In Vivo $^{13}$C NMR Measurements of Hepatocellular Tricarboxylic Acid Cycle Flux*

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A combined isotopic steady state and in vivo isotopic non-steady state analysis was used to calculate tricarboxylic acid cycle flux in livers of anesthetized rats infused with ethanol. In vivo $^{13}$C NMR spectroscopy was used to non-invasively observe label turnover of [4-$^{13}$C]glutamate, [4-$^{13}$C]glutamine, and [2-$^{13}$C]glutamate/glutamine in liver following a bolus intravenous infusion of [2-$^{13}$C]ethanol. The isotopic steady state analysis of [2-$^{13}$C], [3-$^{13}$C], and [4-$^{13}$C]glutamate isotope pomer analysis (Malloy, C. R., Sherry, A. D., and Jeffrey, F. M. H. (1988) J. Biol. Chem. 263, 6964–6971) in liver extracts was used to indirectly calculate the anaplerotic flux (0.90 ± 0.07 × citrate synthase flux) and [2-$^{13}$C]acetetyl-CoA fractional enrichment (51.4 ± 3.4%). The [4-$^{13}$C]glutamate, [4-$^{13}$C]glutamine, and [2-$^{13}$C]glutamate fractional enrichments determined in liver extracts were 23.0 ± 1.1, 17.2 ± 1.5, and 7.7 ± 0.5%, respectively. These data in addition to blood [2-$^{13}$C]acetate and [4-$^{13}$C]glutamate enrichment time course data were used in conjunction with a metabolic steady state mathematical analysis designed to account for liver glutamate and glutamine label dilution as a consequence of glutamine exchange with blood to calculate the tricarboxylic acid (tca) cycle flux ($V_{\text{tca}} = 0.33 ± 0.09 \text{ mol/g wet weight/min}$) in liver.

In summary, It is possible to detect $^{13}$C labeling of glutamate and glutamine in liver via non-invasive $^{13}$C NMR. Additionally, the in vivo $^{13}$C labeling kinetics of glutamate and glutamine in liver and glutamine in blood may be used to calculate the liver tricarboxylic acid cycle flux.

In liver, the tricarboxylic acid cycle is not only coupled to mitochondrial energy production but also provides substrates for gluconeogenesis and other metabolic pathways. Therefore, in order to quantify the tricarboxylic acid cycle flux from the labeling kinetics of glutamate and glutamine in vivo, the influx/efflux of labeled substrates coupled to the tricarboxylic acid cycle must be included in the mathematical analysis. Tricarboxylic acid cycle flux measurements in hepatocyte preparations have previously been made using a variety of substrates (1), and more recently measurements of tricarboxylic acid cycle activity in perfused liver have been made using an isotope pomer steady state analysis (2, 3). Magnusson et al. (4) have measured relative metabolic fluxes including the tricarboxylic acid cycle flux in human liver via a steady state isotope pomer analysis of glutamine which conjugates to phenylacetate in liver and is excreted in the urine. Although these in vivo methods provide relative flux information on a number of metabolic pathways in liver, they lack the ability to calculate absolute fluxes unless the absolute flux of one pathway is known. This is possible in perfused systems but may be difficult in the whole body.

In vivo kinetic measurements of tricarboxylic acid cycle activity have been made non-invasively in brain (5–7) and in perfused heart (8–11) and liver (12) by measuring turnover of $^{13}$C label in glutamate by NMR, and absolute tricarboxylic acid cycle fluxes have been calculated from these measurements in the brain (5, 13) and heart (8–11) using a variety of mathematical models. In kinetic experiments of glutamate isotope labeling in brain, label dilution of the brain glutamate/glutamine pool via exchange with the blood glutamate/glutamine pool is limited by the blood-brain barrier (14). Therefore, the tricarboxylic acid cycle flux has been calculated from the brain [4-$^{13}$C]glutamate/glutamine turnover data using a linear metabolic pathway analysis (5, 13) in which a first approximation the exchange with blood metabolite pools is neglected. However in liver, glutamine is in active exchange with blood causing label dilution of the liver glutamate and glutamine pool (15). Thus in order to calculate the tricarboxylic acid cycle flux in the liver using in vivo NMR measurements of $^{13}$C label turnover in glutamate and glutamine, the mathematical analysis must incorporate labeled liver glutamate exchange with blood. [2-$^{13}$C]Ethanol was used as the labeled precursor in this study, because its metabolism is localized in the liver, and the decreased redox state (NAD$^+$/NADH) associated with ethanol metabolism significantly reduces FFA oxidation in the liver thereby limiting label dilution of the [2-$^{13}$C]acetetyl-CoA pool (Fig. 1).

