Indexing Tricarboxylic Acid Cycle Flux in Intact Hearts by Carbon-13 Nuclear Magnetic Resonance

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Although the tricarboxylic acid (TCA) cycle is the prime means of carbon metabolism for energy generation in normal myocardium, the noninvasive quantification of TCA cycle flux in intact cardiac tissues is difficult. A novel approach for estimating citric acid cycle flux using $^{13}$C magnetic resonance (NMR) is presented and evaluated experimentally by comparison with measured myocardial oxygen consumption over a wide range of cardiac contractile function in intact, beating rat hearts. Continuous series of $^{13}$C NMR spectra, obtained after the introduction of [2-$^{13}$C]acetate as substrate, quantified the time course of $^{13}$C appearance in the carbon positions of myocardial glutamate, which are sequentially enriched via citric acid cycle metabolism. A TCA cycle flux parameter was calculated using the premise that TCA cycle flux is inversely proportional to the time difference between $^{13}$C appearance in the C-4 and C-2 positions of glutamate (glutamate $\Delta t_p$ [minutes]), which are enriched in subsequent “turns” of the TCA cycle. This TCA cycle flux parameter, termed $K_p$, correlated strongly with myocardial oxygen consumption over a range of developed pressures in hearts perfused with 5 mM acetate ($r=0.98$, $p<0.001$), as well as in separate studies in hearts perfused with 5 mM glucose and 0.5–0.8 mM acetate ($r=0.94$, $p<0.001$). Results of numerical modeling of $^{13}$C glutamate kinetics suggest that this TCA cycle flux parameter, $K_p$, is relatively insensitive to changes in metabolite pool sizes that could occur during metabolism of other substrates or during conditions of altered oxygen availability. Additional studies in separate hearts indicated that the time course of $^{13}$C appearance in citrate, which is predominantly mitochondrial in the rat heart, is similar to that in glutamate, further supporting the premise that the described $^{13}$C NMR parameters reflect mitochondrial citric acid cycle activity in intact cardiac tissues. (Circulation Research 1992;70:392–408)

The tricarboxylic acid (TCA) cycle is the principal means of aerobic cardiac energy metabolism, and the rate of carbon flux through this cycle changes with physiological alterations in cardiac contractile performance. Despite the central role of the TCA cycle, there are relatively few methods of quantifying TCA cycle flux in intact hearts. Estimates of TCA cycle flux in isolated cardiac tissues and mitochondrial preparations are typically based on measurements of oxygen consumption, uptake or liberation of radiolabeled tracers, and changes in TCA cycle intermediate pool sizes, many of which are not well suited to studies in intact systems. Another strategy is the use of $^{13}$C nuclear magnetic resonance (NMR) spectroscopy with $^{13}$C-enriched substrates. Together they can be exploited to repetitively and nondestructively quantify the time course of the appearance of labeled carbon nuclei within intracellular metabolite pools in intact organs, animals, or humans. Such an approach has been used with a mathematical simulation of the TCA cycle to calculate absolute steady-state TCA cycle flux from data obtained from rat hearts. The results generated by that model were not, however, rigorously compared with other measures of TCA cycle flux over a range of values, such as might be induced by varied physiological contractile conditions. In addition, utilization of the model requires advanced computing...
facilities and quantification of all TCA cycle intermediate pool sizes; the latter requires tissue extraction and thereby precludes repetitive studies in a given tissue or truly in vivo studies. The recent development of spatially localized $^{13}$C NMR spectroscopic techniques for human heart studies underscores the potential importance of $^{13}$C NMR utilization to non-destructively quantify cardiac TCA cycle activity.

The purpose of the current work was to test the hypothesis that $^{13}$C NMR parameters alone can be used to index TCA cycle flux noninvasively in intact hearts. The experimental approach was to derive $^{13}$C NMR indexes of TCA cycle flux from the time course of $^{13}$C appearance in intracellular metabolite pools of intact beating rat hearts and compare them with measured myocardial oxygen consumption across a range of contractile performance since oxygen consumption is critically linked to TCA cycle flux. The general validity of this method was assessed by studying two different substrate conditions and also by separate mathematical modeling analysis of intermediate pool size conditions beyond those expected for the experimental conditions. Because TCA cycle activity occurs in mitochondria, additional experiments were performed to compare the time course of $^{13}$C appearance in mitochondrial intermediates with metabolites predominantly occurring in the cytosol.

Materials and Methods

Isolated Rat Heart

Nonfasting, ex-breeder, male Wistar rats were anesthetized with 100–150 mg inapritoteraline pentobarbital. After rapid excision of the heart, the aorta was cannulated for retrograde constant-flow perfusion at 15 or 20 ml/min via a peristaltic pump with oxygenated solution at 37°C. The perfusate was pumped through small-diameter tubing to minimize mixing effects during changes in perfusate constituents. The perfusate contained (mM) sodium 144, potassium 5, calcium 1.5, HEPES 6, magnesium 0.9, inorganic phosphate 1, chloride 152, and acetate 5, as well as lidocaine 5 µg/ml. The pH was adjusted to 7.4. Preliminary experiments performed with bicarbonate buffer demonstrated results similar to those performed with HEPES buffer. Perfusate was not recirculated. Hearts were paced at either 140 or 220 beats per minute by using an SD-9 stimulator (Grass Instrument Co., Quincy, Mass.) via a KCl wick electrode placed into the right ventricle. A polyvinyl chloride balloon attached to polyethylene (PE-190) tubing was inserted into the left ventricle through the mitral valve. The balloon was filled in increments to obtain the maximum isovolumic developed pressure during systole, which usually occurred at an end-diastolic pressure of 10–16 mm Hg. The balloon solution contained [6-$^{13}$C]hexanoic acid and phenylphosphonic acid (at pH 7.0), which served as $^{13}$C and $^{31}$P NMR standards. The other end of the polyethylene tubing was connected to a Gould P23Db transducer for continuous measurements of left ventricular pressure. [2-$^{13}$C] Acetate (99% enriched) was obtained from Isotec, Miamisburg, Ohio; [6-$^{13}$C]hexanoate (99% enriched) from Merck Frost Isotopes, Montreal, Canada; and other chemicals from Sigma Chemical Co., St. Louis, Mo., or Aldrich Chemical Co., Milwaukee, Wis.

Nuclear Magnetic Resonance Spectroscopy

The hearts were positioned in a remotely switched dual-tuned ($^{13}$C and $^{31}$P) 20-mm commercial probe of a Bruker AM 360-WB NMR spectrometer (field strength, 8.5 T). Magnetic field homogeneity was optimized while observing the water proton signal with the decoupler coil. Proton-decoupled, minimally saturated $^{31}$P spectra were obtained with a 2.1-second delay between pulses of 22-µsec duration (60°) by using a 2,000-point data table, and proton-decoupled $^{13}$C spectra were collected with a 1.1-second delay between pulses of 22-µsec duration (60°) using 2,000 points and zero-filled to 8,000. Free induction decays were Fourier transformed, and a line broadening of 15–20 Hz was introduced. Intracellular pH was measured by the chemical shift of the inorganic phosphate peak relative to the creatine phosphate (PCr) peak. Peak areas were measured by integrating areas under individual peaks by hand digitization (SIGMASCAN, Jandel Scientific, Corte Madera, Calif.).

Before introduction of a $^{13}$C-enriched substrate in the perfusate, a calibration graph for the determination of absolute metabolite concentrations was constructed for each heart. The intraventricular balloon volume was incremented, in steps, with solution containing known concentrations of phenylphosphonic and [6-$^{13}$C]hexanoic acids. After each increment, $^{31}$P or $^{13}$C NMR spectra were acquired. Each increment in balloon volume placed precise, known amounts of $^{31}$P and $^{13}$C compounds within the sensitive volume of the NMR coil. These acquisitions also provided natural abundant $^{13}$C spectra from the tissue and perfusate. Integrated peak areas of $^{13}$C spectra were determined from difference spectra between these natural abundance and enriched acquisitions. The areas of all peaks were corrected for partial saturation and nuclear Overhauser enhancement by comparison with fully relaxed spectra without nuclear Overhauser enhancement. $^{31}$P and integrated peak areas of heart metabolites were converted to absolute concentrations by comparison with the peak areas of the $^{13}$C- or $^{31}$P-containing substance within the intraventricular balloon, which acted as a standard. Hearts were weighed at the beginning of the experiments, and results are expressed as micromoles of metabolite per gram wet weight. Wet-to-dry weight ratios measured under comparable conditions in glucose-perfused rat hearts range from 6.5 to 7.0.

