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Communication

Transfer of hyperpolarization from long T_1 storage nuclei to short T_1 neighbors using FLOPSY-8

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ABSTRACT

Nuclei with long T_1s are optimal targets for dynamic nuclear polarization (DNP). Therefore, most of the agents used in metabolic imaging and spectroscopy studies are based on carboxylic acid moieties that lack protons, a strong source of dipolar relaxation. Metabolic flux information encoded into spectra of small molecule metabolites in the form of the ^{13}C isotopomer data cannot be accessed using standard ^{13}C hyperpolarization methods because protonated carbons relax too quickly through T_1 dipolar relaxation. It is shown here that the longitudinal mixing sequence FLOPSY-8 can be used to transfer polarization from a long T_1 storage nucleus to adjacent protonated carbons so that they may be detected with high sensitivity. We demonstrate that FLOPSY-8 allows a direct readout of isotopomer populations in butyrate and glutamate *in vitro*.

1. Introduction

Detection of flux in metabolic pathways using ^{13}C enriched tracers and ^{13}C NMR spectroscopy offers numerous advantages over alternative technologies, in part because the information yield provided by the combination of chemical shift and spin–spin coupling [1]. Hyperpolarization of nuclear spins using dynamic nuclear polarization (DNP) followed by fast dissolution with superheated water was first reported in 2003 [2]. Since the first studies of Golman and colleagues, numerous investigators have taken advantage of the tremendous gain in signal-to-noise to make a variety of kinetics measurements *in vitro*, *ex vivo*, and *in vivo* [3–12]. These advances enable new imaging modalities that contain not only structure but also metabolic information not available by any conventional clinical imaging method. One limitation of the hyperpolarization technique is that the enhanced magnetization decays with the time constant T_1 of the nucleus, making observation of protonated carbons difficult at best. Ultra-fast transfer procedures have made *in vitro* analysis possible, but this method cannot be generalized for *in vivo* work due to the time needed for injection and tissue distribution of the hyperpolarized substrate [4]. Consequently, most metabolic imaging reports to date have used [1-^{13}C]pyruvate and detected only those metabolic products that occur in a single step, conversion of [1-^{13}C]pyruvate to [1-^{13}C]lactate or [1-^{13}C]alanine or release of $^{13}CO_2$ followed by rapid conversion to [^{13}C]bicarbonate when [1-^{13}C]pyruvate passes through the pyruvate dehydrogenase (PDH) reaction. A technique that would allow detection of protonated carbons late after the initial dissolution would substantially expand the range of metabolic pathways that can be probed using hyperpolarized biological substrates. In this report, a method is demonstrated for transferring polarization from a long T_1 storage nucleus to distal ^{13}C-enriched short T_1 carbons, thereby allowing detection of these nuclei with high sensitivity long after their initial polarized states have decayed.

FLOPSY-8 (FLip-flOP SpectroscopY) polarization transfer was first introduced as a broadband homonuclear recoupling method for liquid state NMR in 1990 [13]. FLOPSY is one of the most broadbanded polarization transfer schemes, and recent modifications using adiabatic pulses have further improved its performance [14]. FLOPSY is now part of standard pulse sequence libraries on most NMR spectrometers. A potential limitation for translating FLOPSY to hyperpolarized imaging of molecules containing ^{13}C enrichment at multiple positions in a metabolite is the requirement for multiple mixing cycles to achieve the transfer. Since T_1 and T_2 decay is active during the sequence, it was not known whether polarization transfer could be performed in an acceptably short time period. Here, polarization transfer from a long T_1 site through multiple short T_1 carbons has been evaluated. Preliminary

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results showed success, and suggest a new algorithm for making *in vivo* estimates of anaplerosis using uniformly labeled hyperpolarized precursors of acetyl-CoA.