The present study demonstrates that in vivo $^{13}$C NMR detection of glutamate and glutamine turnover in the liver can be achieved non-invasively and that a quantitative metabolic steady state analysis used to calculate the tricarboxylic acid cycle flux in liver must incorporate glutamine exchange with blood. Additional flux measurements associated with liver such as citrate lyase, $\alpha$-ketoglutarate $\leftrightarrow$ glutamate exchange, glutamine synthetase/glutaminase, and glutamine influx/efflux may be calculated as well. Applications of this method to study liver metabolism using different labeled precursors may be possible.

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1 The abbreviations used are: FFA, free fatty acid; Glu, glutamate; Glu$_{\text{in}}$, blood glutamine; Glu$_{\text{out}}$, glutamine; AcCoA, acetyl-CoA; Lac, lactate; Pyr, pyruvate; Acet, acetate; Asp, aspartate; Oaa, oxaloacetate; Cit, citrate; $\alpha$-KG, $\alpha$-ketoglutarate; FE, fractional enrichment; ETOH, ethanol; RF, radio frequency.
EXPERIMENTAL PROCEDURES

Animal Preparation for in Vivo NMR Experiments—Eight male Sprague-Dawley rats (Charles River, Kingston) weighing 280–380 g were fasted 16–24 h before the experiment. They were anesthetized intraperitoneally with Inactin (Byk-Guiden Pharmazeutika, Germany) (80 mg/kg). Once anesthetized, an intramuscular injection of xylazine (15 mg/kg) and atropine sulfate (61 μg/kg) were given.

Prior to the NMR experiment, rats were anesthetized with a mixture of room air and oxygen. Both carotid artery and jugular vein were catheterized. An arterial blood sample was initially taken to measure blood gases and pH on an ABL-30 physiological monitor (Radiometer, Copenhagen, Denmark). Oxygen flow and ventilator volume were adjusted to provide a physiological pO2 (75–100 mm Hg), PCO2 (35-45 mm Hg), and pH (7.3–7.4). A non-magnetic blood pressure transducer was performed, and rats were anesthetized with a 3-way stopcock to monitor blood pressure.

Rats were placed prone on a Lucite platform with the liver positioned over the NMR surface RF coils. After the base-line spectrum, rats were infused with [2-13C]ethanol (99% enriched, 1 g/kg) (Cambridge Isotope Laboratories, Woburn, MA) diluted to a 33% v/v solution in 0.9% NaCl over a 4-min period. A 4-min infusion period was necessary to minimize blood pressure loss during this period. Arterial blood samples were drawn at 0, 5, 10, 15 min, and every 15 min thereafter for blood metabolite measurements. Blood samples were immediately centrifuged for 5 min in heparinized tubes at 10,000 rpm. Plasma was removed and refrigerated at −20°C until further use.

Livers were in situ freeze-clamped and removed at the end of the experiment. Rats were euthanized by administering sodium pentobarbital intravenously.

NMR Spectroscopy—In vivo NMR spectroscopy was performed on a Bruker Biospec 7.0T system (horizontal/22 cm diameter magnet). The magnet was equipped with actively shielded gradients (Bruker, Oxford, UK). Dual concentric surface RF coils were used. The outer 1H (30 mm) was tuned to 300.81 MHz, and the inner 13C coil (18 mm) was tuned to 75.65 MHz. The RF isolation between the two coils was 43 dB.

Global 1H shimming of water was performed to obtain reasonable line widths for imaging. Axial multislice spin-echo scout images (8 interleaved slices, 2 mm thickness, repetition time 24 ms) were taken to ensure placement of the liver in the magnet isocenter. Respiratory gating of the NMR pulse sequence was triggered using a 2-way switch on the respirator responding to each piston stroke. This reduced motion artifacts by limiting signal acquisition to the same period in the respiratory cycle.

Additional localized shimming was performed with a respiratory gated STEAM sequence (16) over a 2 × 2 × 1-cm volume of the liver. Water line widths of 35–60 Hz were observed.

