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^{13}C Isotopomer Analysis of Glutamate by *J*-Resolved Heteronuclear Single Quantum Coherence Spectroscopy

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^{13}C NMR isotopomer analysis is a powerful method for measuring metabolic fluxes through pathways intersecting in the tricarboxylic acid cycle. However, the inherent insensitivity of ^{13}C NMR spectroscopy makes application of isotopomer analysis to small tissue samples (mouse tissue, human biopsies, or cells grown in tissue culture) problematic. ^{1}H NMR is intrinsically more sensitive than ^{13}C NMR and can potentially supply the same information via indirect detection of ^{13}C providing that isotopomer information can be preserved. We report here the use of *J*-resolved HSQC (J-HSQC) for ^{13}C isotopomer analysis of tissue samples. We show that J-HSQC reports isotopomer multiplet patterns identical to those reported by direct ^{13}C detection but with improved sensitivity. © 2001 Academic Press

Key Words: **indirect detection; ^{1}H NMR; ^{13}C isotopomer analysis; 2D NMR; HSQC.**

^{13}C isotopomer analysis has become an important technique for exploring metabolic pathways that intersect in the tricarboxylic acid (TCA)[2] cycle (1–5). When ^{13}C-labeled substrates are oxidized by tissue they contribute enriched carbons to all intermediates of the TCA cycle and to all metabolites in exchange with cycle intermediates. The ^{13}C isotopomer distribution in a metabolite such as glutamate collected from tissue at isotopic and metabolic steady-state contains quantitative information about substrates contributing to the acetyl-CoA pool as well as relative flux information about substrates that pass through all cycle intermediate pools via anaplerotic pathways. ^{1}H decoupled ^{13}C ($\{^{1}H\}^{13}C$) NMR is commonly used to decode this metabolic information by analysis of multiplets arising from ^{13}C–^{13}C spin–spin coupling (1–6). While powerful, isotopomer analysis requires that $\{^{1}H\}^{13}C$ NMR spectra have sufficient signal-to-noise so that multiplet peak areas can be accurately quantified. Thus, in situations where tissue sample size may be limiting such as organs or fluids from a single mouse, cells grown in tissue culture (7), or human fluids (8), analysis of metabolic fluxes using isotopomer analysis could become limited by the inherent insensitivity of $\{^{1}H\}^{13}C$.

Indirect detection of ^{13}C offers a substantial gain in sensitivity over $\{^{1}H\}^{13}C$ (theoretically about 32-fold (9)). We recently applied the HMQC-TOCSY experiment to derive ^{13}C isotopomer information from rat tissue extracts and found a 5- to 6-fold increase in sensitivity over $\{^{1}H\}^{13}C$ (10). This 2D technique reports isotopomer information via ^{1}H–^{13}C spin–spin couplings rather than ^{13}C–^{13}C couplings and therefore reports different groups of ^{13}C isotopomers compared to the $\{^{1}H\}^{13}C$ experiment. Heteronuclear single-quantum coherence (HSQC) spectroscopy is an alternative for the indirect detection of ^{1}H–^{13}C correlated resonances (11). Highly resolved HSQC spectra of ^{13}C-enriched extracts display cross-peaks with splitting patterns in the ^{13}C dimension due to ^{13}C–^{13}C scalar couplings (12–16) that are identical to the multiplets in a ^{13}C NMR spectrum. This would be an advantage over HMQC-TOCSY because these data could be directly analyzed by using previously published isotopomer models. One disadvantage of the highly resolved HSQC method, however, is the

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[2] Abbreviations used: TCA, tricarboxylic acid; HSQC, heteronuclear single-quantum coherence; J-HSQC, *J*-resolved HSQC; RF, radiofrequency; HMBC, heteronuclear multiple bond correlation.

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large number of t1 increments required to provide adequate resolution of all glutamate C2, C3 and C4 multiplets simultaneously. In this work, we have used *J*-resolved HSQC spectroscopy (J-HSQC) (17) to obtain ^{13}C isotopomer data from extracts of mouse hearts, from extracts of β-cells grown in tissue culture, and from glucose isolated from human plasma. In each case, the amount of sample available and the level of ^{13}C enrichment tested the limits of $\{^1H\}^{13}C$, yet J-HSQC spectra adequate for isotopomer analysis were obtained. The J-HSQC method requires fewer t1 increments to resolve the multiplet information and this results in a substantial savings in time required to collect the data. We show that the J-HSQC spectra provide information identical to that of $\{^1H\}^{13}C$ NMR for a wide range of isotopomer mixtures.

MATERIALS AND METHODS

Materials

[2-^{13}C]Acetate and [1,2-$^{13}C_2$] acetate (all 99%) were purchased from Cambridge Isotopes (Andover, MA). Uniformly labeled (99%) long-chain fatty acids (predominantly palmitate, palmitoleic, oleic, linoeic, and linolenic acid) were obtained from Isotec (Miamisburg, OH). Other common materials were obtained from Sigma (St. Louis, MO). Perfusions were conducted with wild-type mice weighing 17–20 g.