The quantity of each $^{13}$C metabolite was plotted versus time after introduction of $^{13}$C-enriched substrate. The maximal enrichment of each glutamate isotopomer (micromoles of $^{13}$C per gram wet weight) was calculated as the mean of three or four of the final concentrations obtained after enrichment had
reached a plateau. The time to half-maximal enrichment \((t_{\text{50}})\) for each metabolite of each heart was interpolated from a least-squares fit to the experimental \(^{13}\text{C}\) data of a sum of exponentials of the form

\[
[13\text{C}]\text{glutamate}(\text{time}) = A + (B \times \exp(-\text{time}/t_1)) + (C \times \exp(-\text{time}/t_2))
\]

where \([13\text{C}]\text{glutamate}(\text{time})\) is the \(^{13}\text{C}\) enrichment of a given glutamate isotopomer as a function of time and \(A, B, C, t_1\), and \(t_2\) are constants. For glutamate C-4 enrichment curves that appeared to have a monoexponential form, \(C\) (typically <0) was taken to be zero.

**Tricarboxylic Acid Cycle Flux Calculations**

After substitution of enriched or unenriched substrate, \(^{13}\text{C}\) nuclei from \([2,^{13}\text{C}]\text{acetate}\) enter the TCA cycle as acetyl coenzyme A (acyetyl-CoA) and appear in the C-4 positions of citrate and, subsequently, of 2-oxoglutarate (see Figure 1). At subsequent steps in the TCA cycle, the C-4 of 2-oxoglutarate becomes the C-2 or C-3 of succinate, and \(^{13}\text{C}\) is thereby incorporated into the C-2 or C-3 position of fumarate, malate, and oxaloacetate. After the condensation of \([2,^{13}\text{C}]\text{oxaloacetate}\) or \([3,^{13}\text{C}]\text{oxaloacetate}\) and acetyl-CoA, \(^{13}\text{C}\) is incorporated into the C-2 or C-3 of citrate and 2-oxoglutarate.

Most citric acid cycle intermediates occur in concentrations too low to permit \(^{13}\text{C}\) NMR detection. Glutamate is an amino acid in rapid equilibrium with 2-oxoglutarate and is present in sufficiently high concentrations to permit \(^{13}\text{C}\) NMR quantification. After the administration of \([2,^{13}\text{C}]\text{acetate}\), the time course of \(^{13}\text{C}\) enrichment of the C-4 position of myocardial glutamate (Figure 1, denoted by “\(x\)”) reflects flux through multiple metabolic steps including the uptake of acetate, formation of acetyl-CoA (step 1), flux through citrate synthase (step 2), aconitase (step 3), isocitrate dehydrogenase (step 4), and the aminotransferase reaction (step 5) with formation of glutamate C-4 from 2-oxoglutarate C-4 (see Figure 1, solid line). The enrichment time course of the C-2 of myocardial glutamate (Figure 1, denoted by “\(l\)”) reflects flux through all of the above steps and, in addition, flux through the 2-oxoglutarate dehydrogenase (step 6), succinate thiokinase (step 7), succinate dehydrogenase (step 8), fumarase (step 9), and malate dehydrogenase (step 10) reactions (with carbon randomization at succinate/fumarate steps) and a second pass through citrate synthase to glutamate (reactions 2–5) (Figure 1, broken line).

Because \([4,^{13}\text{C}]\text{glutamate}\) and \([2,^{13}\text{C}]\text{glutamate}\) are both derived from the same intermediate in the TCA cycle, we propose that, for any TCA cycle pool size, the time difference \((\Delta t)\) between the \(^{13}\text{C}\) appearance in glutamate C-4 and glutamate C-2 (or C-3) (Figure 1, the difference between the dotted line and the solid line) is inversely proportional to flux through reactions 2–10 or one turn (“lap”) of the TCA cycle. In other words, we hypothesize that TCA cycle flux calculations can be based on the premise that the \(^{13}\text{C}\) appearances in isotopomers of TCA cycle–derived metabolic intermediates can serve as “lap counters” for \(^{13}\text{C}\) TCA cycle flux.

Because of the inherent limited sensitivity of NMR spectroscopic detection, it is not possible to directly track the movement of each \(^{13}\text{C}\) nucleus through metabolic pathways. Rather, one can measure the time course of \(^{13}\text{C}\) enrichment in intermediate pools, such as glutamate. Determination of the time of initial \(^{13}\text{C}\) enrichment of each glutamate isotopomer is somewhat imprecise. One approach would be to fit each isotopomer enrichment curve and extrapolate to the point in time where the enrichment was last equal to zero. A more precise approach is to measure the time to half-maximal enrichment, \(t_{50}\). Thus the glutamate \(\Delta t_{50}\) the difference between the \(t_{50}\) of glutamate C-4 and that of glutamate C-2 (or C-3), is directly proportional to the time for a \(^{13}\text{C}\) “lap” in this simplified convention and therefore inversely
proportional to $^{13}$C-labeled flux. This time is dependent not only on TCA cycle activity, however, but also on the sum total of the TCA cycle intermediate pool sizes and the pools of amino acids (glutamate and aspartate) in rapid equilibrium with cycle intermediates since for any given flux the larger the pools the longer it will take for label to move through the cycle. A parameter, $K_l$, (micromoles of $^{13}$C per minute per gram of tissue), can therefore be defined that relates directly to $^{13}$C-labeled flux through the TCA cycle

$$K_l = \frac{[\Sigma \text{TCA cycle--derived metabolites (}\mu\text{mol/g)}]}{\text{glutamate } \Delta t_{50} \text{ (min)}}$$

(2)

where $\Sigma$ TCA cycle--derived metabolites is the sum of the steady-state pools of the TCA cycle intermediates (e.g., citrate, isocitrate, 2-oxoglutarate, succinate, fumarate, malate, and oxaloacetate) and the sum of the amino acid pools (glutamate and aspartate) that are in rapid equilibrium with the cycle via the transaminase reactions. The glutamate $\Delta t_{50}$ is the difference in $t_{50}$ for the glutamate--4 and --2 isotopomers (minutes). Intermediates that can be quantified by $^{13}$C NMR dominate the numerator of this relation as shown in prior studies, which demonstrated that the combined glutamate, aspartate, and citrate pools account for 96% and 97% of the sum total of all TCA cycle--derived pools in glucose- and acetate-perfused hearts, respectively. Therefore, empirical simplification of Equation 2 to include only metabolite pools large enough to be detectable by $^{13}$C NMR would be

$$K_l = \frac{[\Sigma \text{ }^{13}\text{C NMR--detected TCA metabolites (}\mu\text{mol/g)}]}{\text{glutamate } \Delta t_{50} \text{ (min)}}$$

(3)

For anaerobic hearts or normoxic hearts using other substrates including exogenous odd-numbered fatty acids, the numerator may also include additional terms for citrate, succinate, and possibly malate, if these are present in adequate concentrations to permit NMR detection. The concentration of the $^{13}$C glutamate pool is determined from the C-4 isotope since it is fed directly by acetyl-CoA and is not influenced by anaplerotic contributions. The concentrations of the $^{13}$C-enriched aspartate and citrate pools are determined from the C-3 (or C-2 for aspartate) isotopomers and require correction for unlabeled anaplerotic flux.