1H-decoupled 13C NMR spectroscopy was used during the experiment. Localized carbon spectroscopy was necessary to eliminate strong signals from the liver in the 1H 13C NMR spectra. The ISIS excitation slice was generated using a 10-ms hyperbolic tangent pulses for imaging. Axial multislice spin-echo scout images (8 interleaved slices, 2 mm thickness, repetition time 1000 ms, echo time = 24 ms) were taken to ensure placement of the liver in the magnet isocenter. Respiratory gating of the NMR pulse sequence was triggered using a 2-way switch on the respirator responding to each piston stroke. This reduced motion artifacts by limiting signal acquisition to the same period in the respiratory cycle.

A 10-min base-line spectrum was acquired (repetition time = 0.5 s, scan number = 1200, 4K data) with broadband WALTZ-16 decoupling turned on during the acquisition. Subsequent spectra were accumulated in 10-min acquisition periods over 60–75 min. All spectra were Lorentzian-filtered, Fourier-transformed, and baseline subtracted to eliminate residual lipid signals. Chemical shifts were referenced to C-2 ethanol at 17.9 ppm. The average signal to noise (root mean square) for the C-4 glutamine and C-4 glutamate peaks at the last time point were 16.5 ± 1.3 and 15.0 ± 1.4, respectively.

Pseudo-enhanced 1H spectroscopy was performed on liver and plasma extracts for fractional enrichment (FE) as described previously (19). The fractional enrichments (given as atom % excess) of [4-13C]glutamate (2.34 ppm), [4-13C]glutamine (4.44 ppm), [2-13C]acetate (1.93 ppm), [3-13C]lactate (1.32 ppm), and [3-13C]alanine (1.48 ppm) were calculated.

13C NMR analysis of liver tissue extracts was performed on a Bruker AM 500 spectrometer system to obtain C-2,3,4 glutamate relative enrichments and isotopomer data from their respective carbon multiplets. Spectra were acquired with repetition time = 0.5 s, scan number = 10,000, 16K data, and WALTZ-16 broadband proton decoupling. Peak intensities were corrected for saturation and nuclear Overhauser effect contributions (with respect to 14 Nl to 40 min (n = 2) C-2 Glu and 0.98 × C-3 Glu with respect to C-4 Glu) 1.06 × C-2 Glu and 0.81 × C-3 Glu).
FIG. 1. $^{13}$C label flow schematic in liver originating from $[2-^{13}$C]ethanol. This schematic illustrates the flow of $^{13}$C label (in bold) from $[2-^{13}$C]ethanol to $[4-^{13}$C]glutamate and $[4-^{13}$C]glutamine which are coupled to the tricarboxylic acid cycle. Label from $[4-^{13}$C]α-ketoglutarate becomes incorporated into $[2-^{13}$C] or $[3-^{13}$C]malate and oxaloacetate, and in subsequent turns of the tricarboxylic acid cycle, $[2-^{13}$C] and $[3-^{13}$C]citrate, α-ketoglutarate, glutamate, and glutamine become labeled (not all shown). $[2-^{13}$C]Alanine and aspartate are not shown, but will represent label flow.
In vivo \(^{13}\text{C}\) NMR superficial lipid suppression techniques used in the label turnover experiments. A illustrates a 10-min acquisition base-line spectrum of the intact rat liver and superficial tissues after infusion with 1 g/kg \([2-^{13}\text{C}]\)ethanol. The C-2 ethanol (EtOH) signal was assigned to 17.9 ppm. The predominantly superficial aliphatic lipid signals appeared at approximately 23–34 ppm. B illustrates a 10-min acquisition spectrum using a modified one-dimensional ISIS localized pulse sequence on the same liver. This was accomplished by using a 10-ms hyperbolic secant inversion pulse during a \(\delta\) G/cm magnetic field gradient orthogonal to the RF coil plane. This sequence accounted for an approximately 50% \((\text{CH}_2)\) lipid signal reduction with no reduction in the C-2 ethanol signal. Spectrum C was the result of the combined modified one-dimensional ISIS and magnetic field spoiler coil localized pulse sequence. The result was an approximately 68% reduction of the \((\text{CH}_2)\) resonance from that of spectrum A. All measurements were made postmortem to ensure ethanol signal stability.