Heart Perfusions

Hearts were rapidly excised from wild-type mice and perfused using conventional Langendorff methods (retrograde) at 100 cm H_2O with Krebs-Henseleit bicarbonate buffer (KH) bubbled continuously with a 95/5 mixture of O_2/CO_2 at 37°C. The buffer (250-mL recirculating volume) contained 3 mM unenriched glucose and 0.5 mM unenriched acetate. Four different substrate enrichment schemes were used. Either (1) 4 mM [2-^{13}C]acetate + 1 mM [1,2-$^{13}C_2$]acetate, (2) 3 mM [2-^{13}C]acetate + 2 mM [1,2-$^{13}C_2$]acetate, (3) 2 mM [2-^{13}C]acetate + 3 mM [1,2-$^{13}C_2$]acetate, or (4) 1 mM [2-^{13}C]acetate + 4 mM [1,2-$^{13}C_2$]acetate was added to the perfusate to create a wide variety of ^{13}C multiplet patterns. The heart rate was monitored using an open-ended cannula placed across the mitral valve in the left ventricle and attached to a pressure transducer. After 50 min, hearts were freeze-clamped with aluminum tongs precooled in liquid nitrogen.

Tissue Measurements

The freeze-clamped tissue was stored in liquid nitrogen until used. The frozen tissue was ground to a powder and extracted with 1 ml of 3.6% ice-cold perchloric acid. The extract was neutralized (pH ~2.6 for ^1H and 7.0 for ^{13}C analysis) with KOH, freeze-dried, dissolved in 150 μl of D_2O to collect the J-HSQC spectra (3-mm indirect detection probe), and then diluted to 500 μl with D_2O to acquire the ^{13}C spectra (5-mm broadband probe).

NMR Spectroscopy

A J-HSQC pulse sequence was created as described in Davison *et al.* (17) with some modification. A standard gradient selected HSQC sequence was modified by removing the ^1H refocusing pulse in the t1 evolution period and replacing it with a shaped pulse on the carbon channel. All J-HSQC spectra were collected using a 3-mm inverse probe (Nalorac, Inc. Martinez, CA) on a 14.1 T Varian INOVA spectrometer (Varian Instruments, Palo Alto, CA). Spectra were acquired with 2048 points in F2 (proton dimension), covering a spectral width of 3600 Hz, and 64 increments in F1 (carbon dimension), covering a spectral width of 140 Hz. The 2048 * 64 matrices were zero-filled to 4096 * 1024 and multiplied by gaussian functions in both dimensions before Fourier transformation. The first 3 points of the t1 FID(s) were discarded and recalculated by backward linear prediction to obtain a flatter baseline in both dimensions.

Proton pulse widths were determined by using the 180° null method for a signal on resonance. ^{13}C pulse widths in the inverse configuration were determined by using the null of the ^{13}C satellites of an internal ^{13}C-enriched acetate standard (18). GARP ^{13}C decoupling was on during acquisition using a field strength of 12 kHz, offset at about 40 ppm. A total of 256 steady state pulses were acquired to ensure temperature equilibration. Phase selection was accomplished by an initial *z* gradient of 37.54 G/cm for 2 ms and a second *z* gradient of −37.69 G/cm for 0.5 ms. The evolution times were set to a single bond $^1J_{CH}$ of 135 Hz (the average of $^1J_{C2H2} = 145$ Hz, $^1J_{C3H3} = 130$ Hz, and $^1J_{C4H4} = 127$ Hz). These small differences between the experimental versus true coupling constants resulted in slight distortion of the C2H2/C3H3 resonance area ratio but not the C4H4/C3H3 resonance area ratio. More importantly, it did not affect the relative areas of the multiplets contributing to a single resonance.

REBURP (19)-shaped refocusing pulses (applied during t1) were customized for each sample using the pulse shaping routine (Pandora's Box) supplied with the Varian software (VNMR 6.1b). The carbonyl (210–150 ppm) and aliphatic (70–10 ppm) regions were irradiated simultaneously. Bloch simulations indicated that the frequency separation between these simultaneous soft pulses was adequate to avoid distortions in the excitation profile. Since imperfect shaped refocusing pulses could alter the multiplet intensities (see Fig. 1), each sample was spiked with a 5 μl 84 mM 2:1 mixture of [1,2-^{13}C]acetate (56 mM) and [2-^{13}C]acetate

(28 mM) as an internal pulse-calibration standard to ensure accurate calibration of the 180° shaped pulses. J-HSQC multiplets were measured with the VNMR integration routine after projecting the 2D-multiplet region into a 1D spectrum.

After J-HSQC spectra were acquired on a sample volume of 150 μl in the 3-mm inverse probe, the samples were removed, diluted to 500 μl, and placed in a 5-mm NMR tube. $\{^1H\}^{13}C$ NMR spectra were acquired on a 5-mm broadband probe using a 45° pulse and a 3-s repetition time. A WALTZ-16 sequence was used for broadband proton decoupling. The multiplet areas in the $\{^1H\}^{13}C$ NMR spectra were determined by using the line-fitting algorithm in the PC-based NMR software, NUTS (Acorn NMR, Freemont, CA).

