Use of a single $^{13}$C NMR resonance of glutamate for measuring oxygen consumption in tissue

F. MARK H. JEFFREY, ALEXANDER RESHETOV, CHARLES J. STOREY, RUI A. CARVALHO, A. DEAN SHERRY, AND CRAIG R. MALLOY

Department of Radiology, The Mary Nell and Ralph B. Rogers Magnetic Resonance Center, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9085; Intel, Santa Clara, California 95052-8119; and Department of Chemistry, University of Texas at Dallas, Richardson, Texas 75083-0688

FUNCTIONAL IMAGING STUDIES have stimulated interest in measurement of tissue oxygen consumption, yet few methods exist that provide this measurement noninvasively. Nuclear magnetic resonance (NMR) spectroscopy in combination with $^{13}$C tracers could in principle fulfill this need. A number of recent papers have analyzed the rates of appearance of $^{13}$C in metabolic processes and have presented methods for calculating oxygen consumption from $^{13}$C nuclear magnetic resonance (NMR) multiplet data. We have developed a model for calculating oxygen consumption from $^{13}$C NMR data that is based on a kinetic analysis of glutamate metabolism. The model is able to estimate oxygen consumption directly from intact tissue with $^{13}$C multiplet data and to compare this with the more typical measurement of total resonance areas (a reflection of $^{13}$C fractional enrichment). Furthermore, the glutamate C-4 resonance appears in a relatively uncrowded region of the spectrum and the doublet component of this resonance (referred to here as C4D34) is resolved in spectra of human brain (4). Therefore, it was decided to develop a model for calculating oxygen consumption directly from intact tissue with $^{13}$C multiplet data and to compare this with the more typical measurement of total resonance areas (a reflection of $^{13}$C fractional enrichment). Furthermore, because the glutamate C-4 resonance appears in a relatively uncrowded region of the spectrum and the doublet component of this resonance is resolved in spectra of human brain, our kinetic analysis was also applied to the temporal evolution of the C4D34 doublet as the only NMR measurement.

A necessary component of the analysis of the time course of glutamate enrichment is the rate of exchange between $\alpha$-ketoglutarate and glutamate ($V_{\alpha}$) (33). However, estimates of this parameter have varied considerably, ranging from 0.2 (2) to 4 (1) times the citric acid cycle flux ($V_{\text{C4A}}$) in the perfused rat heart. The effect of uncertainty in the exchange rate on citric acid cycle flux estimates must be evaluated because these two parameters are correlated (33), and it has been recognized that the exchange parameter is responsible for much of the uncertainty in estimating citric acid cycle flux (8).

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MEASURING OXYGEN CONSUMPTION WITH 13C NMR

The purpose of this work was to develop a kinetic model capable of analyzing 13C NMR multiplet data, as well as fractional enrichment data, and to evaluate which data variables provide the most reliable estimation of oxygen consumption. Experiments were performed under both “ideal” conditions (13C NMR of solution of oxygen consumption. Experiments were performed to determine the benefits of including multiplet data, the correlation between parameters, and the influence of uncertainty in $V_x$ on $V_{TCA}$ and hence oxygen consumption. It was found that the addition of 13C multiplet data significantly improved the measurement of oxygen consumption. The same benefit was found in the analysis of the C4D34 doublet as the only NMR measurement. Because the glutamate C-4 resonance is reasonably well resolved from other carbon resonances in vivo spectra, temporal measurement of the C4D34 doublet may offer certain advantages for in vivo studies. The C4D34 doublet could be detected with single-frequency 13C excitation and narrow-band 1H decoupling, thereby making spatial localization schemes simpler and potential concerns about tissue heating less of an issue.

MATERIALS AND METHODS

[2-13C]sodium acetate, [3-13C]sodium pyruvate, and [1-13C]glucose (all 99%) were purchased from Cambridge Isotopes (Andover, MA). [3-13C]sodium propionate (99%) was obtained from Isotec (Miamisburg, OH). Other common materials were obtained from Sigma (St. Louis, MO). Perfusions were conducted with Sprague-Dawley rats weighing 350–400 g and fed ad libitum.

Heart perfusions. Rat hearts were perfused by the Langendorff method with standard Krebs-Henseleit bicarbonate buffer bubbled with 95% O2-5% CO2 at 37°C and a column height of 100 cm. Hearts were placed inside the magnet, and the temperature of perfusate surrounding the heart was monitored with a fiber optic thermometry system (Luxtron, Santa Clara, CA). An IonPac AS11 analytical column was used with an anion self-regenerating suppressor and CD20 conductivity detector. Intermediates were eluted with a gradient of NaOH (0.5 mM, increased linearly to 5 mM over 7 min). Concentration measurements were made after the calibration curves with standards were prepared. The malate and succinate peaks coeluted at the same time, and hence their combined tissue contents were obtained. Chromatography peak areas were measured with the PeakNet software supplied with the instrument.