The total TCA cycle flux is the sum of the $^{13}$C-labeled flux and the unlabeled $^{12}$C flux, the latter commonly arising from endogenous triglycerides and glycogen. The relative contributions of the labeled and unlabeled flux can be calculated with the knowledge of the fraction of total acetyl-CoA (FEa) entering the TCA cycle that is $^{13}$C enriched. This proportion can be calculated by analysis of the $^{13}$C-$^{12}$C spin-spin splitting pattern of the glutamate C-4 resonance and the steady-state glutamate C-2/C-4 ratio after correction for nuclear Overhauser enhancement and partial saturation effects. The parameter $K_T$ (micromoles per minute per gram), which relates to total TCA cycle flux, can therefore be defined as

$$K_T = \frac{K_l}{FE_a} = \frac{[\Sigma \text{ }^{13}\text{C NMR--detected TCA metabolites (}\mu\text{mol/g)}]}{FE_a \text{ [glutamate } \Delta t_{50} \text{ (min)}]}$$

(4)

This parameter is directly proportional, but not necessarily equal, to total TCA cycle flux. This approach to TCA cycle flux calculation is based on the time difference between the appearance of tracer in various positions of the same TCA cycle--derived metabolite and is, therefore, independent of substrate-tracer input considerations. For instance, differences in isotope delivery to organs or in cellular uptake would alter equally both the $t_{50}$ for glutamate C-4 and the $t_{50}$ for glutamate C-2 but not affect the glutamate $\Delta t_{50}$.

The assumptions in this method include 1) $^{13}$C entry into the TCA cycle from $[2-^{13}$C]acetate is via acetyl-CoA, 2) $^{13}$C NMR accurately quantifies $^{13}$C enrichment of the noncarbonyl carbons of the glutamate isotopomers (see “Results”), 3) glutamate and TCA cycle intermediate pool sizes are not changing during $^{13}$C infusion under steady-state conditions, 4) the metabolism of $^{13}$C substrates is the same as that of $^{12}$C substrates, and 5) exchange of intermediates between mitochondrial and cytosolic compartments is rapid (see “Results”).

**Numerical Model of Tricarboxylic Acid Cycle Kinetics**

Because a vast number of substrate combinations could occur physiologically and could not all be reasonably evaluated experimentally, a mathematical model was developed to help assess the general validity of the proposed analytic method. This mathematical model was also used to assess the potential impact of different intermediate pool sizes, which might be induced by changes in substrate availability or oxygen delivery, on the glutamate $\Delta t_{50}$ and calculated $K_T$. These were calculated because differences in intermediate pool sizes, independent of any changes in citric acid cycle activity, may influence the time course of glutamate $^{13}$C enrichment. The model itself is presented in the "Appendix," and the findings from the model that are pertinent for interpreting the potential impact of differences in intermediate pool sizes on glutamate $\Delta t_{50}$ and $K_T$ are presented in “Results.”

**Myocardial Oxygen Consumption**

The oxygen tension of perfusate sampled from the aortic inflow and from the coronary effluent of the right ventricle was measured with a blood gas analyzer (Corning pH/Blood Gas Analyzer Model 178, Medfield, Mass.). The mean value was determined
from two to four samples for each level of contractile function. Myocardial oxygen consumption, MVo₂ (micromoles of O₂ per minute per gram wet weight), was calculated by the equation\textsuperscript{17,18}

\[ \text{MVo}_2 = \frac{\text{arteriovenous difference (mm Hg)}}{760 \text{ (mm Hg)}} \times \text{[solubility of O}_2 (\mu\text{mol/ml}] \times \text{[coronary flow (ml/min/g wet wt)]} \] (5)

**Tissue Extracts**

Some hearts underwent tissue extraction. These hearts were frozen by rapid compression between tongs cooled to the temperature of liquid nitrogen. The frozen tissue was pulverized in a mortar under liquid nitrogen in 6% perchloric acid and centrifuged for 10 minutes at 5,000g. The supernatant was neutralized with 2 M potassium hydroxide and centrifuged again. Hearts undergoing amino acid analysis by high-performance liquid chromatography (HPLC) had 0.73 mM tyramine added as an internal standard before centrifugation. Amino acid concentrations were evaluated by gradient elution on a 150 x 4.6 mm i.d. analytic column packed with 5-μm-diameter Excellopak ODS with quantitative fluorometric elute analysis. The peak ratios of glutamate, aspartate, and internal tyramine standard were compared as previously described.\textsuperscript{19,20}

**Protocols**

\textsuperscript{13}C nuclear magnetic resonance estimates of tricarboxylic acid cycle flux. First principles suggest that myocardial oxygen consumption rates parallel those of net carbon movement through the TCA cycle and therefore valid indexes of TCA cycle flux should correlate with myocardial oxygen consumption over a wide range. Two sets of independent experiments were performed to test whether these proposed \textsuperscript{13}C NMR estimates of TCA cycle flux correlate with measured myocardial oxygen consumption over a wide range of contractile performance. Because of concerns about the reliability of myocardial oxygen consumption measurements obtained from hearts within a magnet by sampling effluent outside the magnet through nonmagnetic, oxygen-permeable tubing,\textsuperscript{21} myocardial oxygen consumption was quantified in parallel experiments in separate hearts under contractile conditions identical to those in the magnet in the first series of experiments. In a second series of experiments, myocardial oxygen consumption was measured in the same hearts studied by \textsuperscript{13}C NMR on removal from the magnet at the end of each experiment. So that the results were not limited to a single set of experimental conditions, the substrates used and the manner in which developed pressure was varied also differed in these two series of experiments. All hearts were initially perfused with solution containing 1.5 mM Ca\textsuperscript{2+} while \textsuperscript{31}P and \textsuperscript{13}C standard calibration data were acquired. Each heart was subsequently studied with \textsuperscript{13}C NMR at a single developed pressure as described below. Because prior work had shown that pool sizes of various TCA cycle intermediates may change and that flux through different spans of the TCA cycle may be transiently unequal\textsuperscript{22,23} immediately after acute changes in work load or substrates, each heart was stabilized at the new developed pressure with unlabeled substrates for 12–15 minutes after the baseline period to ensure that a new steady state was reached before [2,\textsuperscript{13}C]acetate introduction.\textsuperscript{2} Thus, \textsuperscript{13}C enrichment occurred when pool sizes were stable.

In the first series of experiments (n=16), hearts were paced at 140 beats per minute, and the perfusion rate was 15 ml/min. The perfusate contained 1 mM inorganic phosphate and the sole substrate was 5 mM acetate. Each heart was studied at one of four contractilities, determined by changing perfusate calcium concentration. These were perfusion with 1.5 mM Ca\textsuperscript{2+} (n=6), 0.8 mM Ca\textsuperscript{2+} (n=3), 0.5 mM Ca\textsuperscript{2+} (n=4), or 1.5 mM Ca\textsuperscript{2+} and 20 mM K\textsuperscript{+} (n=3). Myocardial oxygen consumption was measured in separate hearts under identical conditions and at matched developed pressures.

In the second series of experiments (n=8), hearts were paced at 220 beats per minute, and the perfusion rate was increased to 20 ml/min. Inorganic phosphate was omitted from the perfusate, and the substrates were unlabeled 5 mM glucose and 0.5–0.8 mM [2,\textsuperscript{13}C]acetate. The latter allowed for exogenous substrate competition and evaluation of markedly reduced acetate concentrations while the higher heart rate permitted studies across a wider range of contractile performance. Left ventricular developed pressure was altered by changes in left ventricular end-diastolic volume accomplished by varying the intraventricular balloon volume. Myocardial oxygen consumption was measured at the end of each \textsuperscript{13}C NMR experiment.

