I and II are recorded at hematocrits of 58 and 88%, respectively. Total, in, and out: total, intracellular, and extracellular sodium peaks, respectively.

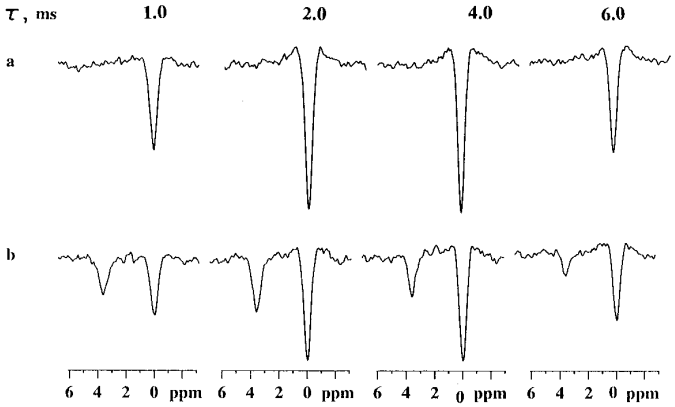

FIG. 3. ^{23}Na DQJB NMR spectra of a suspension of human RBC at a hematocrit of 88% as a function of the creation time τ (a) without SR and (b) in the presence of 2.3 mM SR. Peak assignments are as in Fig. 2.

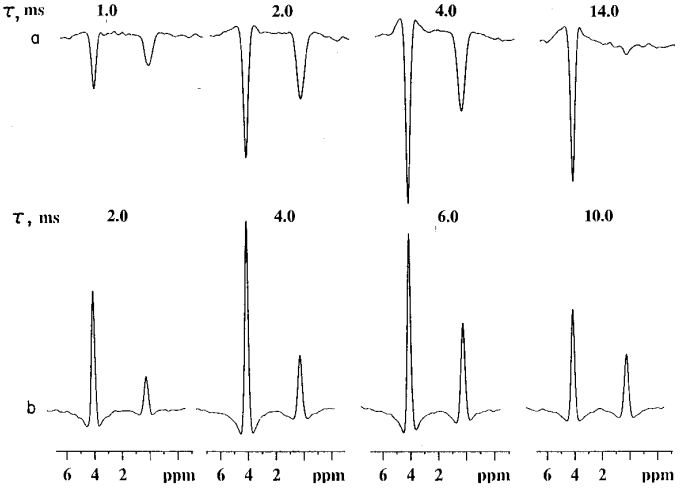

FIG. 5. ^{23}Na DQJB (a) and TQF (b) NMR spectra of a suspension of human RBC at a hematocrit of 58% in the presence of 2.3 mM SR as a function of the creation time, τ. Peak assignments are as in Fig. 2.

double-quantum filtering as compared to the original Jeener–Broekaert experiment. This eliminates contributions from $T_{1,0}$ which may be formed by a 45° pulse due to the nonequal contributions of $T_{1,-1}$ and $T_{1,1}$ created during τ.

RESULTS

The ^{23}Na SQ spectra for suspensions of human RBC at different hematocrits are given in Fig. 2. The signal of the extracellular sodium ions has a major contribution to the total spectrum even at high hematocrits, due to the high extracellular sodium concentration. Typical DQJB and TQF spectra of the RBC suspension at a hematocrit of 88% as a function of the creation time, τ, with and without SR are given in Fig. 3 and 4, respectively. As one can see, the intracellular sodium has the dominant contribution to the total DQJB spectrum (Fig. 3). On the other hand for the same sample it is the contribution of the extracellular sodium that dominates the TQF spectrum (Fig. 4). At a hematocrit of 58%, both DQJB and TQF spectra of the RBC suspension originate mainly from the extracellular sodium ions (Fig. 5). While the relaxation parameters of the intracellular sodium do not change upon changing the hematocrit, those of the extracellular sodium are hematocrit dependent. This can be seen from the creation time, at which the extracellular DQJB signal has its maximum, τ_{max}, which changes from 2.4 to 7.0 ms for hematocrits of 88 and 58%, respectively (see Figs. 3 and 5). In the case of the TQF spectrum the change of the relaxation of the extracellular sodium as a function of hematocrit is small as can be judged from essentially the same τ_{max} values at hematocrits of 88 and 58% (see Figs. 4 and 5).