NMR methods. A General Electric Omega 400-MHz spectrometer (Bruker Instruments, Billerica, MA) was used to collect 13C spectra at 100.6 MHz and 1H spectra at 400.1 MHz. 13C NMR spectra obtained from extracts were collected in 32K data blocks with a sweep width of 20,000 Hz and WALTZ-16 broad-band proton decoupling in a 5-mm broad-band probe. Pulses (45°) were applied every 3 s. These pulsing conditions were optimal for achieving maximal signal-to-noise without differential saturation of the protons of glutamate resonances. The number of scans acquired varied from 4,000 (hearts freeze-clamped after 30 min) to 18,000 (hearts freeze-clamped at 3 min). [3-13C]propionate (2 µmol) was added as an internal standard. 13C NMR spectra of intact hearts were acquired in a 20-mm broad-band probe. Data were collected in 16K data blocks with 45° pulses with a 3-s interpulse delay. Spectra were collected in 5-min blocks (100 scans). Broad-band proton decoupling was performed with
WALTZ-16 with bievel decoupling; 3 W were applied during acquisition of the free induction decay (0.82 s) and 0.3 W between pulses. An external standard consisting of a small bulb with 80 µl dioxane was placed close to the aorta of the hearts. 1H NMR spectra of extracts were obtained with 45º pulses with a 7.0-s interpulse delay and presaturation of water; 256 transients were collected in 8K data blocks.

Free induction decays were analyzed by baseline correction (to remove direct current voltage offsets), exponential multiplication, and Fourier transformation. A line broadening of 0.5 Hz was used to analyze the data collected from extracts. To measure the multiplet areas from spectra collected from intact hearts, 4-Hz line broadening was used. The total area of signals corresponding to the glutamate carbons collected from intact hearts was measured after subtracting the initial background spectrum from all subsequent spectra. These were then analyzed after being processed with 35-Hz line broadening. To measure directly the fractional enrichment in glutamate C-4, 1H NMR spectra were analyzed in spectra processed with 0.5-Hz line broadening. The area of the 13C satellite signals was divided by the total area of the H4 resonance (sum of the 12C and 13C components).

Peak areas were measured by line fitting to the Voigt line shape (18) with custom-written software. The line widths of peaks arising from individual carbons were set to a single (optimized) value. The areas of the multiplets contributing to each resonance were expressed as a fraction of total resonance area, as described elsewhere (6, 15, 16). For example, the glutamate C-4 resonance always consisted of a singlet (C4S) and a doublet (C4D34) in the experiments reported here, with C4D34 representing the area of the doublet expressed as a fraction of the total C-4 resonance area (i.e., C4S + C4D34 = 1). Because only one of these fractional measurements is independent, the C4D34 was chosen for analysis. The peaks corresponding to the C2D12 multiplet were small in hearts perfused with enriched acetate and not very well resolved from lines corresponding to the C2D23 multiplet. Therefore, only the values for the quartet (Q) and singlet from the C-2 spectrum (C2Q and C2S, respectively) were included in the data for kinetic analysis. Similarly, the C-3 resonance consists of an overlapping singlet (typically small) and triplet plus a well-resolved doublet. Rather than introducing error by attempting to separate the C3S and C3T areas, the C-3D multiplet was included as the only independent measurement from this resonance.

Model. A schematic of the model used for the kinetic analysis of the citric acid cycle is shown (Fig. 1). This model is similar to the original used by Chance et al. (1) except that the exchange reaction between alanine and pyruvate was not included, the pathways of anaplerosis were added, and the four-carbon intermediates of the cycle are represented by malate only. The exchange between alanine and pyruvate was not included because pyruvate is not a citric acid cycle intermediate, so the rate of alanine flux is limited to the labelling of intermediates feeding into acetyl-CoA and involved in anaplerotic pathways is fast with respect to citric acid cycle turnover; 2) all reactions can be treated as irreversible without influencing the rate of labeling of glutamate, except of course for exchange between oxaloacetate, α-ketoglutarate, aspartate, and glutamate; 4) no incorporation of 13CO2; and 5) rapid equilibration of enrichment between the inner carbons and between the outer carbons of the symmetrical four-carbon intermediates.

The parameters that can be estimated from the model are listed in the caption to Fig. 1. In principle, the model can be extended to analyze the other labeling patterns in acetyl-CoA ([1-13C]acetate) and pyruvate ([3-13C]pyruvate); PyrD, fraction of anaplerotic substrate, from, for example, [3-13C]pyruvate in the glucose + [3-13C]pyruvate group; V_{TCA}, citric acid cycle flux; V_s, exchange flux; and Y, influx of anaplerotic substrate relative to V_{TCA}. Rate of disposal pathways is equal to anaplerotic flux.
unlabeled isotopomers with time

\[
\frac{d}{dt} AC_0 = V_{TCA} \left( F_{C0} - AC_0 \right) / \left[ AC \right]
\]

\[
\frac{d}{dt} CIT_0 = V_{TCA} \left( AC_0 [OAA_0 + OAA_1] - CIT_0 \right) / \left[ CIT \right]
\]

\[
\frac{d}{dt} \alpha\text{-KG}_0 = \left( V_{TCA} \left( CIT_0 - \alpha\text{-KG}_0 \right) \right) + V_x \left( GLU_0 - \alpha\text{-KG}_0 \right) / \left[ \alpha\text{-KG} \right]
\]