RESULTS

A localized \(^{13}\text{C}\) NMR pulse sequence was used to suppress the strong superficial lipid signals that overlapped the peaks of interest. Fig. 2A represents a non-localized carbon spectrum from the liver, abdominal muscle, and superficial lipid (large lipid signals present at 23–34 ppm) in rat postmortem. These peaks obscure the C-4 resonances of glutamate and glutamine at 34.4 and 31.3 ppm, respectively. The modified one-dimensional ISIS localized pulse sequence was used to produce the spectrum in Fig. 2B. Additional lipid suppression was provided using the combined magnetic field spoiler coil one-dimensional ISIS localization sequence as shown in Fig. 2C. No decrease in the C-2 EtOH peak at 17.9 ppm in Fig. 2, B and C, indicates that there was excellent signal localization to the liver.

A series of base-line subtracted 10 min acquired spectra is shown in Fig. 3. The times indicated are the median time of acquisition. All experiments showed an immediate increase in the \(^{13}\text{C}\) NMR signal at 17.9 ppm as a result of the ethanol bolus. In subsequent spectra, the ethanol signal decreased indicating ethanol clearance. C-4 Glu and C-4 Gln signals appeared at 34.4 and 31.3 ppm, respectively, in the first spectrum and increased until steady state signal intensities were achieved. Labeling of C-4 Glu and C-4 Gln was the result of [\(^2\text{H}\text{C}^\text{13}\text{C}\)acetyl-CoA condensing with oxaloacetate to produce \([4-^{13}\text{C}]\)citrate which in turn labeled \(\alpha\)-ketoglutarate, glutamate, and glutamine at the C-4 position (Fig. 1). C-2 Glu and C-2 Gln at 55.5 and 55.0 ppm, respectively (unresolved), began to appear at 5–15 min and increased at a slower rate than C-4 Glu and C-4 Gln. These peaks appeared during subsequent turns of the tricarboxylic acid cycle which results in scrambling the C-4 label of Glu and Gln to C-2 and C-3 Glu and Gln. The peaks of C-3 Glu and Gln at 27.9 and 27.0 ppm, respectively, and C-2 acetate at 24.2 ppm were difficult to observe, because these frequencies are in the area where the ISIS suppression was optimized. The ISIS suppression was optimized by placing the downfield edge of the frequency selective inversion pulse at the C-2 fatty acyl chain peak (\(\sim 34.2\) ppm) of the triglycerides. Therefore, using a magnetic field gradient of \(\sim 6\) G/cm during the frequency selective pulse, signal suppression at 27.9 and 27.0 ppm was 0.46 and 0.52 cm deep into the liver, respectively, if the ISIS suppression was optimized at the liver/abdominal muscle interface. The C-4 Glu, C-4 Gln, and C-2 Glu/Gln peaks of all the base-line subtracted spectra were integrated, and the time point enrichments of C-4 Glu, C-4 Gln, and C-2 Glu extrapolated from tissue extract enrichment and in vivo label turnover data are shown in Fig. 4A.

The individual C-2, C-3, and C-4 Glu multiplet peak areas (Fig. 5) represented as a fraction of entire isotopomer peak area are given in Table I. Steady state isotopomer analysis of these glutamate peaks was used to calculate the C-2 acetyl-CoA enrichment \((E_{2a})\), labeled anaplerotic substrate enrichment \((E_{a1})\), and ratio of anaplerotic versus citrate synthase flux \((y)\) (Table I).

Fig. 6 illustrates the three spectra obtained when performing Proton-observed carbon-enhanced spectroscopy. Spectrum A represents the heteronuclear non-\(J\)-inverted spectrum, and subspectrum B represents the heteronuclear \(J\)-inverted spectrum. Spectrum C represents the difference (A and B). These spectra were used to calculate the fractional enrichments of C-4 Glu = 23.0 \(\pm\) 1.1%, C-4 Gln = 17.2 \(\pm\) 1.5% \((p < 0.005\) versus C-4 Glu FE), C-3 Ala = 1.9 \(\pm\) 0.4%, C-3 Lac = 2.0 \(\pm\) 0.6%, and C-2 Acet = 49.9 \(\pm\) 3.9% (Table II). The C-2 Glu (7.7 \(\pm\) 0.5%) FE
was calculated from the known C-4 Glu FE and relative C-2 to C-4 Glu was measured in tissue extracts. The Glu and Gln concentrations were $4.02 \pm 0.35$ and $4.64 \pm 0.35 \, \text{mol/g wet weight}$, respectively.