\textsuperscript{13}C appearance in mitochondrial and cytosolic metabolites. Another series of experiments was designed to compare the time course of \textsuperscript{13}C appearance in citrate, a TCA cycle intermediate that occurs primarily in heart mitochondria (R.G. Hansford, personal communication),\textsuperscript{24–26} with its time course of appearance in glutamate, which is present in both mitochondria and cytoplasm. All hearts (n=5) were initially perfused with solutions containing 1.5 mM Ca\textsuperscript{2+} and both unlabeled 5 mM acetate and 0.8 mM pyruvate. Pyruvate was added to increase citrate concentrations\textsuperscript{27} to enhance its quantification by \textsuperscript{13}C NMR. Subsequently, equimolar [2,\textsuperscript{13}C]acetate was substituted for the unlabeled form in the presence of unlabeled pyruvate. Series of \textsuperscript{13}C spectra were acquired, and the time courses of [2,\textsuperscript{13}C]glutamate and [3,\textsuperscript{13}C]citrate appearance were compared.

**Statistics**

All results are expressed as mean±SEM. The data are compared using the unpaired t test or repeated-measures analysis of variance.\textsuperscript{28} Linear regression and nonlinear fitting were performed with commer-
cially available software (SIGMAPLOT, Jandel Scientific, and SYSTAT, SYSTAT, Inc., Evanston, Ill.).

**Results**

*Estimates of Tricarboxylic Acid Cycle Flux by $^{13}$C Nuclear Magnetic Resonance*

Hearts perfused with 5 mM acetate alone. Mean left ventricular developed pressure (developed pressure = systolic-end-diastolic pressure) in all 16 hearts studied with NMR spectroscopy during the initial high developed pressure period was 142±8 mm Hg. A representative $^{31}$P NMR spectrum acquired during this initial period is shown in Figure 2, and analysis of the peak areas reveals high levels of PCr and ATP. Most of the inorganic phosphate peak (chemical shift, 5.35 ppm relative to PCr) arises from 1 mM phosphate contained in the perfusate (pH 7.4) and relatively little from intracellular phosphate (chemical shift, 4.95 relative to PCr). No attempt was made to quantitatively distinguish these forms of inorganic phosphate. The initial mean PCr level was 7.5±0.6 µmol/g wet wt, ATP level was 3.2±0.3 µmol/g wet wt (from the β phosphorus of ATP), and intracellular pH was 7.22±0.02. These are similar to previously published values for perfused rat hearts.29-31 Both high contractile function and normal levels of high energy phosphates suggest that perfusion was adequate at this high initial contractility.

Developed pressures varied over a wide range for the four subsequent contractile conditions and were comparable for hearts studied for oxygen consumption and with NMR spectroscopy at each perfusate calcium concentration: 133±14 versus 138±3 mm Hg at 1.5 mM Ca$^{2+}$, 80±3 versus 80±1 at 0.8 mM Ca$^{2+}$, 37±9 versus 42±4 at 0.5 mM Ca$^{2+}$, and 0 versus 0 for K$^{+}$-arrested hearts (p=NS for each). The closely matched contractile indexes (Table 1) strongly suggest that metabolic flux rates were similar in these parallel experiments. Oxygen consumption increased with contractile performance and was 1.36±0.11 µmol/min/g wet wt in K$^{+}$-arrested hearts and 1.92±0.22, 2.86±0.37, and 3.68±0.35 µmol/min/g wet wt at perfusate Ca$^{2+}$ concentrations of 0.5, 0.8, and 1.5 mM, respectively (Table 1). This range of oxygen consumption values closely agrees with those previously reported for rat hearts under comparable conditions.32

Figure 3 presents a representative $^{13}$C NMR difference spectrum obtained from an intact heart 30 minutes after the substitution of [2-$^{13}$C]acetate for the nonenriched form. This spectrum was acquired during approximately 5 minutes (280 acquisitions) by using 60° pulses with a 1.1-second interpulse delay and shows the prominent peaks of the glutamate isotopomers (C-2, C-4, and C-3; peaks A, D, and E, respectively), the [2-$^{13}$C]acetate substrate (peak F), and the [6-$^{13}$C]hexanoic acid contained within the intraventricular balloon (peak H). In addition, smaller resonances attributed to aspartate (C-2 and C-3; peaks B and C) and acetylcarnitine (C-2; peak G) can be identified, as previously described.7,33,34 Citrate resonances are usually not identified in hearts perfused with acetate alone.

Figure 4 shows the time course of $^{12}$C enrichment of the glutamate isotopomers from a representative heart perfused with 1.5 mM Ca$^{2+}$ (upper panel) and that of another heart perfused with 20 mM K$^{+}$ (lower panel). The effect of reduced contractile function is to delay the time course of glutamate enrichment and to increase the time difference between the enrichment of the C-4 and C-2 glutamate isotopomers. Note that the glutamate $\Delta t_{50}$, the difference between $t_{50}$ for glutamate C-4 and glutamate C-2 denoted by the width of the shaded region, is markedly prolonged at the lower contractile performance, indicating slower citric acid cycle flux. The glutamate $\Delta t_{50}$ increased with decreasing developed pressure and
FIGURE 3. A representative proton-decoupled $^{13}$C nuclear magnetic resonance spectrum acquired over 5.1 minutes (280 pulses, 60° flip angle, 1.1-second interpulse delay) from a heart functioning at a high developed pressure (perfsate Ca$^{2+}$, 1.5 mM) 30 minutes after the substitution of 5 mM [2-$^{13}$C]acetate for the unenriched form. A spectrum acquired before introduction of the [2-$^{13}$C]acetate over an equivalent time period has been subtracted to remove the natural abundance $^{13}$C signals. Peak identification: A, glutamate C-2; B, aspartate C-2; C, aspartate C-3; D, glutamate C-4; E, glutamate C-3; F, [2-$^{13}$C]acetate; G, acetylcarminine C-2; H, [6-$^{13}$C]hexanoic acid contained within the intraventricular balloon.

FIGURE 4. Glutamate isopomer $^{13}$C enrichment versus time after substitution of nonenriched acetate by [2-$^{13}$C]acetate in representative hearts perfused with 1.5 mM Ca$^{2+}$ (upper panel) and with 20 mM K$^{+}$ (lower panel). The time to half-maximal enrichment ($t_{50}$) for each isopomer is demarcated by the dotted lines extending down to the abscissa. The time difference between glutamate C-4 and glutamate C-2 enrichment, glutamate $\Delta t_{50}$, is the width of the shaded area in each plot. The effect of decreased contractile performance is to increase the glutamate $\Delta t_{50}$ reflecting slower citric acid cycle flux.

TABLE 1. Contractile Function, Oxygen Consumption, $^{13}$C Nuclear Magnetic Resonance Data, and Calculated Tricarboxylic Acid Cycle Flux Parameters

<table>
<thead>
<tr>
<th>Perfusate (mM)</th>
<th>1.5 Ca$^{2+}$</th>
<th>0.5 Ca$^{2+}$</th>
<th>0.8 Ca$^{2+}$</th>
<th>1.5 Ca$^{2+}$</th>
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<tbody>
<tr>
<td>DP (mm Hg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NMR studies</td>
<td>0</td>
<td>37±9</td>
<td>80±3</td>
<td>133±14</td>
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<tr>
<td>MVo$_2$ studies</td>
<td>0</td>
<td>42±4</td>
<td>80±4</td>
<td>138±3</td>
</tr>
<tr>
<td>MVo$_2$ (µmol/min/g wet wt)</td>
<td>1.36±0.11</td>
<td>1.92±0.22</td>
<td>2.86±0.37</td>
<td>3.68±0.35</td>
</tr>
<tr>
<td>Glutamate C-4 $t_{50}$ (min)</td>
<td>13.9±0.6</td>
<td>4.6±0.2</td>
<td>3.9±0.4</td>
<td>3.0±0.2</td>
</tr>
<tr>
<td>Glutamate C-2 $t_{50}$ (min)</td>
<td>33.1±1.5</td>
<td>12.9±1.4</td>
<td>10.4±0.6</td>
<td>7.8±0.5</td>
</tr>
<tr>
<td>Glutamate $\Delta t_{50}$ (min)</td>
<td>19.2±1.0</td>
<td>8.3±1.2</td>
<td>6.5±0.6</td>
<td>4.8±0.4</td>
</tr>
<tr>
<td>[4-$^{13}$C]Glutamate (µmol/g wet wt)</td>
<td>5.6±0.6</td>
<td>3.9±0.5</td>
<td>4.3±0.6</td>
<td>4.2±0.5</td>
</tr>
<tr>
<td>[3-$^{13}$C]Aspartate (µmol/g wet wt)</td>
<td>0.9±0.2</td>
<td>0.8±0.2</td>
<td>1.1±0.3</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>FE$_a$</td>
<td>0.88±0.04</td>
<td>0.89±0.03</td>
<td>0.97±0.03</td>
<td>0.94±0.03</td>
</tr>
<tr>
<td>K$_t$ (µmol/min/g wet wt)</td>
<td>0.39±0.04</td>
<td>0.69±0.06</td>
<td>0.87±0.06</td>
<td>1.27±0.15</td>
</tr>
</tbody>
</table>