Standard Curves

Samples containing known but variable amounts of natural abundance and [U-$^{13}C_5$]glutamate were used to demonstrate the response linearity for low enrichment samples. Stock solutions containing [U-$^{13}C_5$]glutamate and natural abundance glutamate were prepared in 99.9% D_2O. Aliquots from these standards were combined to give samples between 0.24 and 8% excess enrichment (total glutamate was 150 mM).

RESULTS

Calibrations and Comparison of $\{^1H\}^{13}C$ versus J-HSQC Spectra

J-HSQC spectroscopy was recently used to analyze multiply ^{13}C-labeled biomolecules (17). The J-HSQC experiment is similar to the common HSQC sequence except that a π pulse is implemented on the ^{13}C channel during the evolution time. This results in refocusing of all ^{13}C chemical shifts, leaving only ^{13}C–^{13}C coupling information in the indirect dimension. However, to provide quantitative multiplet areas in a molecule like glutamate that has a large chemical shift difference among the C1 and C5 (carbonyl) and C2, C3, and C4 (aliphatic) carbons, all carbons must be inverted equally by the π pulse. This was accomplished by using a shaped rather than a hard refocusing pulse.

The time domain portion of the REBURP refocusing pulses are calculated by the Varian software based on the calibration of the ^{13}C radiofrequency (RF) field (90° pulse width at a given RF power), and the frequency domain information (offsets and band widths of the carbonyl and aliphatic regions). If the RF field is properly calibrated, the resulting refocusing pulse will be applied equally to the carbonyl and aliphatic regions. However, if improperly calibrated, the aliphatic and carbonyl spins would experience the refocusing pulse to different extents. This would result in proper refocusing of C3–C4 (aliphatic–aliphatic) coupled spins, for example, improper refocusing of the C4–C5 (aliphatic–carbonyl) coupled spins, and C4 multiplets that do not correctly report accurate isotopomer populations. To avoid this, we added an internal calibration standard consisting of a 1:2 mixture [2-^{13}C]:[1,2-$^{13}C_2$]acetate to each experimental sample. The resulting acetate C2 (methyl carbon) multiplet is a doublet (from [1,2-$^{13}C_2$]acetate) and a singlet (from [2-^{13}C]acetate) of 1:1:1 relative intensities. Since this multiplet also arises from the coupling between aliphatic and carbonyl carbons it is used to verify that the REBURP refocusing pulse has been applied correctly. Figure 1 illustrates how imperfect refocusing pulses (implemented by varying pulse power) affect the multiplet intensities of the [2-^{13}C]/[1,2-^{13}C]acetate internal standard. Based on the accurate calibration of the ^{13}C RF field, the Varian pulse-shaping software suggested a power of 59 dB for the REBURP pulse. When that power was used, the experiment resulted in the expected acetate C2 1:1:1 multiplet. However, when improper RF powers were used to simulate poor calibration of the ^{13}C RF field, erroneous multiplet ratios were observed. Thus, the internal acetate standard is used to monitor problems with the pulse sequence and ensure accurate measurement of the glutamate multiplets.

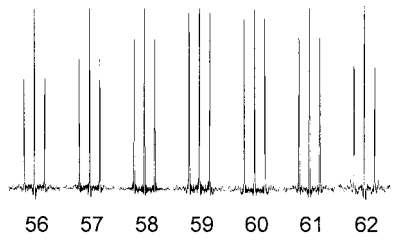

FIG. 1. The effect of imperfect refocusing pulses on the multiplet intensities of the 2:1 [1,2-$^{13}C_2$]:[2-^{13}C]acetate standard. The appropriate RF power was calculated to be 59 dB by the pulse-shaping software.

Figure 2b shows a typical J-HSQC spectrum of a mouse heart extract compared to a $\{^1H\}^{13}C$ spectrum of the same sample in Fig. 2a. In this metabolic situation, the signals of glutamate dominate both the $\{^1H\}^{13}C$ and the J-HSQC spectra. As illustrated, the projections of H2, H3, and H4 in the J-HSQC spectrum exactly match the multiplet patterns observed in the $\{^1H\}^{13}C$ spectrum. No direct information about C1 and C5 isotopomers is reported in the J-HSQC spectrum. The loss of information about unprotonated carbons is inherent in most indirect detection experiments since it is the protons that are detected. In principle, indirect detection in the form of a heteronuclear multiple bond correlation (HMBC) experiment could be utilized to get information from the carbonyl carbons, but in practice the experiment suffers from low sensitivity due to long mixing times and confounding correlations as a result of ^{13}C–^{13}C couplings in highly enriched samples (un-