\[
\frac{d}{dt} MAL_0 = V_{TCA} \left( [MAL] \cdot \alpha\text{-KG}_0 + \alpha\text{-KG}_0 - MAL_0 \right) / \left[ MAL \right]
\]

\[
\frac{d}{dt} OAA_0 = V_{TCA} \left( MAL_0 + Y \cdot Pyr_0 - [1 + Y] \cdot OAA_0 \right) + V_x \left( ASP_0 - OAA_0 \right) / \left[ OAA \right]
\]

\[
\frac{d}{dt} GLU_0 = V_x \left( ASP_0 - OAA_0 \right) / \left[ GLU \right]
\]

\[
\frac{d}{dt} ASP_0 = V_x \left( OAA_0 - ASP_0 \right) / \left[ ASP \right]
\]

where AC, CIT, \(\alpha\text{-KG},\) MAL, OAA, GLU, and ASP are acetyl-CoA, citrate, \(\alpha\text{-ketoglutarate,}\) malate, oxaloacetate, glutamate, and aspartate, respectively; and subscripts denote the position of \(^{13}\text{C}\) in the carbon backbone (thus, for example, OAA\(_0\), OAA\(_1\), and OAA\(_4\) are the fractional amount of unlabeled, [1-\(^{13}\text{C}\)]citrate, and [4-\(^{13}\text{C}\)]oxaloacetate, respectively). The unlabeled fraction of external substrates is represented by F\(_{C0}\) for the oxidized substrate and Pyr\(_0\) for the anaplerotic substrate. Because isocitrate dehydrogenase was assumed to be irreversible, [6-\(^{13}\text{C}\)]citrate (formed from the condensation of [1-\(^{13}\text{C}\)]oxaloacetate with unlabeled acetyl-CoA) was treated as unlabeled citrate, because the C-6 is lost as CO\(_2\). Equations similar to the above can be written for all the isotopomers containing \(^{13}\text{C}\).

The model consists of a system of 88 equations (16 for each of citrate, \(\alpha\text{-ketoglutarate,}\) glutamate, and aspartate; 12 for both malate and oxaloacetate). The model was implemented as a Fortran program, with the routine LSODE (5) for the solution of stiff ordinary differential equations. This provided a solution of the system of equations, with the routine LSODE (5) for the solution of stiff ordinary differential equations. This provided a solution of the system of equations, which was used to obtain kinetic parameters for the glutamate pool from the model.

The model consisted of the total glutamate C-4 and C-3 (or C-2) resonance areas. The C-2 was used in spectra from hearts perfused with glucose plus pyruvate (the signal from [3-\(^{13}\text{C}\)]pyruvate present in the perfusate overlapped somewhat with glutamate C-3, see Fig. 4A), whereas the C-3 resonance was used for hearts perfused with glucose plus acetate. Values for F\(_{C2}\) and Y were used as fixed values.

Finally, the actual fractional enrichment in glutamate C-4 measured with \(^1\text{H}\) NMR of extracts from hearts at steady state was input as a fixed value in the model. As explained in RESULTS, the C-4 fractional enrichment measured by \(^1\text{H}\) NMR can differ from the value for F\(_{C4}\), indicating that the entire glutamate pool was not involved with the citric acid cycle. This would affect the absolute flux value calculated by the model. Therefore, the measured glutamate content was adjusted by the ratio of F\(_{C4}\) over the absolute fractional enrichment before calculation of the change in glutamate isotopomers with time.

Because not all of the glutamate pool is involved with the citric acid cycle, this would also introduce error if fractional enrichments were measured with an external \(^{13}\text{C}\) standard. Therefore, the C-4 and C-3 (or C-2) total resonance areas were not converted to actual \(^{13}\text{C}\) fractional enrichments before input of the data into the kinetic model. Instead, these experimentally determined resonance areas were converted to fractional enrichment before each iteration of the fitting process, with the values for F\(_{C4}\) and Y (as provided as input, as in the case of analysis of data sets 1 and 2, or estimated by the model). The fractional enrichment of glutamate C-4 is equal to [2-\(^{13}\text{C}\)]acetyl-CoA at steady state, whereas the enrichment of C-2 and C-3 is equal to F\(_{C4}/(2Y + 1);\) Ref. 16.

An additional benefit of the above process was avoidance of additional experimental error introduced by the extra step of measuring the area of an external standard. This step requires correction for differences in nuclear Overhauser effects and relaxation between glutamate and the standard. Even after avoiding such steps, the standard deviation of the fit contributed by the C-4 and C-3 (or C-2) resonance areas tended to be higher (6-7%) than that of the multiplet data (1-4%). This indicates that fractional multiplet areas (given adequate resolution for area measurements) are less subject to the uncertainties associated with \(^{13}\text{C}\) fractional enrichment measurements.

Error and parameter sensitivity analysis. Parameter errors are reported as confidence levels (analysis of extract data) or actual range (analysis of intact heart data), both being more accurate descriptions of the uncertainty in the estimates than.