2. Methods

A standard solution of 5.5 M sodium [U–^{13}C]butyrate in de-ionized (DI) H_2O was prepared by neutralization of [U–^{13}C]butyric acid (Sigma–Aldrich, Isotec) with 10 M NaOH (aq). For DNP, trityl radical (Tris(8-carboxyl-2,2,6,6-tetra[2-(1-hydroxyethyl)]-benzo(1,2-d:4, 5-d′)bis(1,3)dithiole-4-yl)methyl sodium salt) (Oxford Instruments Molecular Biotools Ltd., Oxfordshire, UK) was added to a final concentration of 15 mM. The samples were placed in the 3.35 T Oxford HyperSense (Oxford Instruments Molecular Biotools Ltd., Oxfordshire, UK) DNP system and polarized for ~1.25 h. The electron irradiation frequency was set to the positive lobe of the DNP enhancement curve (94.072 GHz). The sample was held at 1.4 K during the polarization. Samples were dissolved by injecting 4 mL of boiling DI H_2O. Three different protocols were used to assess the effect of delays in delivering the sample from the HyperSense to the detection magnet. For absolute enhancement measurements (protocol 1), the entire volume was transferred in 8 s directly to a 10 mm NMR probe positioned in a VNMRS 400 MHz (89 mm bore Oxford magnet) spectrometer (Agilent Instruments, Santa Clara, CA). To examine the effects of collecting the sample in the stray field of the magnet prior to injection (protocol 2), the sample was dissolved and deposited in a beaker near the bore of the spectrometer, where 0.8 mL was transferred to a 5 mm outer diameter NMR tube, then placed in the spectrometer immediately. To allow for the near total destruction of the polarization of the protonated carbons (protocol 3), the sample was allowed to stand for an additional 15 s at high field (center of the magnet) prior to the first detection pulse.

After transfer, a 30° excitation pulse was applied to the ^{13}C channel for detection without proton decoupling to eliminate possible confounding enhancements from NOE effects as compared to DNP. Two seconds after the first detection pulse, the FLOPSY-8 sequence was initiated for a variable number of cycles, followed by detection with another 30° ^{13}C excitation pulse. The RF field strength during the FLOPSY mixing was 13.8 kHz.

A standard solution of 100 mM sodium [U–^{13}C]glutamate in (1:1) DI H_2O:glycerol was prepared by neutralizing [U–^{13}C]glutamic acid (Cambridge Isotope Laboratories) with concentrated NaOH (aq). An equal volume of glycerol was added to the aqueous sodium [U–^{13}C]glutamate solution to attain the final standard solution. For DNP, trityl radical was added to a final concentration of 15 mM. The samples were placed in the 3.35 T Oxford HyperSense DNP system and polarized for ~6 h. The electron irradiation frequency was set to the positive lobe of the DNP enhancement curve (94.072 GHz). The sample was held at 1.4 K during the polarization. Protocol 3 was followed after samples were dissolved by injecting 4 mL of boiling DI H_2O. FLOPSY data was acquired similarly to the [U–^{13}C]butyrate samples. All experiments were repeated four times ($n = 4$). Spectra were processed using ACD NMR processor (Toronto, Canada) in phased mode for the enhancements in protocol 1 and 2, and in the magnitude mode for protocol 3. Data are presented as mean ± standard deviation and two-tailed *t*-tests were used to assess differences for Fig. 2. All statistical analyses were performed with 95% confidence level using GraphPad Prism version 5.03 (GraphPad Prism Software, Inc., La Jolla, CA).

3. Results

Absolute enhancements for the [U–^{13}C]butyrate were ~3500 above the thermal polarization when measured with a minimum transfer time of 8 s (protocol 1). For experiments where the sample was loaded into a 5 mm NMR tube inside the fringe field of the magnet and then placed in the magnet, enhancements were ~30% lower (Table 1) for the carboxyl carbon and even lower for the protonated carbons. Adding an additional ~15 s delay (protocol 3) resulted in an enhancement of only ~600 for the carboxyl carbon. It was at this time point that the FLOPSY-8 sequence was initiated. The effect of FLOPSY-8 on the ^{13}C NMR spectrum of hyperpolarized [U–^{13}C]butyrate is illustrated in Fig. 1. The bottom spectrum shows the protonated carbons of butyrate after the first 30° pulse. The $^1J_{CC}$ for C1 to C2 is 51 Hz, while the $^1J_{CC}$ between the C2 and C3 and C3 and C4 is degenerate, being ~35 Hz in both cases. Therefore, the apparent triplet of triplets for the C3 resonance is due to a $^1J_{CH}$ coupling as well as coupling to its two carbon neighbors, resulting in overlapping resonances associated with the central peaks of the doublet of doublets arising from the uniform ^{13}C labeling. In the top spectrum (Fig. 1), polarization was transferred from the C1 to the C2, C3, and C4 carbons in a total mixing time of ~22 ms (13 FLOPSY-8 cycles). FLOPSY-8 is a longitudinal mixing sequence; therefore an excitation pulse is still required after the mixing to excite transverse magnetization. Since butyrate has only a single long T_1 nucleus, this result demonstrates that this experimental protocol can transfer polarization along a chain of at least three protonated ^{13}C-enriched carbons. The choice of mixing time for optimal polarization transfer was explored empirically (Fig. 2). Mohebbi et al. showed that for the two spins of [U–^{13}C]acetate at 75 MHz detection frequency, the optimal mixing time consists of a total of 10 cycles of FLOPSY-8 evolution. For our case (100 MHz ^{13}C frequency) longer mixing times (13 cycles) were needed for optimal transfer. Fig. 2 plots both the absolute enhancement (top) of the protonated carbons as compared to the thermal NMR signal versus the total number of cycles as well as the relative enhancements (bottom) as measured compared to the initial 30° pulse on the hyperpolarized sample. The absolute and relative enhancements were significantly different for the C2 and C4 carbons at each choice of number of mixing cycles. The C3 was significantly lower for 4 and 7 cycles, but an evolution period of 10, 13, or 16 cycles did not produce a significant difference in C3 enhancement. Table 1 includes both the T_1s of the butyrate carbons as well as the absolute enhancements of each prior to the beginning of the mixing sequence. As can be seen, FLOPSY-8 restores the absolute enhancement of the C2 and C4 carbons to a level that is about half that of the carbonyl carbon. The measured C3 carbon enhancement is lower, primarily due to the anomalous phase of the central peak associated with the overlapping doublet of doublets. This appears to be a phenomenon associated with the hyperpolarization of the signals, as FLOPSY-8 experiments using a thermally polarized sample of [U–^{13}C]butyrate did not show the out of phase character typically seen here (see Supplementary material).