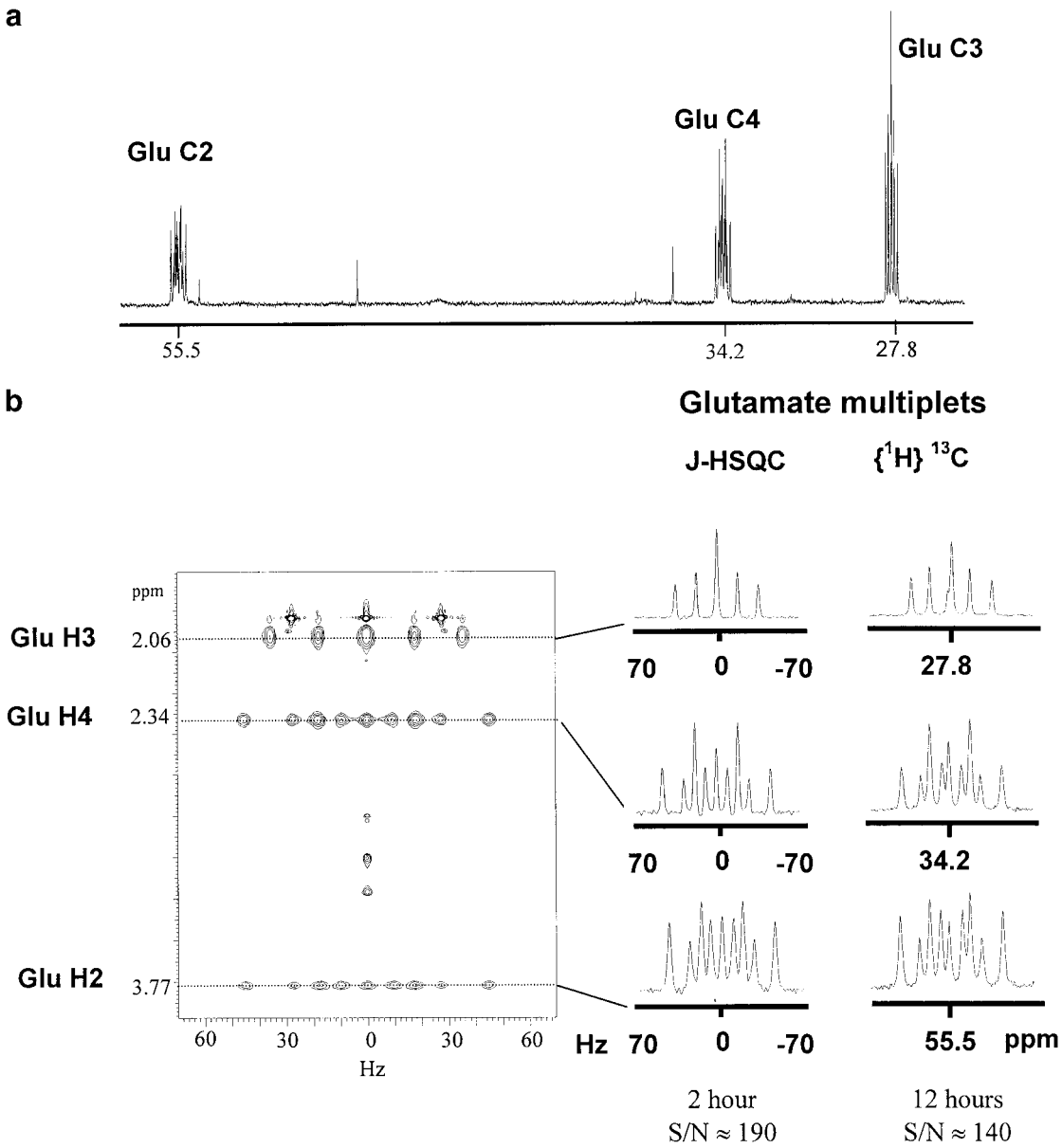

FIG. 2. (a) $\{^1H\}^{13}C$ NMR spectrum and (b) J-HSQC spectrum collected at 14.1 T of an extract of a mouse heart perfused to steady state with a mixture of [2-^{13}C]acetate and [1,2-$^{13}C_2$]acetate. The projection of the J-HSQC and the $\{^1H\}^{13}C$ spectrum give the same multiplet data. Note the loss of isotopic shift in the J-HSQC spectra.

published observations). Unlike the HMQC-TOCSY experiment, the J-HSQC experiment does provide some information on carbonyl enrichment (C1 and C5) as a result of 1-bond *J* coupling to the nearest neighbor C2 or C4 resonances (H2 and H4 in the J-HSQC spectrum).

To demonstrate that this technique is reliable over a range of isotopomer conditions, mouse hearts were perfused with varying concentrations of [2-$^{13}C_1$]acetate and [1,2-$^{13}C_2$]acetate. The range selected covers the wide variety of conditions that may be encountered in a typical tracer experiment involving multiply enriched substrates. Figure 3a shows that the correlation between *J*-resolved HSQC and $\{^1H\}^{13}C$ is excellent for a range of multiplet contributions. The slope of the correlation shown in Fig. 3b for the C4 quartet contribution is 0.99 ± 0.05. The other C4 multiplets and all C2 and C3 multiplets displayed equally good correlations (data not shown: R = 0.96–0.99, slope = 0.94–1.08).