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\(^1\)The program tcaFLUX is available to others as a service to the scientific community. Please contact the authors for further information.
standard deviations (7). The $^{13}$C NMR data from heart extracts were combined for analysis, giving one time course with replicates at each time point, from which one set of parameter values was estimated. The error in these parameters was obtained with Monte Carlo simulation, a robust technique for error estimation that is subject to fewer assumptions than other methods (24, 29). The simulation was used to determine the 5 and 95% confidence levels. The $^{13}$C NMR data obtained from intact hearts provided the complete time course for each heart. Rather than combine measurements from all hearts, the data from each heart were analyzed to obtain parameter estimates. The error in the parameters was then reported with the actual range of values obtained from hearts in each experimental group. (Confidence levels could not be determined because of the small sample size).

A parameter sensitivity analysis was also performed with Monte Carlo simulation. Synthetic $^{13}$C measurements were generated and subdivided into the three data sets (described in Data used with model) and analyzed with the model. This was used to test the effect of available data on parameter estimates. Different values for $V_r$ were also used in generating the synthetic data to examine the consequences of uncertainty in determination of this parameter. (Further details are given in RESULTS section.) The results of these simulations were used to determine the confidence levels and correlation between model parameters.

Calculation of oxygen consumption from model parameter estimates. This was performed with

$$MV_{O_2} = V_{TCA}(1 - F_0)R_0 + F_0R_2 + YR_A$$

where $R_0$ was the ratio of oxygen consumption ($MV_{O_2}$) to $V_{TCA}$ ($MV_{O_2}/V_{TCA}$) for endogenous substrate utilization and was considered to be 2.8; $R_2$ was the $MV_{O_2}/V_{TCA}$ for exogenous substrate oxidation and was 2.0 or 2.5 for hearts perfused with [2-$^{13}$C]acetate or [3-$^{13}$C]pyruvate, respectively; and $R_A$ was $MV_{O_2}/V_{TCA}$ for the anaplerotic substrate and was 0.5 (14).

Statistical analysis. Data are reported as means ± SD or, in the case of parameter estimates, means (5–95% confidence levels in the case of extract data, actual range in the analysis of data from intact hearts). Absolute flux rates are reported as micromoles per minute per gram of dry weight of tissue. Tissue contents are reported as micromoles per minute per gram of dry weight. Statistical analysis was conducted with paired two-tailed $t$-tests.

RESULTS

Size of intermediate pools involved in the citric acid cycle. The tissue content values of the more readily measured intermediates are shown in Table 1. The total amount of the citric acid cycle intermediates was only 5–7% of all measurements (citric acid cycle intermediates + amino acids) combined. Hence, the content of the amino acids must be the more accurately determined, as errors in measuring the amounts of citric acid cycle intermediates have little effect on flux estimates.

The extent to which the intermediates are involved in the citric acid cycle also affects the calculation of citric acid cycle activity. It has been reported that a fraction of glutamate remains unlabeled in rabbit (9, 10) and rat hearts (28). The actual fractional enrichment in glutamate C-4 (measured by $^1H$ NMR spectroscopy) was compared with that calculated from acetyl-CoA enrichment (estimated by $^{13}$C isotopomer analysis) with extracts of hearts freeze-clamped at the final point in the time series. With the use of paired $t$-tests, a significant difference ($P = 0.0010$) was found in hearts perfused with 1 mM [3-$^{13}$C]pyruvate and 5 mM glucose (0.70 ± 0.01 and 0.84 ± 0.02, $n = 6$, $^1H$ NMR and $^{13}$C NMR, respectively). Hence, the value obtained for the extractable amount of glutamate in this group (Table 1) was adjusted by the ratio 0.70 to 0.84 for use in the kinetic analysis. In hearts perfused with 5 mM [2-$^{13}$C] acetate plus 5 mM glucose, the difference was not significant: 0.92 ± 0.05 and 0.95 ± 0.02, $n = 8$. Similar measurements were not possible for the other intermediates, but because their tissue contents were much lower than glutamate and assuming the required correction factor (~10% for glutamate) is the same for all intermediates, this will have little effect on the measurement of citric acid cycle flux.

Kinetic analysis of data. Spectra from the first 30 min of exposure to glucose plus [2-$^{13}$C]acetate are shown in Figs. 2A (data collected from extracts) and 3A (data collected from intact hearts). Figure 2B shows the multiplet measurements made from the extract data, along with the curves generated after fitting the data to the kinetic model. All of the data shown were used in the analysis; the values measured in triplicate at each time point were included as individual values. Figure 3B shows the same measurements obtained from intact hearts. The signal-to-noise of the spectra in this case was lower, as expected, so the scatter in the measurements is higher and measurements at the earlier time points were not always possible. The curves in this figure show the result of fitting the combined data. However, this is for demonstration purposes only; the data from individual hearts were analyzed separately to allow statistical evaluation of the parameter estimates. Figure 4A shows hearts perfused with glucose plus [3-$^{13}$C]pyruvate. Hearts perfused with these substrates had less glutamate (Table 1) and a lower enrichment in acetyl-CoA. As a result, the signal-to-noise in spectra collected from hearts in this group was worse, and the scatter in the NMR measurements was greater (compare Fig. 4B with Figs. 2B and 3B).