Values are mean±SEM. DP, developed pressure; NMR, nuclear magnetic resonance; MVo$_2$, myocardial oxygen consumption; $t_{50}$, time to half-maximal enrichment; glutamate $\Delta t_{50}$, difference in time for half-maximal enrichment of glutamate C-4 and glutamate C-2; FE$_a$, fractional enrichment of acetyl coenzyme A entering the tricarboxylic acid cycle; K$_t$, total tricarboxylic acid cycle flux parameter.

was 4.8±0.4, 6.5±0.6, 8.3±1.2, and 19.2±1.0 minutes for hearts perfused with 1.5 mM Ca$^{2+}$, 0.8 mM Ca$^{2+}$, 0.5 mM Ca$^{2+}$, and 1.5 mM Ca$^{2+}$/20 mM K$^{+}$, respectively (see Table 1). Within each group the time course and steady-state levels of glutamate C-2 and C-3 did not differ significantly from each other.
Steady-state levels of the $^{13}$C-enriched glutamate C-4 isotopomer (micromoles per gram wet weight) were comparable at perfusate Ca$^{2+}$ concentrations of 1.5 mM (4.2±0.5), 0.8 mM (4.3±0.6), and 0.5 mM (3.9±0.5) and slightly higher in K$^{+}$-arrested hearts (5.6±0.6 μmol/g wet wt).

$^{13}$C NMR quantification of total glutamate pools was also compared with HPLC quantification in heart extracts. High contractility hearts (perfusion Ca$^{2+}$, 1.5 mM) had steady-state $^{[4,13]}$C glutamate levels of 4.2±0.5 μmol/g wet wt, which when corrected by FE$\alpha$ (see below) predict total glutamate levels of 4.5±0.5 μmol/g wet wt. Total glutamate measured from extracts of four separate hearts under identical conditions with the use of HPLC was 4.2±0.4 μmol/g wet wt, which agrees well with the NMR results. Values of total glutamate pool sizes derived from $^{13}$C NMR data (4.5±0.5 μmol/g wet wt×[mean wet weight/dry weight =6.75]=30 μmol/g dry wt) are also in close agreement with those previously reported for acetate-perfused rat hearts as determined by conventional biochemical techniques (23–33 μmol/g dry wt).2,35

The different plateau values of glutamate C-4 and C-2 are due to a small amount of anaplerotic flux (e.g., the addition of unlabeled carbons to the total TCA cycle carbon pool via a mechanism other than the citrate synthase reaction). The relative anaplerotic to acetyl-CoA–derived flux was 8% for all of these hearts and agrees with previously measured values in acetate-perfused hearts.14,15

Total TCA cycle flux can be derived from the $^{13}$C-labeled flux by calculation of FE$\alpha$, the fraction of acetyl-CoA entering the TCA cycle that is $^{13}$C enriched (see Equation 4). The $^{13}$C-$^{13}$C spin-spin splitting pattern of the glutamate C-4 resonance was similar in spectra of intact hearts and of their perchloric acid extracts (not shown), and therefore intact heart data were used. FE$\alpha$ was largely unchanged over the contractile performance range studied with a mean for all hearts of 92±2% (see Table 1). This is in close agreement with previously published values of 88–97% for acetate-perfused hearts.14,15 Thus, during perfusion with $^{[2,13]}$C acetate as sole substrate, labeled flux accounts for most of the total TCA cycle flux, and KT nearly equals K$T$.

Table 1 compiles the results of contractile function, oxygen consumption, $^{13}$C NMR data, and calculated TCA cycle flux parameters for all hearts studied. In summary, increased contractile performance resulted in graded increases in oxygen consumption and decreases in glutamate ΔGσ. The calculated total TCA cycle flux constant, K$T$, increased from 0.39±0.04 to 1.27±0.15 μmol/min/g wet wt over the developed pressures studied and parallels the increase in oxygen consumption from 1.36±0.11 to 3.68±0.35 μmol/min/g wet wt.

Because a linear relation between developed pressure and oxygen consumption has previously been recognized in the isolated Langendorff-perfused rat heart,17 this relation was also analyzed in these hearts (Figure 5). The oxygen consumption/developed pressure (upper panel) and the KT/developed pressure (lower panel) relations are presented in Figure 5. Both oxygen consumption [MVO$\gamma$ = 0.0176(DP) + 1.305; r = 0.87, p < 0.01] and KT [KT = 0.00583(DP) + 0.439; r = 0.89, p < 0.01] correlate well with developed pressure (DP) over the wide range of contractile function studied. The y axis intercept indicates basal metabolic oxygen consumption (upper panel) or the basal tricarboxylic acid cycle flux parameter (lower panel).

![Graph](image-url)
zero and predicts the anticipated relation that oxygen consumption ceases when TCA cycle flux is halted.

**Hearts perfused with both glucose and acetate.** The baseline mean developed pressure for the eight hearts studied during glucose and acetate perfusion was $184 \pm 7$ mm Hg and the mean end-diastolic pressure was $3 \pm 1$ mm Hg. A representative $^{31}$P NMR spectrum is shown in Figure 7 and evidences, as in the earlier series, high levels of high energy phosphates. Note that inorganic phosphate was not present in the buffer in these experiments and therefore the small peak at approximately 5.0 ppm, relative to PCR, represents intracellular inorganic phosphate. A phosphonate standard was not used in several of these hearts, so absolute high energy phosphate levels could not be determined. However, the mean PCR/ATP ratio in these hearts was $1.9 \pm 0.1$ and the mean intracellular pH, $7.19 \pm 0.01$. Figure 8 shows a $^{13}$C NMR spectrum also obtained from a heart during perfusion with unlabeled 5 mM glucose and 0.8 mM $[^{13}$C]acetate.

The mean steady-state $^{13}$C pool size of glutamate C-4 was $3.5 \pm 0.3$ mmol/g wet wt and that of aspartate C-3, $0.9 \pm 0.2$ mmol/g wet wt. The mean contribution of anaplerotic relative to acetyl-CoA-derived flux was only $6 \pm 1\%$. The calculated mean fraction of acetyl-CoA derived from $[^{2}$H$_3$]acetate was $91 \pm 2\%$, which did not differ from that measured in the hearts perfused by acetate alone. This is likely due to inactivation of pyruvate dehydrogenase by even relatively low concentrations of medium- and short-chain fatty acids, including acetate, which has been reported previously.$^{10,35}$

Figure 9 presents the relation between myocardial oxygen consumption and the TCA cycle flux parameter, $K_T$, measured in each of these eight hearts. Note that under these conditions, with the higher pacing rate, a considerably wider range of flux rates is attained than in the earlier experiments (see Figure 6). Despite differences in experimental conditions, $K_T$ correlates strongly with myocardial oxygen consumption over a wide range [$K_T = 0.477(MVO_2) - 0.245; r = 0.94, p<0.001$] in these hearts as well. Analysis of covariance indicated that there was no significant difference between this $MVO_2/K_T$ relation (Figure 9) and that observed in the first series of experiments (Figure 6). It is important to note that under two separate experimental conditions that differ in substrate availability, heart rate, coronary flow, ionic milieu, and the manner in which contractile function was varied the $^{13}$C NMR parameter $K_T$, which we have proposed to index total TCA cycle flux, correlates strongly with myocardial oxygen consumption across a wide range.