^{13}C-enrichment levels in a typical human tracer experiment can be as low as 0.5% above natural abundance (8). In this situation, the singlet due to natural

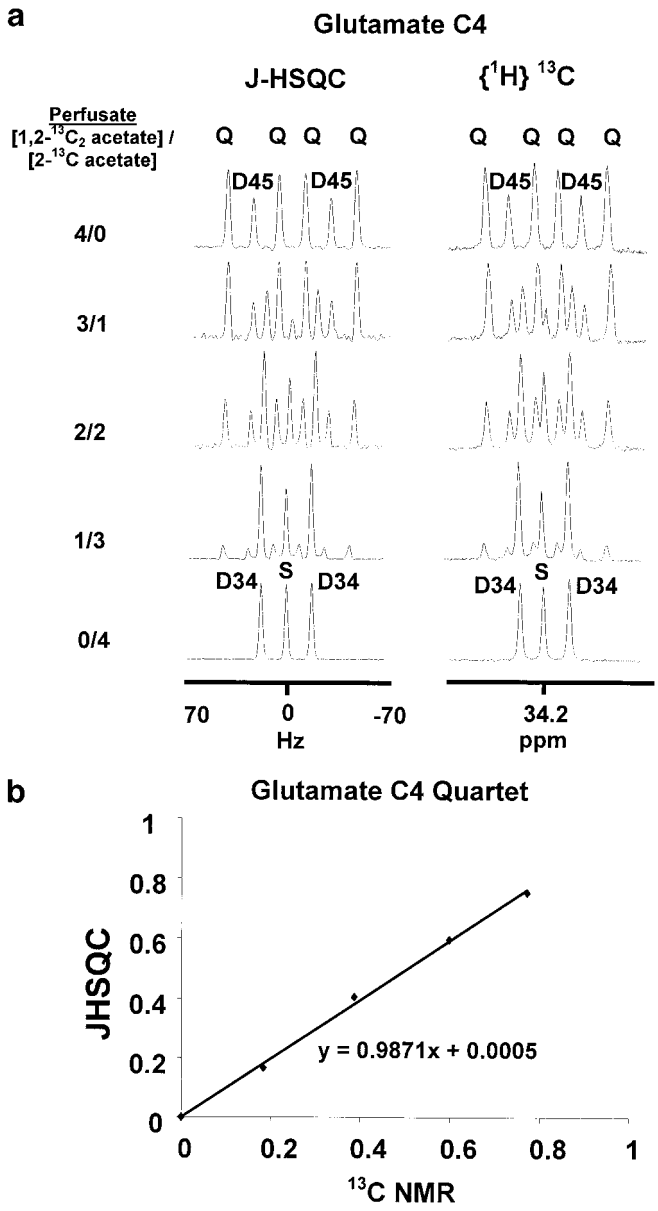

FIG. 3. (a) J-HSQC projections and $\{^1H\}^{13}C$ NMR spectra collected at 14.1 T of extracts of mouse hearts perfused to steady state with different ratios of [2-^{13}C]acetate and [1,2-$^{13}C_2$]acetate. Concentrations of [2-^{13}C]acetate and [1,2-$^{13}C_2$]acetate are shown in mM to the side. (b) J-HSQC vs $\{^1H\}^{13}C$ NMR. Correlation of the glutamate C4 multiplet distribution by J-HSQC and $\{^1H\}^{13}C$ NMR gives a slope of 0.99.

abundance can dominate the spectrum of a metabolite compared to the multiplets arising from metabolism of a ^{13}C-enriched tracer. Figures 4a and 4b illustrate a series of J-HSQC spectra collected on samples of glutamate having variable excess ^{13}C enrichment. These data demonstrate that the area of the quartet can be accurately measured in the presence of a much larger singlet even in a sample containing only 0.24% excess [U-$^{13}C_5$]glutamate. Although spectra shown were collected on samples containing 150 mM total glutamate for ease of data collection, this same experiment could have been performed at a much lower total glutamate concentrations and longer NMR scan times.

Another parameter of interest in isotopomer analysis is the ratio of ^{13}C enrichment in the various carbons of a metabolite. For example, some simple relationships have been derived (20) which allow the measurement of the fractional contribution of a ^{13}C-enriched substrate to acetyl-CoA under non-steady-state conditions. This technique is especially useful *in vivo* where it is often difficult to prove that a system is at isotopic steady state. This method requires a measure of the fractional enrichment of glutamate C4 relative to C3, or simply the C4/C3 intensity ratio in a ^{13}C spectrum (after small corrections for differences in nOe and T_1