Table 2 lists the means and ranges of $V_{TCA}$ and $V_x$ estimated by analysis of the three different NMR data sets. Not shown in this table are the estimates for $F_{c2}$

<table>
<thead>
<tr>
<th>Table 1. Tissue contents</th>
<th>Glucose + acetate (n = 5)</th>
<th>Glucose + pyruvate (n = 6)</th>
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<tbody>
<tr>
<td>Citrate</td>
<td>0.69 ± 0.19</td>
<td>0.81 ± 0.21</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>0.29 ± 0.02</td>
<td>0.30 ± 0.06</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.11 ± 0.09</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.45 ± 0.03</td>
<td>0.51 ± 0.08</td>
</tr>
<tr>
<td>Malate + Succinate</td>
<td>0.92 ± 0.22</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>Total</td>
<td>2.36 ± 0.16</td>
<td>2.00 ± 0.34</td>
</tr>
<tr>
<td>Amino acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartate</td>
<td>2.28 ± 0.25</td>
<td>7.11 ± 0.81</td>
</tr>
<tr>
<td>Glutamate</td>
<td>39.5 ± 2.54</td>
<td>20.3 ± 3.26</td>
</tr>
<tr>
<td>Total</td>
<td>41.7 ± 2.51</td>
<td>27.4 ± 3.89</td>
</tr>
</tbody>
</table>

Values are means ± SD μmol/g dry wt.
The analysis of data set 3 from each experimental group gave values for Fc2 of 0.96 (0.94–0.97), 0.94 (0.88–0.96), and 0.84 (0.81–0.87) and values for Y of 0.04 (0.03–0.05), 0.03 (0.02–0.05), and 0.05 (0.00–0.10) for hearts perfused with glucose plus acetate (extracts and intact hearts) and glucose plus pyruvate, respectively. No evidence was found for the entry of labeled pyruvate as anaplerotic substrate (Pyr3 < 0.0). Because Fc2 and Y were not as well determined in the analysis of data sets 1 and 2, these parameters were estimated from spectra of tissue extracts at steady state and were used as fixed values during the fitting. The values of Fc2 and Y obtained from extract spectra were identical to those listed above as determined by the fitting of data set 3.

The range in VTCA indicates that there were only minor differences between the results of fitting data sets 2 and 3, whereas the range was greater for the fit of data set 1. This reflects correlation of VTCA and Vx, which is apparently magnified in the fitting of glutamate C-4 and C-3 (or C-2) resonance areas (see Numerical evaluation of model).

**Oxygen consumption measurement.** Table 2 shows experimental MV302 and values estimated by the kinetic model. In no case was the calculated MV302 statistically different from the experimental measurement. There was a ~30% difference in experimental MV302 between hearts perfused with acetate and glucose vs. glucose plus pyruvate. Note that the ranges for these two groups do not overlap. However, the kinetic model did not predict differences in MV302 for these two substrate groups when data sets 1 and 3 were analyzed. An analysis of data set 2 did, however, successfully predict differences in MV302 for these two substrate groups.

**Numerical evaluation of model.** Monte Carlo simulation was used to determine the extent of correlation between parameters, measuring this for the three different subsets of the data. Because the uncertainty in Vx increases as the size of this flux increases, four
series of simulated data sets were generated in which \( V_x \) was varied from 3 to 60 \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{g dry wt}^{-1} \). The values of other parameters were the same for each simulation (\( V_{TCA} = 10 \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{g dry wt}^{-1} \), \( F_{C2} = 0.95 \), and \( Y = 0.05 \)). From each of these, a further 300 data sets were constructed by adding random noise to give standard deviations of 3\% for multiplet data and 6\% for C-4 and C-3 resonance areas (to compare with the experimental finding). Finally, each data set was analyzed with the kinetic model in three ways, selecting resonance areas only (corresponding to experimental data set 1), the C4D34 multiplet only (corresponding to experimental data set 2), or resonance areas plus multiplet data combined (corresponding to experimental data set 3). The model parameter estimates generated by this procedure were then used for correlation and regression analysis. These parameter estimates were also used to calculate oxygen consumption and hence the influence of \( V_x \) on the error in this measurement.

Table 3 compares the degree of correlation between parameters \( V_{TCA} \) and \( V_x \), both as a function of different subsets of the data and as the value of \( V_x \) used in the simulations increased. In the analysis of data set 1, the correlation was negative, declining \(-20\%\) as \( V_x \) increased. The absolute value of the correlation coefficient determined from data set 2 was initially one-half of that obtained with data set 1, fell to 35\% of its absolute value at \( V_x = 10 \), and then gradually increased as \( V_x \) increased. These correlations, initially positive, were negative for values in \( V_x \) of 10 \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{g dry wt}^{-1} \) and greater. The correlation coefficients obtained by analysis of data set 3 were positive and smaller than in the analysis of the other two data sets when \( V_x \) was 3 \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{g dry wt}^{-1} \), showed a small increase at \( V_x = 10 \), and then decreased with increasing \( V_x \).