[U–^{13}C]glutamate differs from butyrate in that it has two long T_1 carboxylic acid groups at the C1 and C5 positions of the molecule, both with T_1s ~ 14 s [15]. The spectrum following the initial 30° excitation shows both the C1 and C5 carbons as well as the glycerol from the glassing matrix (Fig. 3). Residual magnetization from the protonated carbons of glutamate was not observed using this relatively small flip angle. Thirteen cycles of FLOPSY-8 mixing results in the spectrum shown in the top of Fig. 3, which displays some of the anomalous intensities for the C2 carbon at 55 ppm as seen for the C3 carbon of [U–^{13}C]butyrate. In this case $^1J_{CC}{}^{12}$ (54 Hz) and $^1J_{CC}{}^{23}$ (35 Hz) are not degenerate. The relative and absolute enhancements for C2, C3 and C4 were 75, 54, and 76 times the thermal spectrum. Due to the short T_1 of the protonated glutamate carbons, the bottom spectrum of Fig. 3 represents the thermal spectrum of these resonances; these carbons have returned to thermal polarization prior to the FLOPSY-8 mixing.

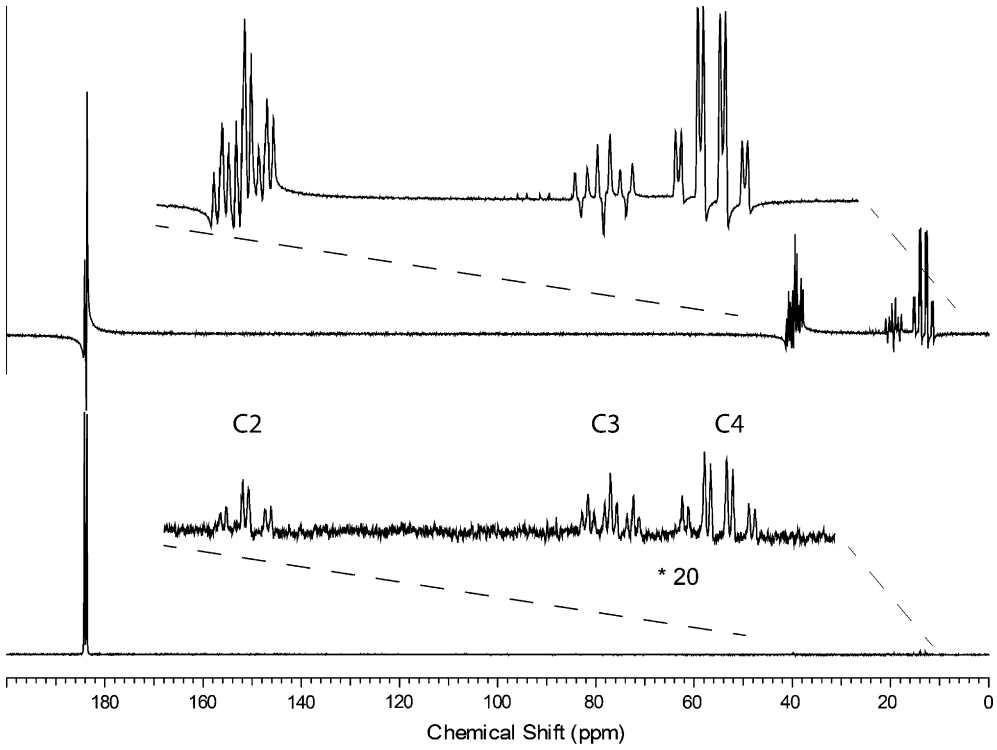

Fig. 1. Enhancement of protonated butyrate carbons following FLOPSY-8 mixing. The ^{13}C NMR spectra of hyperpolarized [U–^{13}C]butyrate after a 30° excitation pulse (bottom) was acquired 2 s prior to the FLOPSY-8 enhanced spectrum (top). The top spectrum shows the impact of 13 cycles of evolution, followed by the same 30° excitation pulse. The absolute phase is not preserved due to the non-zero transverse components of the average Hamiltonian that describes the FLOPSY-8 sequence. In addition, the C3 resonance exhibits a change in phase for the central peaks of the multi-component resonance.

Table 1
Calculated spin–lattice relaxation and pre-FLOPSY enhancement of sodium [U–$^{13}C_4$]butyrate.

T_1 (s)[a]	Pre-FLOPSY enhancement[b]	
	1 s delay	15 s delay
30	1123 ± 194	636 ± 108
6	233 ± 54	14 ± 3
6	230 ± 57	14 ± 3
6	265 ± 76	16 ± 4

[a] Determined using standard inversion-recovery methods at 37 °C.
[b] Determined after a single 30° excitation pulse and comparing the peak area to a thermally acquired peak area with a 90° excitation pulse.

4. Discussion

FLOPSY-8 is effective for transferring polarization from long-T_1 storage nuclei to other J-coupled carbons in both [U–^{13}C]butyrate and [U–^{13}C]glutamate. The presence of two long T_1 carbons in glutamate does not apparently provide any benefit in increasing the polarization of the protonated carbons. However, the condition of having both the C1 and C5 carbons of glutamate in a