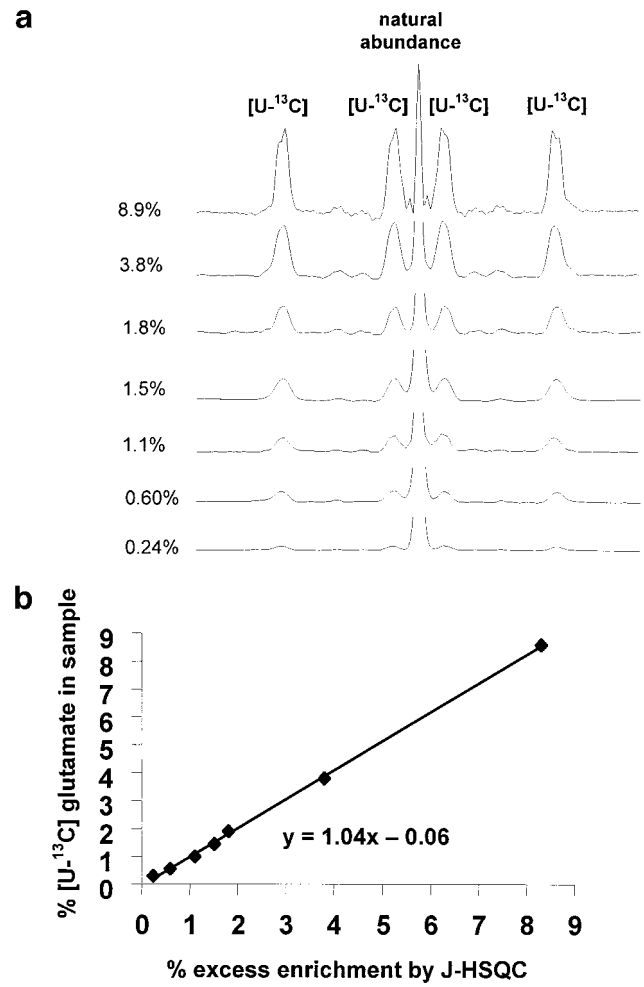

FIG. 4. (a) J-HSQC spectra of glutamate containing various amounts of added [U-^{13}C]glutamate. Total glutamate was 150 mM. (b) Plot of known chemical excess enrichment of [U-$^{13}C_5$]glutamate as measured analytically versus excess enrichment as measured by J-HSQC. The latter values were obtained by measurement of quartet versus singlet resonance areas in each spectrum.

values). Proton spectroscopy has the advantage of shorter T_1 relaxation times so individual intensities are less dependent on postacquisition delays. We have found that the measured C4/C3 ratio (as detected by the ratio of H4/H3 multiplet areas) was identical in J-HSQC spectra of [U-$^{13}C_5$]glutamate using no postacquisition delay (1.05 ± 0.08) versus a 2-s delay (1.01 ± 0.05). It should be noted that resonances with substantially different $^1J_{CH}$ couplings may need correction to obtain accurate resonance area ratios. This is because incorrect evolution times ($1/2J$) in the pulse sequence cause a decrease in sensitivity for those correlations. Fortunately, the $^1J_{CH}$ for glutamate C3 and C4 are 127 and 130 Hz, respectively, so the correction here is inconsequential.

Applications of J-HSQC to Tissue Samples Having Limited ^{13}C Signal

{^{1}H}^{13}C NMR methods have been used to probe intermediary metabolism in extracts of cultured cells (21–23). While some studies have utilized ^{13}C–^{13}C coupling information (22, 23), to our knowledge ^{13}C isotopomer analysis has not been used to investigate oxidation of physiological substrates in cultured cells. We have recently found that glutamate becomes highly enriched with ^{13}C in INS-1 β-cells incubated with [U-$^{13}C_6$]glucose and that ^{13}C spectra with sufficient signal-to-noise can be collected on an extract of $\sim 1.5 \times 10^8$ cells (7). Long-chain fatty acids are not oxidized at a high rate in these cells, so cellular glutamate is not highly enriched when INS-1 β-cells are incubated with [U-^{13}C]long-chain fatty acids. Figure 5a compares {^{1}H}^{13}C versus J-HSQC spectra of the glutamate C4 resonance in an extract of INS-1 β cells after a 2-h incubation with [U-^{13}C]long-chain fatty acids. The determination of the fraction of acetyl-CoA derived from the long-chain fatty acids in this experiment requires a measure of the quartet contribution to the C4 resonance (C4Q). Clearly, this is not possible with the S/N shown in the {^{1}H}^{13}C spectrum but can be performed with ease with the quality of data obtained by J-HSQC spectroscopy. In this experiment, a non-steady-state analysis (20) showed that 17% of acetyl-CoA was derived from the fatty acids. Perhaps equally important is that the J-HSQC data were collected in about half the time needed to acquire the {^{1}H}^{13}C spectrum.

The J-HSQC experiment can also be applied to other metabolites. Glucose isotopomers have been measured from human blood samples to determine fluxes into pathways feeding gluconeogenesis (24). Since abnormalities of hepatic glucose metabolism are common, such measurements may ultimately prove valuable clinically. However, the long turnaround times for {^{1}H}^{13}C make them impractical for routine clinical analysis. The J-HSQC experiment can significantly reduce the experimental requirements from overnight to a couple of hours. Figure 5b compares the {^{1}H}^{13}C and J-HSQC spectra of a derivative of glucose prepared from plasma glucose after oral ingestion of [U-$^{13}C_3$]propionate (25). Again, the quartet component of the glucose C2 resonance in the J-HSQC spectrum was easily analyzed, while those from the {^{1}H}^{13}C spectrum could not be estimated reliably.