**Numerical modeling findings.** To gain insight into the general applicability of this method to other
conditions such as altered substrate availability and oxygen delivery, which can be associated with different TCA cycle pool sizes, a mathematical model of the TCA cycle was developed. It is based on the work of Chance et al,7 in which pool sizes can be altered independently of TCA flux and the impact on the time course of glutamate $^{13}$C enrichment assessed (see “Appendix”). The model was used to assess the impact on glutamate $^{13}$C enrichment of changes in intermediate pool sizes, which can often be quantified by $^{13}$C NMR (i.e., glutamate, aspartate, and citrate) and of those that are usually too small to be quantified by $^{13}$C NMR spectroscopy (i.e., oxaloacetate, 2-oxoglutarate, succinate, and malate).

First, the impact of separately varying the pool sizes of glutamate, aspartate, and citrate over broad ranges while TCA cycle flux was held constant on glutamate $\Delta_{50}$ and on $K_T$ was calculated from Equation 4. It was assumed that pool sizes below 0.5 $\mu$mol/g wet wt were not quantified by $^{13}$C NMR and thus neglected in calculation of $K_T$, as in the earlier experiments. Figure 10 shows that at a constant cycle flux the glutamate $\Delta_{50}$ increases as expected with increases in the glutamate, aspartate, and citrate pools and importantly demonstrates that calculated $K_T$ is relatively unchanged by these large changes in pool sizes. The mean $K_T$ was 1.01±0.01 $\mu$mol/min/g wet wt for this range of glutamate, aspartate, and citrate pools, which extend beyond those previously measured in hearts exposed to substrates other than acetate.2,4,7,35 Second, the potential impact of changes in the smaller pools of oxaloacetate, 2-oxoglutarate, succinate, and malate on $K_T$ was assessed in a similar manner. The extent to which these smaller pools, which typically cannot be quantified by $^{13}$C NMR and hence are neglected in calculation of $K_T$, are changed by conditions of altered substrate or oxygen availability has been less well studied. Therefore, an extreme example of a fourfold increase in the pool sizes of all of these intermediates was chosen. Such changes considerably exceed, for example, the roughly threefold increases in succinate and malate, unchanged fumarate, and 80% decrease in 2-oxoglutarate reported after 20 minutes of total ischemia in glucose-perfused rat hearts.13 Figure 11 presents the time course $^{13}$C-enrichment curves for the glutamate C-4 and C-2 isotopeomers derived from the model for the control (solid line) and increased pool size (dotted line) conditions and demonstrates that for both

### Figure 8
A representative proton-decoupled $^{13}$C nuclear magnetic resonance spectrum acquired in a heart perfused with unlabeled 5 mM glucose and 0.8 mM [2-$^{13}$C]acetate. Peak identification: A, glutamate C-2; B, glutamate C-4; C, glutamate C-3; D, hexanoic acid C-6 (contained within intraventricular balloon). Note that a resonance from the buffer [2-$^{13}$C]acetate is barely detectable.

### Figure 9
Total tricarboxylic acid cycle flux parameter, $K_T$, versus myocardial oxygen consumption (MVO$_2$) as studied in the same eight hearts. Variations in developed pressure were achieved by changes in intraventricular balloon volume except for the two lowest, which were observed during near-contracitile arrest with perfusate calcium of 0.2 mM. There is a strong linear correlation of $K_T$ with MVO$_2$ [$K_T=0.477(MVO_2)-0.245$; $r=0.94$, $p<0.001$] over a wide range of contractile performance.

---

**Notes:**
- Weiss et al. Indexing Krebs Cycle Flux by Carbon-13 NMR.  
- Figures 8 and 9 are included as illustrations.
- The text discusses the impact of changes in glutamate, aspartate, and citrate pool sizes on the calculation of $K_T$.
- The model is used to assess the impact on glutamate $^{13}$C enrichment of changes in intermediate pool sizes.
- The significance of the change in pool sizes is compared to physiological changes.
high contractility (perfusate Ca$^{2+}$, 1.5 mM) and non-beating (perfusate K$^+$, 20 mM) hearts perfused with 5 mM acetate, the resultant changes in glutamate $^{13}$C enrichment are small. This simultaneous fourfold increase in the oxaloacetate, 2-oxoglutarate, succinate, and malate pools alters the glutamate $\Delta t_{50}$ and calculated $K_T$ by only 5-7%.

**Compartmentation and $^{13}$C Labeling Kinetics**

Although TCA cycle activity occurs in mitochondria, a significant fraction of myocardial glutamate is located in the cytoplasm. To investigate whether the time course of glutamate $^{13}$C enrichment reflects that of mitochondrial TCA cycle intermediates, additional experiments were performed. Because rat heart mitochondria, unlike liver or adipocyte mitochondria, are relatively impermeable to citrate (R.G. Hansford, personal communication), we compared the time course of $^{13}$C appearance in the glutamate isotopomers with that in citrate derived from the same “turns” of the TCA cycle. Because previous work has shown that pyruvate perfusion increases cellular citrate concentrations approximately 20-fold,$^{27}$ unlabeled 0.8 mM pyruvate was added to the perfusate in this series of hearts ($n=5$) to increase citrate $^{13}$C resonances and enhance quantification.

Figure 10 presents an expanded region of a $^{13}$C difference spectrum from an intact heart acquired 25 minutes after perfusion with unlabeled 0.8 mM pyruvate and 5 mM [2-$^{13}$C]acetate. In comparison to hearts perfused with acetate alone (Figure 3), prominent peaks of citrate C-4 and citrate C-2 at 45 ppm and citrate C-3 at 76 ppm are readily identified.$^{37}$ Note that the glutamate resonances are much less prominent than those in hearts perfused solely with
acetate (Figure 3) because of both the decrease in $^{13}$C enrichment of acetyl-CoA entering the citric acid cycle and the previously reported smaller glutamate pool size during unlabeled pyruvate supplementation. Because of the symmetry of the citrate molecule, citrate C-4 and C-2 cannot be resolved. Hence the citrate C-3 resonance, which is well resolved, was quantified and the enrichment time course compared with that of the equivalent glutamate isotopomer (C-2 or C-3), which is also enriched in the later "turns" of the cycle. Figure 13 displays citrate C-3 and glutamate C-2 enrichment versus time after [2-$^{13}$C]acetate was substituted for the unenriched form. The time courses of $^{13}$C enrichment of citrate C-3 and glutamate C-2 are similar and not statistically different. Because rat heart mitochondria are relatively impermeable to citrate, these findings indicate that, within the limits of the signal-to-noise ratio and temporal resolution of these experiments, the time course of $^{13}$C glutamate enrichment is similar to that of citric acid cycle intermediates confined to the mitochondria and further suggest that quantification of myocardial glutamate $^{13}$C enrichment provides valid information regarding citric acid cycle kinetics.