Although the correlation coefficient indicates the fraction of the error in a parameter estimate that can be explained by another, it does not indicate the magnitude of the influence. This was determined by examin-

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**Table 2. Parameter estimates and calculated oxygen consumption**

<table>
<thead>
<tr>
<th></th>
<th>Data Set 1</th>
<th>Data Set 2</th>
<th>Data Set 3</th>
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<tbody>
<tr>
<td></td>
<td>Heart extracts</td>
<td></td>
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<tr>
<td></td>
<td>Glucose + acetate; ( \dot{MV}_O_2 = 24.9 ) (17.7–30.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{TCA} )</td>
<td>12.1 (10.7–14.2)</td>
<td>11.0 (10.4–11.4)</td>
<td>11.3 (11.0–11.6)</td>
</tr>
<tr>
<td>( V_x )</td>
<td>34.1 (18.3–62.4)</td>
<td>122.0 (58.0–206)</td>
<td>65.5 (49.1–83.7)</td>
</tr>
<tr>
<td>( \dot{MV}_O_2 )</td>
<td>25.2 (22.2–29.4)</td>
<td>22.8 (21.7–23.7)</td>
<td>23.5 (22.7–24.2)</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>Intact hearts</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Glucose + acetate; ( \dot{MV}_O_2 = 24.7 ) (22.8–27.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{TCA} )</td>
<td>12.0 (8.2–21.2)</td>
<td>10.9 (8.3–13.5)</td>
<td>11.2 (9.1–14.3)</td>
</tr>
<tr>
<td>( V_x )</td>
<td>15.4 (8.6–24.6)</td>
<td>22.6 (31.3–31.6)</td>
<td>14.9 (7.6–20.6)</td>
</tr>
<tr>
<td>( \dot{MV}_O_2 )</td>
<td>25.0 (17.5–44.0)</td>
<td>22.7 (17.6–28.1)</td>
<td>23.3 (18.7–29.8)</td>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Glucose + pyruvate; ( \dot{MV}_O_2 = 17.0 ) (12.0–22.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{TCA} )</td>
<td>8.44 (4.79–14.8)</td>
<td>5.32 (4.81–6.51)</td>
<td>6.07 (4.41–7.64)</td>
</tr>
<tr>
<td>( V_x )</td>
<td>5.53 (3.20–14.6)</td>
<td>28.6 (7.6–64.2)</td>
<td>14.6 (3.9–54.3)</td>
</tr>
<tr>
<td>( \dot{MV}_O_2 )</td>
<td>22.2 (12.6–39.2)</td>
<td>13.9 (12.6–17.3)</td>
<td>15.9 (11.6–20.2)</td>
</tr>
</tbody>
</table>

Values are either means (5–95\% confidence levels) in \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{g dry wt}^{-1} \) for model parameter values obtained from extracts or means (range) for values obtained from intact hearts. The former were obtained by Monte Carlo simulation using as error the SD from fit (typically 3\% for multiplet and 6\% for fractional enrichment data). \( \dot{MV}_O_2 \), measured oxygen consumption; \( V_{TCA} \), citric acid flux; \( V_x \), exchange between \( \alpha \)-ketoglutarate and glutamate.
Table 3. Correlation and regression coefficients

<table>
<thead>
<tr>
<th></th>
<th>Data Set 1</th>
<th>Data Set 2</th>
<th>Data Set 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vx</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-0.814</td>
<td>0.463</td>
<td>0.132</td>
</tr>
<tr>
<td>10</td>
<td>-0.807</td>
<td>-0.163</td>
<td>0.293</td>
</tr>
<tr>
<td>20</td>
<td>-0.774</td>
<td>-0.226</td>
<td>0.189</td>
</tr>
<tr>
<td>60</td>
<td>-0.663</td>
<td>-0.391</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Regression coefficients

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>-4.374</td>
<td>0.503</td>
<td>0.754</td>
</tr>
<tr>
<td>10</td>
<td>-0.401</td>
<td>-0.040</td>
<td>0.138</td>
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<tr>
<td>20</td>
<td>-0.081</td>
<td>-0.018</td>
<td>0.021</td>
</tr>
<tr>
<td>60</td>
<td>-0.011</td>
<td>-0.003</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values determined from simulations performed as explained in MATERIALS AND METHODS. Vx was varied as shown, whereas other parameters were fixed: Vtca, 10; Fe2, estimate for acetyl-CoA enrichment, 0.95; Y, anaplerotic flux, 0.05.

because this influences the accuracy of the calculated oxygen consumption.