The best fit C-4 Glu, C-4 Gln, and C-2 Glu turnover curves generated by the computer analysis of the data above are shown in Fig. 4A. Table II summarizes the flux results from the mathematical fitting procedure. $V_{\text{tca}} = 0.33 \pm 0.09 \, \text{mol/g wet weight/min}$, and the $\alpha$-KG $\leftrightarrow$ Glu exchange rate was an order of magnitude greater ($V_{\text{glx}}, V_{\text{glu}}^{-1} = 3.80 \pm 1.2 \, \text{mol/g wet weight/min}$). These fluxes were relatively insensitive to changes in the Cit, $\alpha$-KG, and Oaa pools as a doubling of these concentrations had no effect on $V_{\text{tca}}$.

A separate group of rats were sacrificed for base-line and intermediate time point (10, 25, and 40 min) measurements after $[2-\text{13C}]\text{ethanol}$ infusion to obtain liver glutamate and glutamine enrichments and concentrations at various times. The C-4 Glu and C-4 Gln enrichments were 14, 20.3, 24.1, and 23% and 7.2, 14.4, 16.9, and 17.2% at 10, 25, 40, and 55 min, respectively. These enrichments correspond to C-4 Glu and C-4 Gln label turnover rates that are not significantly different than those obtained by in vivo NMR. The glutamate and glutamine concentrations were $4.02 \pm 0.10$ and $5.02 \pm 3.10 \, \text{mol/g}$, respectively, at base line and $3.63 \pm 0.20$ and $3.53 \pm 0.78 \, \text{mol/g}$, respectively, at 10 min and did not vary through-out the remaining time points. Therefore, the maximum over-estimation in $V_{\text{tca}}$, assuming that the Glu pool increased from $2.49 \pm 0.10 \, \text{mol/g}$ at base line to $4.02 \pm 0.35 \, \text{mol/g}$ at the end of the study would be $27\%$ ($0.24 \text{ vs. } 0.33 \, \text{mol/g wet weight/min}$).

**DISCUSSION**

In this study, the non-invasive $^{13}$C NMR detection of glutamate and glutamine labeling in liver was achieved using physiologically allowable concentrations of $[2,^{13}$C]$\text{ethanol}$ ($\sim 0.1\%$ in blood). A metabolic steady state mathematical analysis similar to that previously used by Mason et al. (13) and Yu et al. (10) was applied to the kinetic and steady state labeling data to calculate a number of metabolic fluxes coupled to the tricarboxylic acid cycle in liver. We have combined isotopomer steady state ($^1$H and $^{13}$C NMR of liver extracts) and non-steady state (in vivo $^{13}$C NMR) measurements to calculate the absolute substrate flux through the tricarboxylic acid cycle directly in liver for the first time.

Oxygen consumption rates have previously been measured in perfused liver in situ following ethanol addition to the perfusate (24–26). Thurman and Scholz (24) measured $O_2$ consumption rates of 1.83 $\mu$mol/g/min during a 1 mM EtOH perfusion. Taylor et al. (25) measured $O_2$ consumption rates of approximately 1.6 $\mu$mol/g/min during a 2.5 mM EtOH perfusion, and Eriksson et al. (26) measured $O_2$ consumption rates of approximately 1.5 $\mu$mol/g/min after 20 min during a 4 mM EtOH perfusion. Typically, it is difficult to extrapolate tricarboxylic acid cycle flux rates from tissue oxygen consumption rates unless the exact profile of substrates oxidized by the tricarboxylic acid cycle is known due to differences in stoichiometric coupling. Assuming $O_2$ consumption was aerobically coupled to acetate oxidation in the above-mentioned ethanol perfusion studies, then the oxygen consumption rate ($V_{O_2}$) is stoichiometrically coupled to $V_{\text{tca}}$ by $V_{O_2} = 2 \, V_{\text{tca}}$. Therefore, from these measurements we estimate $V_{\text{tca}}$ equal to 0.92, 0.80, and 0.75 $\mu$mol/g/min, respectively. These rates are more than double our measured value of $0.33 \pm 0.09 \, \mu$mol/g/min. Unfortunately, $V_{O_2}$ extrapolated measurements are not accurate during conditions where there is elevated production of reducing equivalents, and the respiration quotient is very low as is the case during ethanol oxidation. It has been shown that although oxygen consumption rates are maintained, $CO_2$ production is significantly inhibited by ethanol metabolism (27). We estimate that greater than 90% of the acetate formed in our study was not oxidized in the liver, yet 2 NADH reducing equivalents were produced for every acetate formed via ethanol oxidation in the liver. Therefore, the vast majority of reducing potential necessary for ATP synthesis was provided by ethanol oxidation and not tricarboxylic acid cycle turnover as reflected by our low tricarboxylic acid cycle flux measurements.