highly polarized state could not exist *in vivo*. As demonstrated by Tyler et al. hyperpolarized [5-^{13}C]glutamate produced from [2-^{13}C]pyruvate can be detected in the perfused rat heart [7]. This observation indicates that considerable polarization is maintained in at least four downstream metabolites (citrate, isocitrate, α-ketoglutarate and glutamate) as hyperpolarized [2-^{13}C]pyruvate is metabolized in the TCA cycle. Glutamate is produced *in vivo* following the condensation of acetyl-CoA with oxaloacetate, with the two carbons of acetyl-CoA becoming the C4 and C5 carbons of glutamate. The C1 position of glutamate can only be labeled after multiple turns of the TCA cycle, so it is highly probable that all hyperpolarization of the spins would have decayed back to thermal equilibrium by the time the enrichment could arrive in C1. If FLOPSY-8 were to be used for *in vivo* or *in vitro* measurements of Kreb's cycle kinetics, the transfer would therefore come from the C5 position alone. For this reason, we chose to use the [U–^{13}C]butyrate as a mimic of *in vivo* production of glutamate.

Optimization of the mixing time for butyrate empirically produced a maximum at 13 cycles of mixing; this differs with the original FLOPSY-8 papers which restricted the mixing to a two-spin model system [16]. However, FLOPSY-8 mixing has a marked dependence on the shift between the resonances observed, as evidenced in Refs. [12,14]. As confirmation, the same experiment shown here was duplicated for [U–^{13}C]acetate at 4.7 T. With half the B_0 field strength, only 7 cycles of FLOPSY-8 mixing resulted in maximum transfer between the coupled spins (data not shown). Increasing efficiency of mixing at lower frequencies means that carrying out this experiment *in vivo* at 4.7 T, 3 T, or even 1.5 T should become markedly easier to accomplish, requiring progressively less B_1 amplitude. For the experiments shown here at 100 MHz ^{13}C frequency, other TOCSY type mixing sequences such as DIPSI-2 [17] and MLEV-16 [18] performed much worse than FLOPSY-8 in producing polarization transfer. The adiabatic version of the sequence [14] was not attempted for these experiments as currently it is not easily generated in the Agilent software. It is predicted that moving to lower detection frequencies would allow a variety of mixing sequences to become effective in transferring the polarization. Sequential transfer schemes using shaped pulses would also be a viable option provided that the T_2 of the detected carbons was not a limiting factor.