DISCUSSION

The *J*-resolved HSQC experiment has advantages and disadvantages compared to HSQC and HMQC-TOCSY for ^{13}C isotopomer analysis. Szyperski and coworkers (12–15) used HSQC for isotopomer analysis by selecting a small region of the ^{13}C dimension and applying a large number of t1 increments to increase resolution. A HSQC experiment that uses a relatively small spectra width, say 10 ppm, can have sensitivity and resolution that may match or slightly exceed that of the {^{1}H}^{13}C experiment. In cases where signal is abundant but the complexity of the sample requires dispersion in both the ^1H and ^{13}C dimensions, HSQC is the experiment of choice. In many cases however, there is sufficient dispersion in the ^1H dimension, and under these conditions, the *J*-resolved HSQC experiment holds a clear advantage. A 10 ppm ^{13}C spectral width requires 750 increments in t1 in order to achieve a resolution of 2 Hz/increment on a 14.1 T instrument. The J-HSQC experiment requires 10-fold fewer increments since the spectral width in the second dimension need only cover the coupling constant range. As a result, either the time required to complete the experiment or the S/N can be optimized. In the experiments presented here, the signals of interest are cleanly resolved and unambiguously assigned in the proton dimension so J-HSQC holds a sensitivity advantage over HSQC. Even under more complicated circumstances such as liver extracts where the glutamate proton signals are obscured by other labeled metabolites, the sensitivity of the J-HSQC can still be taken advantage of after isolation of glutamate (26).

HSQC has been applied to the *in vivo* human brain after ingestion of [1-^{13}C]glucose using a 2 T imaging system (27). However, at such low field, the limited proton dispersion makes J-HSQC unattractive since the glutamate protons would not be cleanly resolved from other metabolites like glutamine and possibly aspartate. At higher imaging fields (i.e., 4.7 to 9.4 T) glutamate H4 is resolved from glutamine H4, but glutamate H2 and H3 would not be fully resolved from glutamine H2 and H3 (28). Although a full isotopomer analysis would not be possible, we have recently shown that TCA cycle flux, tissue oxygen consumption, and rate of exchange of aspartate and glutamate with the TCA cycle can be accurately measured using glutamate