In perfused liver, the tricarboxylic acid cycle flux has previously been quantified using a mass spectrometric assay (2, 3) of $^{13}$C labeling of glutamate during conditions of steady state labeling and using equations developed by Magnnusson et al. (4). Beylot et al. (2) reported a tricarboxylic acid cycle flux of 0.34 $\mu$mol/g/min following $[3,^{13}$C]$\text{lactate}$ administration, and DiDonato et al. (3) reported a value of 0.25 $\mu$mol/g/min following combined $[3,^{13}$C]$\text{lactate}$ and $[3-^{13}$C]$\text{pyruvate}$ administration. A mathematical model by Vogt et al. (28) was also established to calculate relative tricarboxylic acid cycle flux using data obtained from $^{13}$C NMR isotopomer analysis of glutamine in liver extracts.

The heterogeneous liver acinus is spatially comprised of periportal and perivenous zones, and it has been proposed that the periportal zone is primarily gluconeogenic and the perivenous zone is glycolytic (32). In our experiment, periportal...
and perivenous metabolism cannot be distinguished, but Cline and Shulman (33) have shown in vivo that there is no difference in tricarboxylic acid cycle substrate labeling patterns in periportal versus perivenous zones following a [U-13C]glucose administration. This would imply that our tricarboxylic acid cycle flux measurement was representative of the global hepatocyte tricarboxylic acid cycle.

Glutamine is transported across the cell membrane by the following two glutamine transport systems: the Na\(^+\)-dependent \(N\) transport system, and the Na\(^+\)-independent system which involves facilitated diffusion of glutamine in hepatocytes (34–36). There was a difference in C-4 Glu and C-4 Gln enrichment (23.0 versus 17.2%, respectively) in our experiment which is

### Table I

**13C NMR steady state isotopomer analysis of glutamate in liver extracts (% of multiplet peak areas)**

| C-2 Glu       | % of entire peak area |
|---------------|-----------------------|
| Singlet       | 66.6 ± 1.5            |
| Doublet 1–2   | Not observed          |
| Doublet 2–3   | 33.4 ± 1.8            |
| Quartet       | Not observed          |

| C-3 Glu       |                      |
|---------------|----------------------|
| Singlet       | 36.3 ± 4.0           |
| Doublet 2–3 or 3–4 | 45.3 ± 2.9  |
| Triplet       | 18.4 ± 2.1           |

| C-4 Glu       |                      |
|---------------|----------------------|
| Singlet       | 77.3 ± 2.0           |
| Doublet 3–4   | 22.7 ± 2.0           |

**Steady state analysis**

- C-2 acetyl-CoA FE \((F_{c2})\): 51.4 ± 3.4%
- Anaplerosis \((\gamma)\): 89.8 ± 6.7%
- Labeled anaplerotic substrate FE \((F_{a1})\): 0.4 ± 6.2%

![13C NMR spectrum of a liver extract](image1)

**Fig. 5.** 13C NMR spectrum of a liver extract. Steady state isotopomer analysis of glutamate was used to calculate the C-2 acetyl-CoA enrichment \((F_{c2})\), labeled anaplerotic substrate enrichment \((F_{a1})\), and ratio of anaplerotic versus citrate synthase flux. The individual C-2, C-3, and C-4 glutamate multiplet peak areas (inset) were integrated, and the data were used in the isotopomer analysis (results shown in Table I). Homonuclear \(J\) coupling patterns are denoted above the individual multiplets, and coupled nuclei are indicated.