For [U–^{13}C]butyrate, an absolute enhancement of ~300 was observed for the C2 carbon adjacent to the long T_1 C1 carbon, which itself began with a polarization of ~600 prior to the mixing period (Table 1). FLOPSY-8 can produce higher polarization transfer values

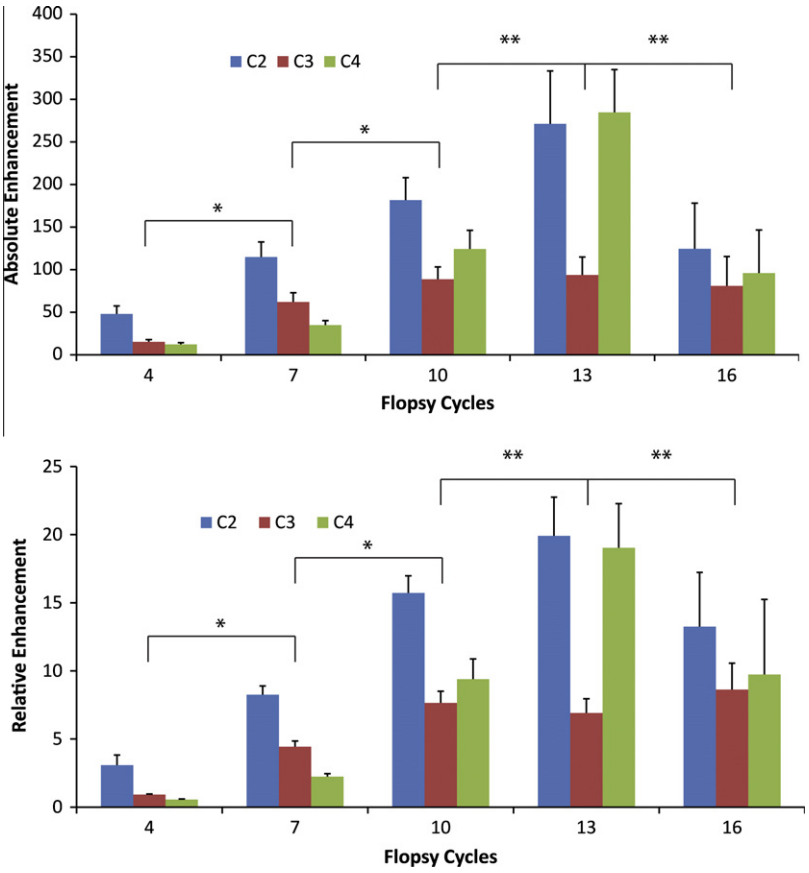

Fig. 2. Absolute (top) and relative (bottom) enhancements of C2, C3, and C4 of [U–^{13}C]butyrate as a function of FLOPSY-8 cycles. A total of $n = 4$ enhancements were used to produce the error bars. $^{*}P < 0.0001$; $^{**}P < 0.05$ (except for C3 values).