Estimating citric acid cycle flux. The first part of this discussion will be limited to the results from the analysis of data set 3 in Table 2. In hearts perfused with glucose plus [2-13C]acetate and extracted for analysis, acetate provided ~95% of the acetyl-CoA pool, and the citric acid cycle flux rate was estimated to be 11.3 µmol/g dry weight. The latter value agrees well with the literature; in hearts perfused with acetate and glucose, Randle et al. (25) reported a value of 14, whereas studies with acetate only as the substrate have found ~11 µmol·min⁻¹·g dry wt⁻¹ (31, 33, 35). These values are somewhat higher than those estimated by Chance et al. (1; 8.3 µmol·min⁻¹·g dry wt⁻¹); this cannot be explained by a lower workload in the hearts used in the Chance et al. study, because the oxygen consumption was higher (30.2 in the Chance et al. study vs. 24.7 µmol·min⁻¹·g dry wt⁻¹ here; Table 2). The difference in Vtca estimates is not due to differences in modeling results: when the kinetic model of this study was used to analyze the time course of fractional enrichments in Fig. 4 of Chance et al., the citric acid cycle flux estimated (8.2 µmol·min⁻¹·g dry wt⁻¹, 5–95% confidence levels: 7.5–8.9) agreed very well with that obtained by Chance et al. (8.3 µmol·min⁻¹·g dry wt⁻¹, 5–95% confidence levels: 7.9–8.7).

When the same analysis was performed with data collected from intact hearts, the estimated citric acid cycle flux agreed very well between the two sets of experiments (Table 2). The difference in the confidence levels obtained from the analysis of extract data (0.6
µmol·min⁻¹·g dry wt⁻¹) is smaller than the range of values obtained from intact heart data (5.2 µmol·min⁻¹·g dry wt⁻¹). This is largely a result of differences in data treatment. Measurements collected from extracts were, by necessity, analyzed as one data set, and the confidence levels calculated represent the uncertainty in parameter estimates resulting from the fit. The data from intact hearts, on the other hand, provided the complete time course for each heart and were analyzed separately. The data range obtained by this method represents both the variation between samples and the uncertainty in estimating parameters from the data. The variation between samples is the larger source of error for \( V_{\text{TCA}} \) but not necessarily \( V_x \). When all of the data from intact hearts were combined into a single data set and fit, the confidence levels were very similar to those found in the analysis of extract data.

In hearts perfused inside the magnet with glucose plus [3-\( ^{13} \)C]pyruvate, pyruvate provided the majority (84%) of the substrate for oxidation and \( V_{\text{TCA}} \) was estimated at 6.07 µmol·min⁻¹·g dry wt⁻¹. This is lower than that found in hearts perfused with acetate plus glucose, which can be explained by the additional NADH produced by oxidation of pyruvate and the lower oxygen consumption measured in this group (Table 2). When these two factors are taken into account, \( V_{\text{TCA}} \) estimated from hearts perfused with these two substrate mixtures agreed well with each other. However, \( V_{\text{TCA}} \) determined in this substrate group was less than one-half of that estimated by the Chance et al. study. As noted above, this discrepancy is not due to modeling differences: when the glutamate measurements taken from Fig. 11 of Chance et al. were analyzed with the present model, \( V_{\text{TCA}} \) was estimated at 10.6 (9.86–11.6) µmol·min⁻¹·g dry wt⁻¹, in good agreement with that estimated by Chance and et al., 11.9 µmol·min⁻¹·g dry wt⁻¹ (11.3–12.6). The higher flux in this case may be partly explained by a higher oxygen consumption (34.9 µmol·min⁻¹·g dry wt⁻¹) reported by Chance et al. for this same substrate mixture.

Exchange between \( \alpha \)-amino- and \( \alpha \)-keto acids. It is apparent from Table 2 that the \( V_x \) is not well determined. This has already been noted by Chance et al. (1) in their 1983 report, where they described a “rather poorly determined value for isotope exchange between \( \alpha \)-ketoglutarate and glutamate.” This results simply from the nature of exchange reactions, where the system is more sensitive to changes at relatively low flux rates. With the use of this model, it was found that the exchange parameter is poorly determined when rates are equal to or greater than the citric acid cycle flux. A review of the literature indicates that values of \( V_x \) determined by fitting glutamate C-4 and C-3 enrichment curves (equivalent to our data set 1) to kinetic models of varying complexity have been quite variable. Chance and et al. reported \( V_x \) to \( V_{\text{TCA}} \) ratios (\( V_x/V_{\text{TCA}} \)) of 2.8 (acetate + glucose) and 4.4 (pyruvate + glucose) (1), whereas Weiss et al. (31, 32), with acetate only, reported values for \( V_x/V_{\text{TCA}} \) ranging 0.85–2.5, and we reported a ratio of 1.0 (28). In a later report (2), the original model of Chance et al. (1) was modified to generate a more complex model that calculated \( V_x \) based on oxygen consumption, which was used as an input to the model. That treatment gave much lower values for \( V_x/V_{\text{TCA}} \) (~0.2). Work with \( ^{14} \)C in the rat heart has also yielded \( V_x/V_{\text{TCA}} \) values of 1.0 (22) and 13 (25). In the rabbit heart, Lewandowski and co-workers (11, 23, 33–35) have reported values for the \( V_x/V_{\text{TCA}} \) that are generally around 1.0. The variability in \( V_x \) was also found to be large, i.e., 10.18 ± 6.57 µmol·min⁻¹·g dry wt⁻¹ (means ± SD), from the analysis of data from individual hearts (33). This variation is not significantly different from the range obtained here in intact hearts with data set 1 (Table 2). Taken together, we conclude that \( V_x \) is not well determined in the heart with either \( ^{14} \)C fractional enrichments or \( ^{13} \)C measurements (enrichments alone or combined with multiplet data).