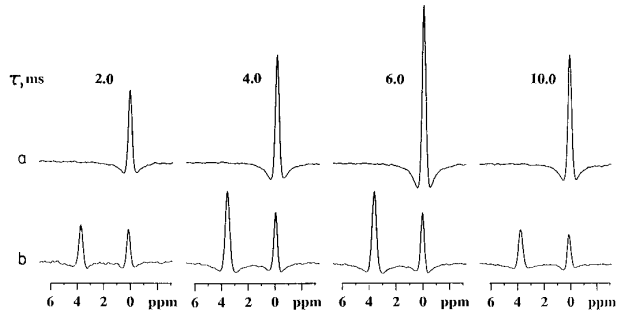

FIG. 4. ^{23}Na TQF NMR spectra of a suspension of human RBC at a hematocrit of 88% as a function of the creation time τ (a) without SR and (b) in the presence of 2.3 mM SR. Peak assignments are as in Fig. 2.

The quantification of the relative contributions of the extra- and intracellular sodium to the total MQF spectra of the RBC suspension was done by comparing the MQF spectra of the human RBC suspension in the presence of the SR to those obtained without SR. In the presence of the SR the intra- and extracellular sodium signals are separated and can be assessed independently. However, the extracellular MQF signal intensity is reduced due to the presence of SR (*1*). Enhanced relaxation induced by the paramagnetic SR tends to equalize the two components of the SQ transverse relaxation, T_{2f} and T_{2s}. The shift reagent, on the other hand, has no effect on the relaxation rates of intracellular sodium. However, some broadening may be induced due to the effect of susceptibility gradients. In order to avoid this problem we chose to use Tm which has a small magnetic moment (*6*). The broadening of the intracellular sodium signal at SR concentrations smaller than 3 mM is found to be negligible. We have thus designed a procedure of subtracting the intracellular peak, obtained in the presence of the SR, from the signal of the total sodium, measured in its absence. This subtraction enables one to obtain the contribution of the extracellular sodium pool. The subtraction of the two spectra was done after adjusting the positions of the intracellular

TABLE 1
Relative Contributions of the Extracellular Sodium to the DQJB and TQF Spectra for Suspensions of Human RBC at Different Hematocrits

Hematocrit, %	[SR], mM	$\frac{TQF^{out}}{TQF^{total}}$	$\frac{TQF^{out}/[Na^{out}]^a}{TQF^{in}/[Na^{in}]}$	$\frac{DQJB^{out}}{DQJB^{total}}$	$\frac{DQJB^{out}/[Na^{out}]}{DQJB^{in}/[Na^{in}]}$
58	1.15	0.72	0.39	0.59	0.21
	2.3	0.74	0.39	0.60	0.20
	3.5	0.79	0.49	0.64	0.24
88	1.15	0.73	1.92	0.28	0.28
	2.3	0.7	1.64	0.30	0.30
	3.5	0.77	2.38	0.33	0.34
92	1.85	0.61	1.92	0.22	0.34
	3.7	0.56	1.41	0.22	0.31
	5.5	0.67	2.13	0.25	0.34

[a] TQF, DQJB, peak intensities; SQ, peak integrals.

and total peaks in the corresponding spectra in order to compensate for signal shifts caused by addition of the SR (up to 20 Hz). The relative contributions of the extracellular sodium to the total DQJB and TQF peaks at the τ values corresponding to the maximum signal intensities of the intracellular sodium are given in Table 1 and Fig. 6.

In order to account for the different sodium concentrations in the extra- and intracellular sodium pools the maximum intensities of the DQJB and TQF signals have also been normalized to the corresponding sodium SQ integrated peak intensities. The ratios of the normalized contributions of the extracellular sodium to those of the intracellular ones for the DQJB and TQF measurements at different hematocrits are also given in Table 1.

DISCUSSION

In the present study the relative contribution of the extracellular sodium to the total sodium MQF spectrum of suspensions of human RBC is found to be hematocrit dependent. At high hematocrits the contribution of the intracellular sodium ions to the overall DQJB signal is dominant, while their contribution to the TQF signal is smaller than that of the extracellular pool. However, at a hematocrit of 58% the extracellular sodium has a dominant contribution not only to the TQF, but to the DQJB signal as well. Thus, at high hematocrits (around 90%) ^{23}Na DQJB NMR spectra of the RBC are dominated by the contribution of the intracellular pool and can be used for studies of sodium binding to the anisotropic binding sites at the inner side of the RBC membrane without SR. However, at lower hematocrits the contribution of the extracellular sodium should be taken into account.