![1H NMR spectra of a liver extract](image2)

**Fig. 6.** 1H NMR spectra of a liver extract. Proton-observed carbon-enhanced NMR analysis was performed on liver extracts to determine the C-4 glutamate, C-4 glutamine, C-2 acetate, C-3 alanine, and C-3 lactate fractional enrichments. Spectrum A represents the homonuclear non-\(J\)-inverted spectrum, and subspectrum B represents the homonuclear \(J\)-inverted spectrum. The broadband 13C inversion pulse frequency was placed between C-4 glutamate and C-3 alanine (≈ 26.2 ppm). Spectrum C represents the difference (A and B). These spectra were used to calculate the fractional enrichment of the relevant enriched metabolites.
TABLE II  
Liver extract metabolite fractional enrichments and calculated metabolic fluxes

| Metabolite enrichments | C-4 Glu FE | C-4 Gln FE | C-2 Acet FE | C-2 AcCoA FE | C-3 Lac FE | C-3 Ala FE |
|------------------------|-----------|-----------|-------------|--------------|-----------|-----------|
| V\text sub{tca}          | 23.0 ± 1.1| 17.2 ± 1.5*| 49.9 ± 3.9  | 51.4 ± 3.4   | 2.0 ± 0.6 | 1.9 ± 0.4 |
| V\text sub{glu}          | 0.33 ± 0.09| 3.8 ± 1.2 | 0.39 ± 0.07 | 0.25 ± 0.05 | 0.45 ± 0.06| 0.70 ± 0.06|
| V\text sub{glu} / V\text sub{gln} | 640       | 3.4       | 6        | 0.6          | 1.9       | 0.6 |

* Significantly different than C-4 Glu FE (p < 0.005). Unidirectional fluxes are defined as follows: V\text sub{tca}, tricarboxylic acid cycle flux; V\text sub{glu}, V\text sub{glu} / V\text sub{gln}, glutamine synthetase and glutaminase flux, respectively; V\text sub{lyase}, citrate lyase flux; V\text sub{pc}, phosphoenolpyruvate carboxykinase flux; V\text sub{pepck}, pyruvate carboxylase flux. Metabolic fluxes are represented as μmol/g wet weight/min.

** Mean ± mean S.D. reflecting the accuracy of the measurement derived from the error in each curve fit. Random error determined from spectral SN measurements was included with each data point in the curve fitting procedure.

Evidence that liver glutamine was in exchange with lower 13C-enriched glutamine in blood. Previous in vivo measurements of net glutamine flux across the liver in fasted rats resulted in a small net uptake of 1.7 (37) to 2.4 (38) nmol/g/min. By using the glutamine extraction data established by Partridge (39) and a plasma glucose concentration of 0.4–0.6 mM (37,38), the unidirectional glutamine influx would be 260–390 nmol/g-min, and efflux would be −258–388 nmol/g-min by using the above net uptake data. These values are similar to our calculated values for V\text sub{in} = 250 ± 50 nmol/g-min assuming 0 net glutamine flux across the membrane. If ethanol metabolism did change the net flux of glutamine, a large increase in net glutamine efflux of, for example, 100 nmol/g-min would have only a small effect on the calculated V\text sub{tca} (1.9%, 0.30 versus 0.33 nmol/min) assuming that endogenous glutamate or glutamine provided the necessary substrate for increased net glutamine efflux.

Häussinger and Gerok (40) measured a glutamate uptake and net glutamate release of approximately 0.03 μmol/g/min for each at physiological glutamine concentrations in perfused livers, and Low et al. (41) measured glutamine influx kinetics (K\text sub{m} = 0.25 mM, V\text sub{max} = 0.046 μmol/g protein/min) suggesting an even lower uptake at physiological concentrations in sinusoidal membrane vesicles. Since these transport rates are 1 to 2 orders of magnitude slower than the calculated V\text sub{tca} and glutamine transport rates, this transport mechanism was omitted from the model (Fig. 1).