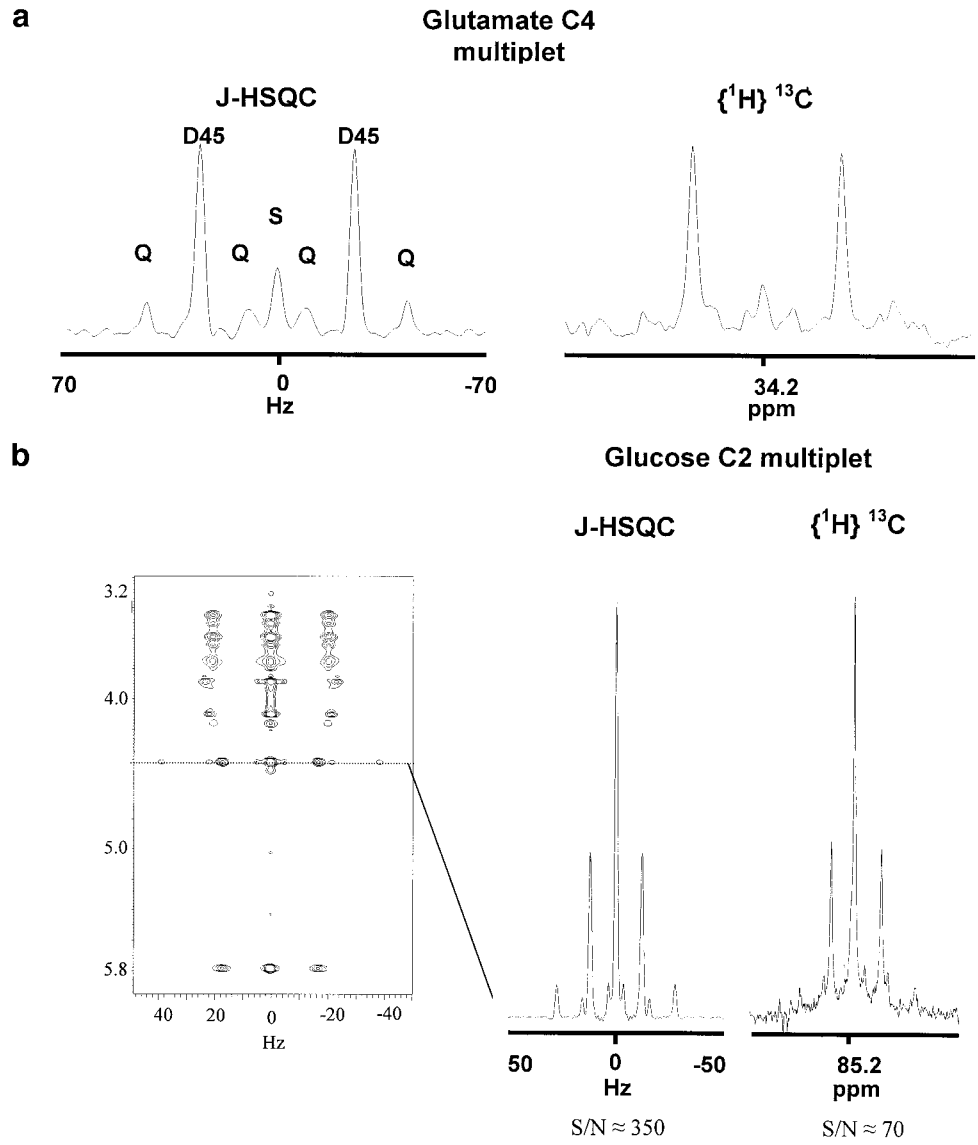

FIG. 5. Examples that demonstrate the advantages of J-HSQC spectroscopy over direct ^{13}C. (a) The glutamate C4 multiplet in an extract of insulinoma β-cells grown in culture then incubated with [U-^{13}C]fatty acids for 4 h. The $\{^1H\}^{13}C$ spectrum was averaged over 17 h while the J-HSQC spectrum was collected in 8 h. (b) J-HSQC and $\{^1H\}^{13}C$ spectra of mono-acetone glucose derived from ~15 ml of human blood plasma. Both spectra were signal-averaged for 12 h.

C4 multiplet data alone (29). Thus, the J-HSQC experiment may be applicable to *in vivo* brain metabolism at the higher imaging fields.

HMQC-TOCSY provides isotopomer information via C–H coupling. This experiment does not require excessive resolution in the ^{13}C dimension, so it offers both added dispersion and significant sensitivity gains. In instances where a full isotopomer analysis is needed but sensitivity and proton dispersion are limited, the HMQC-TOCSY is probably a better choice than the J-HSQC. This may be the case with *in vivo* studies or some perfused organs such as the liver. The primary drawback of the HMQC-TOCSY as a tool for isotopomer analysis is the form in which the information is given. While the information is complementary to ^{13}C multiplet information, treatment of the data is quite different. Models for isotopomer analysis using ^{13}C multiplets have existed for nearly 20 years and are highly recognized and translatable between groups interested in the same metabolic experiments. Since the information and presentation of data from a J-HSQC experiment are identical to a $\{^1H\}^{13}C$ experiment, one can apply existing models to analyze metabolism. Analysis by HMQC-TOCSY also requires extensive calibration of signals before isotopomer populations can be accurately compared to each other (10), but this is

not necessary in the J-HSQC experiment. Additionally, whereas some isotopomer information about the tertiary C1 and C5 carbons can be obtained in the J-HSQC experiment via ^{13}C–^{13}C spin coupling, no such information is available from the HMQC-TOCSY spectrum.

Small isotopic shifts due to neighboring ^{13}C enrichment do complicate $\{^1H\}^{13}C$ spectra of metabolites to some extent, especially in those resonances that are made up of many multiplets. Since chemical shifts are refocused in the *J*-resolved HSQC experiment, all multiplets are symmetrical about their center (see for example Figs. 2, 3, and 5). This is a particular advantage when deconvoluting a 9-line multiplet, commonly seen in isotopomer experiments. While a seemingly obscure advantage, it is actually quite valuable under certain conditions. When two isotopes such as ^{13}C and ^{2}H are incorporated into glutamate or other TCA cycle intermediates, the resulting ^{13}C spectrum can be quite confounding due to the additional coupling and isotope shifts (30). However, since the J-HSQC spectrum does not contain chemical-shift information and ^{13}C decoupling is applied during acquisition, the multiplets are significantly simplified.

The *J*-resolved HSQC experiment has a clear sensitivity advantage over the $\{^1H\}^{13}C$ experiment. Although we have not attempted to explicitly quantify the gain in sensitivity because different probes with different quality factors were used in the two experiments (a 3-mm inverse probe was used in the J-HSQC experiment while a 5-mm broadband probe was used in the $\{^1H\}^{13}C$ experiment), a qualitative comparison has been made. A 3- to 5-fold increase in *S*/*N* was observed in J-HSQC versus ^{13}C spectra collected on the same sample (diluted from 150 μl for the 3-mm probe to 500 μl for the 5-mm probe) using similar pulse repetition rates and data collection times. While not near the theoretical maximum of 32-fold, this value is comparable to the *S*/*N* increase observed elsewhere for indirect detection of metabolites (10, 17, 31). Such an increase in *S*/*N* approximates to a 9- to 25-fold savings in data collection time when the J-HSQC experiment is run compared to the ^{13}C pulse and acquire experiment. Essentially, an overnight ^{13}C scan can be completed in a 1- to 2-h J-HSQC scan. Given this qualitative gain in sensitivity, we are finding that ^{13}C isotopomer methods can be applied in a much richer variety of samples than considered possible previously, i.e., extracts of cells grown in tissue culture or tissue biopsies.

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