**Discussion**

In this study, a method of indexing TCA cycle flux solely by $^{13}$C NMR spectroscopy is proposed and tested. It is hypothesized that TCA cycle flux is inversely proportional to the time difference between $^{13}$C appearance in carbon positions of metabolites that are enriched in sequential "turns" of the TCA cycle. Estimation of TCA cycle flux by this $^{13}$C approach is strongly supported by the correlation of the $^{13}$C-derived TCA cycle flux parameter, $K_T$, with measured myocardial oxygen consumption over a

*Figure 11. The numerical model-generated time course of glutamate isotopomer enrichment for the extremes of cardiac contractile performance studied. The effect on glutamate $^{13}$C enrichment of a simultaneous fourfold increase in the oxaloacetate, 2-oxoglutarate, succinate, and malate pool sizes (dotted lines) over that calculated using pool sizes reported in acetate-perfused hearts (solid lines) is a subtle delay in each isotopomer enrichment. This increase in pool sizes altered the calculated total tricarboxylic acid cycle flux parameter by only 5–7%.*

*Figure 12. Representative proton-decoupled $^{13}$C nuclear magnetic resonance spectrum obtained from an intact heart 30 minutes after perfusion with nonenriched 0.8 mM pyruvate and 5 mM [2-$^{13}$C]acetate (420 acquisitions, 1.1-second interpulse delay, natural abundance resonances subtracted). Peak identification: A, citrate C-3; B, glutamate C-2; C, aspartate C-2; D, citrate C-4 and C-2; E, aspartate C-3; F, glutamate C-4; G, glutamate C-3. In comparison to hearts perfused with [2-$^{13}$C]acetate alone, the citrate resonances (A and D) are increased and the glutamate resonances (B, F, and G) are reduced.*
wide range of contractile function under different experimental conditions.

Most previous investigations with labeled substrates to index oxidative metabolic flux have relied on the detection of tissue or effluent radioactivity after administration of radiolabeled substrates. In a recent report, the rate of the initial rapid phase of tissue and effluent radioactivity from isolated rat hearts correlated with oxygen consumption after bolus administration of tracer [1,14C]acetate. As in the current investigation, several different levels of cardiac contractile performance were studied, a TCA cycle flux parameter but not absolute TCA cycle flux was calculated, and the results correlated with oxygen consumption. Like all radioisotope studies, however, identification of the specific metabolites containing the 14C radioactivity could not be determined nondestructively. Hence, the precise causes of the poor correlation with oxygen consumption when nontracer acetate concentrations were used and the mechanism of the second kinetic component for late 14C washout could only be inferred.

This proposal uses 13C NMR spectroscopy, which can quantify on a continuous and nondestructive basis the time course of appearance of labeled carbon nuclei within various intracellular metabolites and even in specific positions within a given metabolite. A detailed mathematical model of TCA cycle flux with 13C NMR data has been reported previously for extracts from isolated hearts in which the citric acid cycle reactions were mathematically modeled and the solutions to the corresponding 200 differential equations obtained using FACSIMILE software. Some distinct advantages of that method include the absolute estimation of TCA cycle flux as well as the aspartate aminotransferase exchange and acetyl-CoA formation rates. In addition, that model provides the useful ability to mathematically alter metabolic parameters and predict the impact on calculated flux rates. More recently, a less complicated model for estimating citric acid cycle flux in brain by using H-detect, 13C-decoupled NMR spectroscopy has been reported; these results, however, have not been rigorously compared with another index of citric acid flux (e.g., oxygen consumption).

The method proposed in the present study for estimating of TCA cycle flux is a simplified empirical approach to the many reactions that make up the TCA cycle. This method can be used in conjunction with computer modeling and offers some unique advantages. The computer modeling of the TCA cycle by Chance et al. as well as the mathematical model presented in the “Appendix” requires detailed information about metabolite pool sizes for each modeled reaction. Such information can be obtained only from chemical analysis of tissue extracts or from published literature. While the latter is valid if experimental conditions exactly mimic those in previously published work, the former requires tissue destruction, thus precluding serial studies in a single organ as well as in vivo studies. For these reasons, there are considerable potential advantages of indexing TCA cycle flux by 13C NMR measures alone. The parameters can be obtained nondestructively and rely minimally, or not at all, on results from prior studies. In addition, the presented method can be more readily used to quantitatively compare results among different 13C NMR studies since the TCA cycle flux indexes are experimentally determined and not model dependent.

A theoretical limitation of the proposed construct is that changes in TCA cycle intermediate pool sizes that are not detected by NMR could change the time course of 13C enrichment in glutamate C-2 and C-3 relative to that of glutamate C-4 and thereby alter Kf in the presence of an unchanged TCA cycle flux. The strong correlation of derived TCA cycle flux parameters with oxygen consumption and contractile indexes indicates that either the pool sizes of TCA cycle intermediates not detected by 13C NMR do not vary significantly over the range of developed pressures studied at steady state or, if they do, that such differences are not of sufficient magnitude to significantly alter the Kf. Moreover, the results of numerical modeling of the TCA cycle and 13C glutamate isotopomer enrichment (Figures 10 and 11 and “Appendix”) suggest that even marked fourfold simultaneous increases in the oxaloacetate, 2-oxoglutarate, succinate, and malate pools over published values in the absence of altered cycle flux would change Kf by less than 7%. Larger increases in these pools are unlikely to occur physiologically but if present would probably permit NMR detection of the metabolite pools of succinate and malate that could then be used in the calculation of Kf. Thus, the total TCA cycle flux parameter, Kf, is relatively insensitive to changes in pools not detected by 13C NMR. The modeling data also support the general applicability of the proposed empiric method of indexing TCA cycle flux by Kf beyond the experimental conditions studied.
Another potential limitation is that unlabeled anaplerotic flux could affect the time course of glutamate C-2 or C-3 enrichment but not C-4 and thus impact on calculated $K_t$ independent of the steady-state acetyl-CoA–derived TCA cycle flux. Under nearly all physiological conditions the contribution of anaplerotic flux relative to acetyl-CoA–derived flux is relatively small; therefore, one would not anticipate that physiological anaplerosis would significantly alter these $^{13}$C estimates of TCA cycle flux beyond the inherent uncertainty in the constituent measurements.

Acetate was chosen as the $^{13}$C-enriched substrate for these studies since it enters the TCA cycle directly via the citrate synthase reaction as acetyl-CoA. Acetate has been reported to increase myocardial oxygen consumption and TCA cycle flux in hearts previously perfused with glucose.\(^2\) Unequal flux rates through different spans of the cycle were observed in those experiments moments after the increase in flux. Rapid attainment of steady-state occurred, however, and flux through the different spans equalized, as assumed in our studies. The methods presented here could be applied to other substrates that enter the TCA cycle as acetyl-CoA. For instance, the individual $t_{50}$ for each glutamate isotopomer obtained using $[^1\text{--}^{13}]$glucose substrate would be additionally dependent on glucose uptake, glycolytic metabolism, and the pyruvate dehydrogenase reaction. Nevertheless, the difference in the time courses of C-4 and C-2 glutamate enrichment, the glutamate $\Delta t_{50}$, would not be affected by these reactions and would depend solely on TCA cycle metabolism. In a previous report, we described $[^1\text{--}^{13}]$glucose metabolism during graded reductions in coronary flow in isolated rat hearts.\(^11\) A reduction of coronary flow from 15 to 5 ml/min resulted in reduced $[4\text{--}^{13}]$glutamate levels (from 1.5 to 1.0 $\mu$mol/g wet wt) and increased the $t_{50}$ for glutamate C-4 (from 5 to 12 minutes) and for glutamate C-2 (from 9 to 17 minutes). With the current construct, reduction of flow from 15 to 5 ml/min in $[^1\text{--}^{13}]$glucose-perfused hearts resulted in a 47% reduction in calculated $K_t$ (from 0.83 $\mu$mol/g wet wt at 15 ml/min to 0.44 $\mu$mol/g wet wt at 5 ml/min), which closely paralleled the 49% reduction in contractile function.

Although subcellular compartmentation of many metabolic reactions has long been recognized, most quantitative approaches to TCA cycle flux have not addressed the potential impact of bidirectional carbon transport between mitochondrial and cytoplasmic loca
tions.\(^2\)\(^7\) Because $^{13}$C movement from the mitochondria to the cytoplasm is crucial for $^{13}$C NMR quantification of TCA cycle flux by sequential glutamate exchange, the similar time course of $^{13}$C appearance in citrate C-3 and glutamate C-2 (Figure 13), within the limits of the signal-to-noise ratio and temporal resolution of these experiments, suggests that the kinetics of $^{13}$C appearance in predominantly mitochondrial and cytoplasmic pools is comparable and that transaminase activity is rapid relative to that of net TCA cycle flux.