[2-13C]Ethanol was used as the labeled precursor in this experiment to minimize [2-13C]acetate-CoA label dilution resulting from fat oxidation due to the decreased redox potential [NAD+/NADH] (29–31) resulting from ethanol metabolism. Although ethanol is not a preferred substrate used to study normal liver physiology, this choice nevertheless allowed adequate in vivo 13C NMR detection of liver glutamate and glutamine labeling. With the current sensitivity of NMR detection of 13C-labeled glutamate and glutamine in liver, it may be possible to make these 13C NMR measurements using [2-13C]acetate precursor infusion. It may also be possible to observe [2-13C]glutamate turnover if [3-13C]acetate precursor is used. Schumann et al. (42) suggested that the distribution of 13C label in liver glutamate derived from [2-13C]acetate and [2-13C]ethanol precursors was incompatible with [2-13C]acetate metabolism being primarily in the human liver. They suggested that acetate was also metabolized to glutamate by peripheral skeletal muscle and cycled back to the liver via glutamine. Numerous studies have observed acetate metabolism as occurring in peripheral tissues (43–45). At 40 min, we measured a [4-13C]glutamate FE of 11.8% in blood (Fig. 4B) but a [4-13C]glutamine FE of only 3.7% in skeletal muscle. This suggests that the glutamine label in blood was predominantly a result of label contribution from liver rather than from peripheral muscle. Therefore, when comparing the relatively high enrichment of C-4 Glu in liver (30.9%) versus C-4 Glu in muscle (3.7%), we can be reasonably confident that liver and not muscle tricarboxylic acid cycle activity was detected in vivo.

The liver [2-13C]acetate represented in Fig. 3 as a small peak at 24.2 ppm remained stable throughout the experiment, and therefore the [2-13C]acetate-CoA FE most likely remained stable. Additionally, the [2-13C]acetate FE (49.9 ± 3.9%) in liver extracts equaled the [2-13C]acetate-CoA FE (51.4 ± 3.4%). It is not known whether the label dilution of these pools was due to incompletely suppressed FFA oxidation and acetyl-CoA hydrolysis activity in liver or from unlabeled acetate originating in peripheral tissues. Label incorporation into [2-13C]acetate-CoA via pyruvate recycling (pyruvate → oxaloacetate → phosphoenolpyruvate → pyruvate) or via malic enzyme which are both active in liver was most likely negligible as pyruvate dehydrogenase (2, 3, 15) and malic (23) enzyme activity in fasted liver is low. Additionally, [3-13C]lactate and [3-13C]alanine FE in the liver extracts were low (2.0 ± 0.6 and 1.9 ± 0.4%, respectively) and therefore could have had only a minimal effect on varying the [2-13C]acetate-CoA enrichment throughout the experiment. Although we could not explicitly calculate V\text sub{pdc} from the model, independently varying this flux while maintaining the [2-13C]acetate-CoA FE did not alter V\text sub{tca} because of the rapid turnover of this small pool. The dilution of [2-13C]acetate-CoA (51.4%) to [4-13C]glutamate (23.0%) could not be entirely accounted for by liver glutamine exchange with blood. It is possible that endogenous protein synthesis may have occurred. This unexplained dilution was similarly observed by Des Rosiers et al. (46) in liver. The anaplerotic flux (0.9 × V\text sub{pdc} determined from the isotopic steady state analysis of glutamate (20) was set equal to the pyruvate carboxylase (V\text sub{pc}) flux. This may be an overestimation of V\text sub{pc} as malic enzyme (V\text sub{malic}) flux may also contribute to anaplerosis, but the distribution of anaplerotic flux between V\text sub{tca} and V\text sub{malic} has no effect on V\text sub{tca}. Citrate lyase flux (V\text sub{lyase}) provides acetyl-CoA substrate for FFA synthesis, and although this flux contributed negligibly to the rate of C-4 and C-2 Glu turnover in liver, varying this flux affected C-2 Glu FE considerably as pyruvate carboxylase (V\text sub{pc}) coupled to V\text sub{lyase}. The α-KG ↔ Glu exchange flux (V\text sub{glu}, V\text sub{pc} / V\text sub{pc}) which comprises both aminotransferase and glutamate dehydrogenase fluxes and mitochondrial/cytosolic transfer of α-KG and Glu was the source for the greatest error in V\text sub{tca}. It was necessary to model both C-2 and C-4 Glu turnover to calculate this flux. This exchange rate was determined to be very rapid from in vitro kinetic measurements (47) (−640 μmol/g/min) and determined to be much slower in vivo, but an order of magnitude greater than V\text sub{tca} in our experiments (3.8 ± 1.2 μmol/g/min).
In summary, it is possible to detect $^{13}$C labeling of glutamate and glutamine in liver via non-invasive $^{13}$C NMR. The in vivo $^{13}$C labeling kinetics of glutamate and glutamine in liver and glutamine in blood combined with a metabolic steady state mathematical analysis allows for rapid, direct calculation of the liver tricarboxylic acid cycle flux. This study illustrates how non-invasive metabolic flux measurements in tissues other than brain can be made by including label exchange with blood in the mathematical analysis.

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