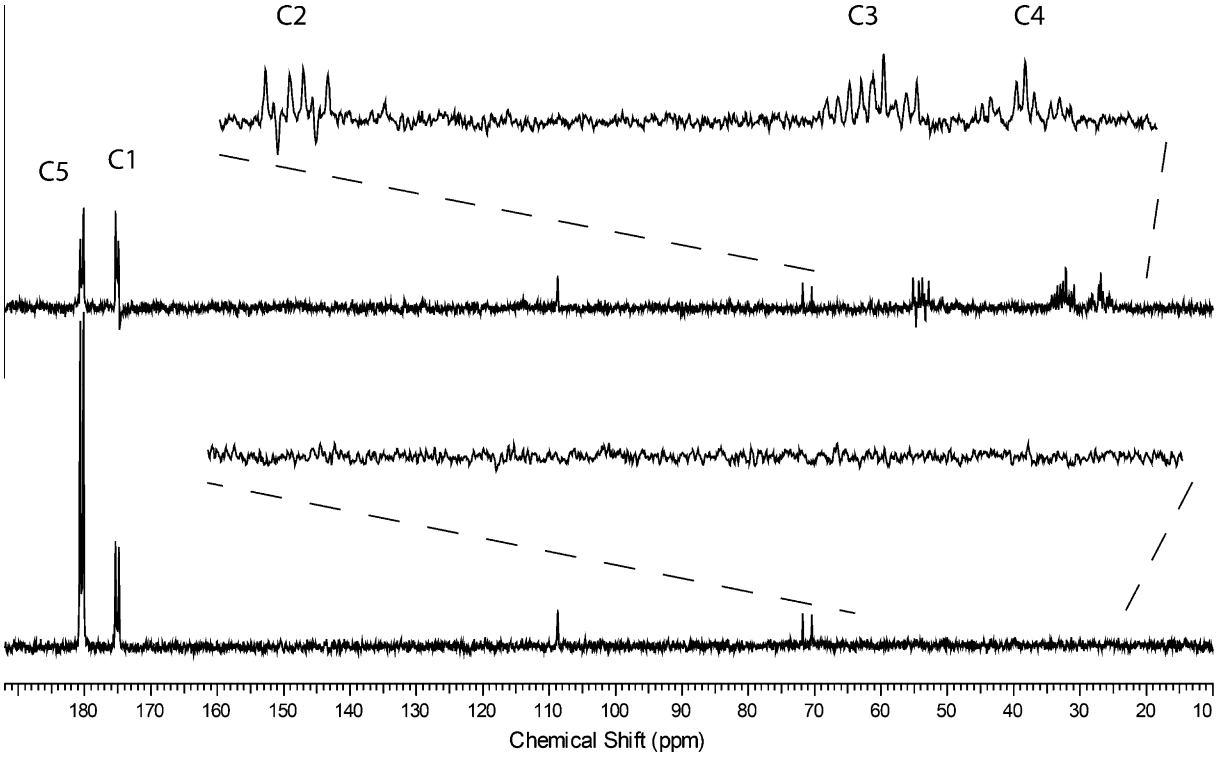

Fig. 3. ^{13}C NMR spectrum of hyperpolarized [U–^{13}C]glutamate. The bottom panel shows the signal after an initial 30° excitation pulse. The top panel shows the spectrum after a 13 cycle FLOPSY-8 mixing period prior to a 30° excitation pulse, 2 s after the bottom spectrum was collected.

for isolated two-spin systems, however, this is not the case for samples which are uniformly labeled in ^{13}C. For [U–^{13}C]butyrate, the transfer achieved here appears in line with that which could be achieved for a thermally polarized sample (see Supplementary material). New experiments that include proton decoupling will explore the effects of the proton J-coupling and NOE upon the absolute enhancements. It is not clear why the polarization transfer drops so precipitously at 13 cycles of mixing (Fig. 2). While an answer to this might be of substantial theoretical interest, the results of mixing for 13 cycles were sufficient to allow us to develop the ideas outlined below.

The polarization transfer results suggest a method for making a fundamentally new *in vivo* measurement of metabolic function using uniformly labeled pyruvate or acetate. In the case where the glutamate in a tissue was pre-labeled using an infusion of ^{13}C labeled substrates prior to the HP injection, isotopomers would be formed by the condensation of the [1,2-^{13}C]acetyl-CoA with ^{13}C labeled oxaloacetate. FLOPSY-8 transfer from the C5 to C3 position, via the ^{13}C labeled C4 position, would provide a direct readout of C3 enrichment, and the J-coupled multiplets there could be used to measure anaplerotic flux into the Krebs cycle via known methods [19]. While the enhancements measured here are an order of magnitude lower than that typically achieved with hyperpolarized [1-^{13}C]pyruvate, that is not necessarily an obstacle to performing the experiment suggested above in cell culture or *in vivo*. The measurement of anaplerosis depends upon relative areas of the peaks in the multiplets, not upon measuring a time course of magnetization evolution; the measurement only needs a single spectrum. With only this precondition, a single 90° pulse that consumes all of the magnetization (and therefore provides maximum sensitivity) would be sufficient for the measurement. In addition, the enhancements achieved here for butyrate are much less than that achievable with pyruvic acid or acetate. Future experiments implementing this scheme with either of these precursors of acetyl-CoA would likely start with polarizations three or four times as high as those shown here for butyrate. With the proper modeling of the polarization transfer through the entire spin system using a program like SPINACH [20], the protocol for measuring anaplerosis suggested above does not need enhancements greater than those shown here.

In conclusion, a method for transferring polarization from hyperpolarized, long T_1 nuclei to J-coupled neighbors has been demonstrated. This method should be amenable to making fundamentally new measurements of metabolism *in vivo*, namely anaplerosis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmr.2011.09.012.

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