We demonstrated that extracellular sodium contributes to the total DQJB spectrum of the RBC suspension and quantified this contribution. The data are in line with the qualitative results of Tauskela and Shoubridge, who have detected the extracellular ^{23}Na DQF (θ = 54.7°) NMR signal for the RBC suspension in the presence of the SR (*12*).

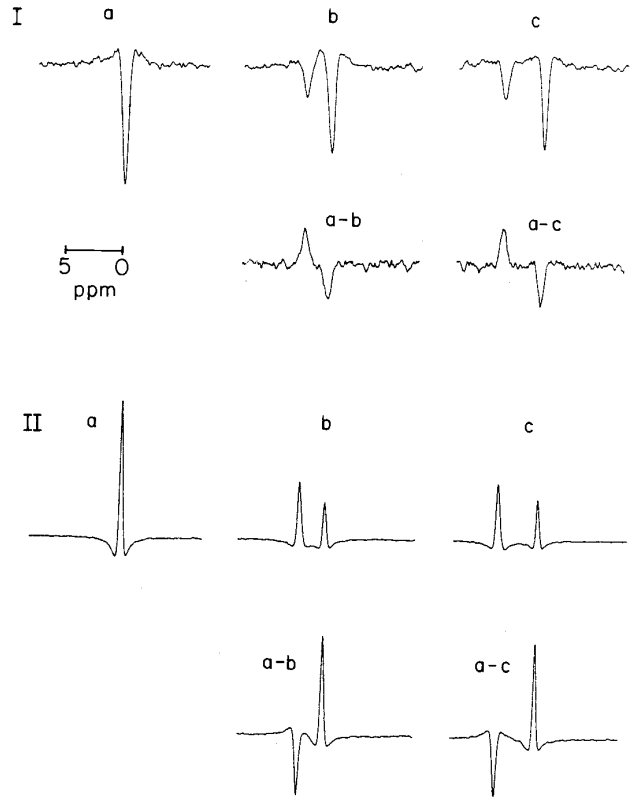

FIG. 6. ^{23}Na MQF NMR spectra of a suspension of human RBC at a hematocrit of 88% (a) without SR, (b) in the presence of 1.15 mM SR, (c) in the presence of 2.3 mM SR, (a − b) the difference spectrum of spectra (a) and (b), and (a − c) the difference spectrum of spectra (a) and (c). (I) DQJB spectra measured with the creation time, τ, of 3 ms and (II) TQF spectra measured with the creation time, τ, of 7 ms. Peak assignments are as in Fig. 2.

At all the hematocrits evaluated in this study, the relative contribution of the extracellular ions to the TQF spectrum is larger than their contribution to the DQJB peak. The large contribution of the extracellular sodium to the TQF spectrum of RBC is in line with the DQF results of Jelicks and Gupta (*1*). Their measurements were performed using $\theta = 90°$ and relatively long creation times, τ, where the signal resulting from the formation of the second-rank tensor has already decayed. Therefore their data are comparable with our TQF results. We have shown that both DQJB and TQF spectra are characterized by an enhanced contribution of the intracellular sodium as compared to the ^{23}Na SQ NMR signals. In this respect ^{23}Na MQF spectra enable a better evaluation of the intracellular sodium without using SR than the SQ spectra.

In the suspensions of RBC measured in this study there is an order of magnitude difference in the sodium concentration between the intracellular (about 15 mM) and the extracellular (about 150 mM) pools. As can be seen from Table 1, the efficiency of the DQJB spectra for the intracellular sodium is higher than that for the extracellular sodium by a factor of 3–5 depending on hematocrit. In contrast, the same ratio for the TQF is consistently lower and varies significantly with hematocrit, so that at the highest hematocrit the efficiency of the TQF^{in} is only half of that of the TQF^{out}. Thus, in the case of unsealed RBC ghosts where the sodium concentration is the same in the extra- and intracellular compartments, the contribution of the extracellular sodium to the total DQJB signal can actually be neglected in agreement with our previous study (*4*).