Parameters estimated from different data subsets. The mean value for \( V_{\text{TCA}} \) determined by kinetic analysis of \( ^{13} \)C NMR data did not vary greatly among the three data subsets (Table 2). However, although the range of values was similar when derived from data sets 2 and 3, the range was consistently higher when analyzed with data set 1. As a result, a statistically significant difference in \( V_{\text{TCA}} \) was found between hearts perfused with the two substrate mixtures when analyzed with data sets 2 and 3, but not when data set 1 was analyzed.

The poorer determination of \( V_{\text{TCA}} \) when analyzed with data set 1 is likely due to correlation between \( V_{\text{TCA}} \) and \( V_x \). The mean and range of \( V_x \) values were higher when estimated with data set 2. However, because the degree of correlation and the influence of \( V_x \) on \( V_{\text{TCA}} \) are the smallest with this data set (Table 3), the uncertainty in \( V_{\text{TCA}} \) was similar to that found by analysis of data set 3. In contrast, the correlation between parameters and their influence on each other is stronger when data set 1 is analyzed.

The nature of the correlation between \( V_{\text{TCA}} \) and \( V_x \) in the analysis of fractional enrichment data has previously been discussed by Yu et al. (33). We believe the correlation exists and is negative simply because both parameters tend to alter the rate of \( ^{13} \)C appearance in glutamate C-4 and C-3 in a similar manner; thus an increase in one or another of these parameters will increase the rate at which multiply enriched molecules appear while causing a correspondingly more rapid decline in sparsely labeled glutamate. Hence, in the \( ^{13} \)C spectrum C2Q, C3T, and C4D34 multiplets increase, whereas the singlets decline to their steady-state values more rapidly at higher \( V_{\text{TCA}} \). An increase in \( V_x \), however, promotes the interchange of newly labeled intermediates with unlabeled pools early in the perfusion period, thus temporarily prolonging the existence of singly labeled molecules at the expense of multiply enriched products: the reverse of increasing the citric acid cycle flux rate. The net outcome of these two opposing effects is a
reduction in the degree of correlation and a tendency toward positive correlation.

Calculating oxygen consumption from kinetic parameters. In hearts perfused with glucose and acetate, $\text{MV}_2$ determined by analysis of data set 1 was not very different, the range of $\text{MV}_2$ values was significantly greater than the other two data sets. The span was approximately five times larger (7.2 µmol·min$^{-1}$·g dry wt$^{-1}$) and approximately two times larger (26.5) in the analysis of extracts and intact hearts, respectively. Thus, even under ideal conditions (data collected from extracts with high signal-to-noise), the addition of multiplet data improved the precision of the $\text{MV}_2$ estimate.

In hearts perfused with glucose plus pyruvate, the signal-to-noise was poor and perhaps represents more closely what may be obtained in vivo. This did not affect the analysis of data sets 2 or 3 because the parameter ranges actually were smaller in this substrate group. However, the range of $\text{MV}_2$ values (26.6 µmol·min$^{-1}$·g dry wt$^{-1}$) estimated by analysis of data set 1 was four times that of the other two data sets.

To conclude, the addition of $^{13}$C multiplets to kinetic analysis of time-dependent data significantly improves estimates of $\text{MV}_2$ in intact tissues. The principal reason for this benefit is the reduced correlation between model parameters involved in calculation of $\text{MV}_2$ in the heart. Under conditions of reasonable signal-to-noise (data collected from intact hearts perfused with acetate + glucose), the use of multiplet data would allow one to detect a 60% increase or decrease in oxygen consumption. Conversely, a twofold increase in $\text{MV}_2$ would not be reliably detected using the appearance of $^{13}$C in glutamate C-4 and C-3 alone. Hence, the use of multiplets will make it easier to distinguish between different physiological and pathological states that influence oxygen utilization. Not only is the addition of multiplet data beneficial, we have demonstrated that analysis of temporal changes in C4D34 alone can reliably predict $\text{MV}_2$ in the heart. For technical reasons, this may be a significant advantage for measuring $\text{MV}_2$ in vivo. Because C4D34 is obtained from a single glutamate resonance, narrow-band frequency excitation may be used and broad-band $^1$H decoupling is unnecessary. It has already been shown that this multiplet is resolved in spectra of human brain at 2.1 tesla (4). Thus, use of the C4D34 multiplet to estimate $\text{MV}_2$ in vivo could extend recent studies that employed appearance of $^{13}$C in glutamate C-4 and C-3 (3, 19, 20, 26) while minimizing the error in $V_{\text{TCA}}$ estimates introduced by the uncertainty in $V_x$ (8).

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Address for reprint requests and other correspondence: F. M. H. Jeffery, The Mary Nell and Ralph B. Rogers Magnetic Resonance Center, Dept. of Radiology, Univ. of Texas Southwestern Medical Center, 5801 Forest Park Road, Dallas, TX 75235-9085 (E-mail: Mark.Jeffery@email.swmed.edu).

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