The application of this method of indexing TCA cycle flux by $^{13}$C NMR to in vivo studies remains untested but may be possible with recently described spatially localized proton-decoupled $^{13}$C NMR cardiac techniques.\(^8\) Inherent uncertainties in in vivo studies of quantification of the time from peripheral injection of tracer to time of target tissue delivery are due to many factors that affect delivery and blood flow but should not be problematic with this approach, which primarily depends on the difference in time between sequential glutamate isotopomer enrichment. The major hurdles to in vivo application of this method will be the temporal resolution for quantification of the glutamate $\Delta t_{50}$ and the resolution of the glutamate C-4 carbon-carbon splitting for quantification of $F_{Fe}$. The combined use of this approach with spatially localized NMR spectroscopic techniques may permit the assessment of regional myocardial TCA cycle flux and, therefore, of approximating regional oxygen consumption.

In summary, a simple approach to index TCA cycle flux in intact, isolated perfused hearts has been presented that uses only $^{13}$C NMR data, namely, the time difference in enrichment of glutamate isotopomers derived from sequential turns of the citric acid cycle, the $^{13}$C NMR–detectable metabolite pool sizes, and the fraction of acetyl-CoA that is $^{13}$C enriched entering the cycle. The validity of the approach was demonstrated by a strong correlation with myocardial oxygen consumption over a wide range of cardiac contractile function and extrapolation to a near-zero intercept when predicted oxygen consumption ceased in two separate experiments with different substrate availability. The general applicability of the approach is suggested by mathematical modeling findings that indicate that $K_t$ is relatively independent of marked differences in TCA cycle pool sizes. In addition, the similar time course of $^{13}$C enrichment in predominantly mitochondrial and cytosolic metabolites provides further evidence of the ability of these techniques to index mitochondrial citric acid cycle flux in the intact heart.

**Appendix**

This empirical method has proposed that citric acid cycle activity can be indexed by the time difference between the labeling of the C-4 and C-2 glutamate isotopomers (the glutamate $\Delta t_{50}$). It is important to note, however, that changes in intermediate pool sizes, independent of any change in citric acid cycle activity, may influence the delta variable. A numerical model of the citric acid cycle was developed to assess the impact of changes in the pool sizes of various intermediates on the time course of glutamate isotopomer $^{13}$C enrichment and on the calculated total cycle flux parameter, $K_t$, at a given cycle flux.

The dynamics of $^{13}$C labeling of citric acid cycle intermediates was computed using a model derived from the analysis of Chance et al.\(^7\) This analysis is based on a simplified scheme of the cycle containing...
citrate, α-ketoglutarate, succinate, malate, and oxaloacetate, connected by effectively irreversible reactions and coupled to pools of glutamate and aspartate by way of the alanine aminotransferase and aspartate aminotransferase reactions.

The full model, as analyzed by Chance et al, requires 176 differential equations for the various possible species labeled (or unlabeled) at all possible positions. Since only the total labeling of carbons 2 and 4 in glutamate is required for comparison with our data, the full model is not needed. Because of the symmetry resulting from the isotopic independence of reaction rates, the equations may be summed over intermediates labeled at a common position to obtain 17 differential equations that describe the evolution of the fractional labeling of individual carbon positions. To demonstrate this algebraically is extremely tedious, but it may be understood intuitively by considering the possible contributions to the rate of change of the fractional labeling of the “pool” representing a particular carbon on a particular intermediate. Because the reaction scheme determines unambiguously the possible sources of this carbon, the fractional labeling can increase by addition of labeled carbon from one of these source pools and decrease by exit of carbon along the reaction path(s) leading away from the intermediate in question. The general form of such an equation is therefore

\[
dL_i/dt = \sum (F_{ij}L_j) - F_{out}L_i/P_i
\]

where \(L_i\) is the fractional labeling of the \(i\)th pool, \(F_{ij}\) is the steady-state absolute reaction flux from the \(j\)th to the \(i\)th intermediate, \(F_{out}\) is the total reaction flux leaving the \(i\)th intermediate, and \(P_i\) is the absolute size of the \(i\)th intermediate pool (regardless of labeling). All reactions are assumed to be at chemical (as opposed to isotopic) steady state, with “known” reaction fluxes, some of which are to be fitted from the data. The actual equations are as follows:

\[
\frac{dK3}{dT} = \frac{-(FAL+FA+F)K3+(FAL+FA)G3+CI3×F}{PK}
\]

\[
\frac{dS2}{dT} = \frac{F(K3−S2)}{PS}
\]

\[
\frac{dS3}{dT} = \frac{F(K4−S3)}{PS}
\]

\[
\frac{dM3}{dT} = \frac{F\left(\frac{(S3+S2)}{2}-(FP+1)M2\right)}{PM}
\]

\[
\frac{dO3}{dT} = \frac{-(FA+F)O3+F×M3+A3×FA}{PO}
\]

\[
\frac{dC12}{dT} = \frac{F(O3−C12)}{PCI}
\]

\[
\frac{dK2}{dT} = \frac{-(FAL+FA+F)K2+(FAL+FA)G2+CI2×F}{PK}
\]

\[
\frac{dG2}{dT} = \frac{(FAL+FA)(K2−G2)}{PG}
\]

\[
\frac{dG4}{dT} = \frac{(FAL+FA)(K4−G4)}{PG}
\]

\[
\frac{dC14}{dT} = \frac{(AC−C14)F}{PCI}
\]

\[
\frac{dK4}{dT} = \frac{-(FAL+FA+F)K4+(FAL+FA)G4+CI4×F}{PK}
\]

Here CI, K, S, M, O, G, and A stand for citrate, α-ketoglutarate (2-oxoglutarate), succinate, malate, oxaloacetate, glutamate, and aspartate, respectively. K4, for example, is the fractional labeling of carbon 4 on α-ketoglutarate (using the numbering scheme of Chance et al?), and PCI, PK, PS, PM, PO, PG, and PA are the absolute total pool sizes of the corresponding intermediates. F is the citric acid cycle flux, and FA and FAL are the exchange fluxes of aspartate aminotransferase and alanine aminotransferase, respectively. AC is the fractional labeling of (carbon 2 of) acetyl-CoA (termed \(F_E\) in the text), and anaplerosis is represented by exchange of malate with an infinite unlabeled pool at a flux rate FP×F, where FP is the dimensionless fractional rate of anaplerosis. Pool sizes and fluxes are in micromoles per gram wet weight and micromoles per gram wet weight per minute, respectively; any consistent units could be
used, however, since the calculated fractional labelings are dimensionless. The pool sizes of glutamate and aspartate were measured; other pool sizes were taken from the literature but were shown to have minimal effect (over a fourfold range) on the time course of glutamate labeling (see Figures 10 and 11). The transaminase fluxes were taken as the mean values measured by Chance et al7 but could equally well have been computed from the pool sizes and assumed rate constants. The citric acid cycle flux F and fractional anaplerotic flux FP were determined by fitting the model to the G4 and G2 data measured from the hearts by using a Marquardt-Levenberg iterative least-squares fitting algorithm (Figure A1). All model computations were performed using the modeling language MATLAB (CiviliZed Software, Bethesda, Md.).

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References

interactions with glycolysis in the perfused rat heart. Biochem J 1972;128:147–159
29. Williamson JR: Glycolytic control mechanisms: Inhibition of glycolysis by acetate and pyruvate in the isolated, perfused rat heart. J Biol Chem 1965;240:2308–2321
35. Latipaa PM, Peuhkurinen KJ, Hiltunen JK, Hassinen IE: Regulation of pyruvate dehydrogenase during infusion of fatty acids of varying chain lengths in the perfused rat heart. J Mol Cell Cardiol 1985;17:1161–1171

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R G Weiss, S T Gloth, R Kalil-Filho, V P Chacko, M D Stern and G Gerstenblith

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