The dependence of the relative efficiency of MQF signals of extracellular sodium on the hematocrit most probably stems from hematocrit-related changes in the percentage of bound sodium. The fraction of bound sodium is one of the factors determining the ratio of the fast transverse relaxation rate to the slow one, which determines the efficiency of the multiple-quantum filtering (*13, 14*). The decrease in the fraction of bound sodium ions at lower hematocrits is most probably also responsible for an extremely narrow DQJB signal of the extracellular sodium at a hematocrit of 58% (Fig. 5). A similar effect has been reported by Tauskela *et al.* for ^{23}Na in perfused rat heart, where they have recorded a narrow ^{23}Na DQF ($\theta = 54.7°$) NMR signal of the extracellular sodium comparable in width with the TQF peak (*15*).

The approach proposed in the present study can be extended for the characterization of sodium interaction with the intracellular anisotropic binding sites in other biological systems. Recently anisotropic motion has been found for sodium in various biological tissues: muscle (*16, 17*), brain (*16, 18*), and connective tissues, including skin, cartilages, and intervertebrae disks (*5*). For connective tissues we have proven that the effect results from sodium interaction with the collagen fibers. Our experiments on RBC (*4*) and blood vessels (*19*) demonstrate that the anisotropic phenomenon is sensitive to the conformational changes in the macromolecules. The proposed approach may prove useful for *in vivo* studies of other biological tissues in order to find the source of the sodium anisotropy and to probe intracellular sodium content upon different physiological conditions.

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REFERENCES

1. L. A. Jelicks and R. K. Gupta, *J. Magn. Reson.* **81,** 586–592 (1989).
2. U. Eliav, H. Shinar, and G. Navon, *J. Magn. Reson.* **98,** 223–229 (1992).
3. H. Shinar, T. Knubovets, U. Eliav, and G. Navon, *Biophys. J.* **64,** 1273–1279 (1993).
4. T. Knubovets, H. Shinar, U. Eliav, and G. Navon, *J. Magn. Reson. B* **110,** 16–25 (1996).
5. H. Shinar, U. Eliav, T. Knubovets, Y. Sharf, and G. Navon, *Q. Magn. Reson. Biol. Med.* **2,** 73–77 (1995).
6. A. D. Sherry, C. R. Malloy, F. M. H. J. Jeffrey, W. P. Cacheris, and C. F. C. C. Geraldes, *J. Magn. Reson.* **76,** 528–533 (1988).
7. R. K. Gupta and P. Gupta, *J. Magn. Reson.* **47,** 344–352 (1982).
8. G. Jaccard, St. Wimperis, and G. Bodenhausen, *J. Chem. Phys.* **85,** 6282–6293 (1986).
9. J. Pekar and J. S. Leigh, *J. Magn. Reson.* **69,** 582–584 (1986).
10. J. Jeener and P. Broekaert, *Phys. Rev.* **157,** 232–240 (1967).
11. R. Kemp-Harper and S. Wimperis, *J. Magn. Reson. B* **102,** 326–331 (1993).
12. J. S. Tauskela and E. A. Shoubridge, *Biochim. Biophys. Acta* **1158,** 155–165 (1993).
13. C.-W. Chung and S. Wimperis, *J. Magn. Reson.* **88,** 440–447 (1990).
14. H. Shinar, U. Eliav, and G. Navon. *Isr. J. Chem.* **32,** 299–304 (1992).
15. J. S. Tauskela, J. M. Dizon, P. J. Cannon, and J. Katz, *J. Magn. Reson. B* **108,** 165–169 (1995).
16. R. Reddy, L. Bolinger, M. Shinnar, E. A. Noyszewski, and J. S. Leigh, *Magn. Reson. Med.* **33,** 134–139 (1995).
17. T. Knubovets, T. Kushnir, Y. Itzchak, U. Eliav, and G. Navon, *in* ''Proceedings, Society of Magnetic Resonance, 4th Annual Meeting'' p. 29 (1996).
18. R. C. Lyon, J. Pekar, C. T. W. Moonen, and A. C. McLaughlin, *Magn. Reson. Med.* **18,** 80–92 (1991).
19. Y. Sharf, Akselrod, and G. Navon, *Magn. Reson. Med.* **37,** 69–75 (1997).