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Evaluation of Carbon Flux and Substrate Selection through Alternate Pathways Involving the Citric Acid Cycle of the Heart by ^{13}C NMR Spectroscopy*

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A previous ^{13}C NMR technique (Malloy, C. R., Sherry, A. D., and Jeffrey, F. M. H. (1987) *FEBS Lett.* 212, 58–62) for measuring the relative flux of molecules through the oxidative *versus* anaplerotic pathways involving the citric acid cycle of the rat heart has been extended to include a complete analysis of the entire glutamate ^{13}C spectrum. Although still simple in practice, this more sophisticated model allows an evaluation of ^{13}C fractional enrichment of molecules entering both the oxidative and anaplerotic pathways under steady-state conditions. The method was used to analyze ^{13}C NMR spectra of intact hearts or their acid extracts during utilization of ^{13}C-enriched pyruvate, propionate, acetate, or various combinations thereof. [2-^{13}C]Pyruvate was used to prove that steady-state flux of pyruvate through pyruvate carboxylase is significant during co-perfusion of pyruvate and acetate, and we demonstrate for the first time that a nine-line ^{13}C multiplet may be detected in an intact, beating heart. Acetate or pyruvate alone provided about 86% of the acetyl-CoA; in combination, about 65% of the acetyl-CoA was derived from acetate, about 30% was derived from pyruvate, and the remainder from endogenous sources. Propionate reduced the contribution of exogenous acetate to acetyl-CoA to 77% and also reduced the oxidation of endogenous substrates. Equations are presented which allow this same analysis on multiply labeled substrates, making this technique extremely powerful for the evaluation of substrate selection and relative metabolic flux through anaplerotic and oxidative pathways in the intact heart.

The heart is capable of oxidizing a wide variety of energy-providing substrates. When the heart is exposed to a mixture of compounds, as it is *in vivo*, the contribution of each substrate to acetyl-CoA depends upon many factors. Although some features of substrate selection are well-established (*e.g.* fatty acids suppress glucose oxidation), the interactions among other potential substrates are controversial or have not been investigated. Two intermediates, pyruvate and propionate, each represent the final common pathway for the metabolism of several compounds. Analysis of the net contribution of these metabolites to the respiratory fuel is difficult because isolated rat heart mitochondria show significant activity of propionyl-CoA carboxylase and pyruvate carboxylase, and both enzymes may be activated in the intact rat heart (1–7). Thus, steady flux of carbon into the citric acid cycle through these pathways, termed anaplerosis, provides for carbon flow from pyruvate or propionate into the cycle without net oxidation. Investigation of the activity of these pathways has previously required purification and carbon-by-carbon degradation of individual citric acid cycle intermediates (5, 8).

Hearts perfused with ^{13}C-enriched substrates produce multiple isotopomers of citric acid cycle intermediates as a consequence of ^{13}C scrambling in the citric acid cycle (9–12). The multiplets observed in the ^{13}C NMR spectrum of glutamate from these hearts are due to ^{13}C-^{13}C spin-spin coupling in the isotopomers of glutamate (10–15). Since the relative concentrations of these isotopomers are sensitive to the various pathways for carbon flow into the citric acid cycle, recent studies have suggested that the ^{13}C NMR spectrum may be interpreted in quantitative physiological terms (10–12, 14). Under steady-state conditions, the doublet/total ratio of the glutamate C4 resonance equals the fraction of acetyl-CoA entering the citric acid cycle that is derived from labeled substrate in the perfusate (11). This measurement is based on the assumption that carbon atoms enter the citric acid cycle solely through citrate synthase. However, activation of the anaplerotic pathways substantially alters the ^{13}C NMR spectrum of the heart (12). When unlabeled carbons enter the cycle via anaplerotic mechanisms, the spectra may be analyzed using models of the citric acid cycle previously developed and validated for analysis of ^{14}C tracer studies (12, 16–19). However, the influence on the glutamate spectrum of simultaneous entry of ^{13}C into the citric acid cycle via citrate synthase and anaplerotic mechanisms has not been described.

The sensitivity of the ^{13}C spectrum in the intact heart to various pathways of carbon flow into the citric acid cycle is also of interest because the integrated regulation of replenishment and disposal of citric acid cycle intermediates is not well understood. It has long been known that various physiologically and clinically relevant states (such as diabetes, fasting, or ischemia) alter the relative rates of the reactions regulating the concentration of citric acid cycle intermediates, but the significance of these changes is unknown (7, 20–23).

We describe herein a complete analysis of the ^{13}C NMR spectrum of glutamate obtained from hearts perfused to steady-state ^{13}C enrichment which permits measurement of the relative activity of anaplerotic and oxidative reactions as well as the ^{13}C fractional enrichment of the anaplerotic and

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oxidative substrates. In this context, the term "oxidative" indicates entry of 2 carbon units into the citric acid cycle followed by net oxidation to CO_2 and water. The correlated nature of the ^{13}C information also allows measurement of these physiological parameters during perfusion with mixtures of ^{13}C-labeled substrates such as [1,2-^{13}C]acetate and [3-^{13}C]pyruvate.

EXPERIMENTAL PROCEDURES

Reagents and Perfusion Conditions—Standard reagents were obtained from Sigma. ^{13}C-enriched substrates were obtained from MSD Isotopes ([2-^{13}C]acetate, 99%; [3-^{13}C]pyruvate, 99.6%; [2-^{13}C]pyruvate, 90%; and [3-^{13}C]propionate, 91.1%) and Kor Isotopes ([1,2-^{13}C]acetate, 90%). Male rats (300–350 g) of the Sprague Dawley strain were allowed free access to water and food. Rats were anesthetized by intraperitoneal injection of pentobarbital. Heparin (200 units) was injected about 1 min before the heart was excised. The heart was perfused using the Langendorff technique at a pressure of 70 cm H_2O with standard Krebs-Henseleit medium ([Ca^{2+}] = 2.5 mM) bubbled continuously with an O_2/CO_2 gas mixture of 19/1. The spontaneous heart rate was 240–300 beats/min. The perfusate temperature was maintained at 37 °C with water jackets and the O_2 tension at the level of the aorta was >550 mm of Hg.

Eight groups of hearts were perfused with the following substrates: group 1, 2.5 mM [3-^{13}C]pyruvate; group 2, 2.5 mM [3-^{13}C]pyruvate, 5 mM glucose and 5 units/liter of insulin; group 3, 2.5 mM [2-^{13}C]acetate; group 4, 2.5 mM [2-^{13}C]acetate and 1.0 mM propionate; group 5, 2.5 mM [2-^{13}C]acetate and 1.0 mM [3-^{13}C]propionate; group 6, 2.5 mM [2-^{13}C]acetate and 2.5 mM pyruvate; group 7, 2.5 mM [3-^{13}C]pyruvate and 2.5 mM acetate; group 8, 2.5 mM [1,2-^{13}C]acetate and 2.5 mM [3-^{13}C]pyruvate. There were four hearts in each group.

Hearts were initially perfused for 10 min without recirculation of the perfusate, followed by 10 min with recirculation. During this initial 20-min period, natural abundance substrates were present at the final concentrations indicated to allow citric acid cycle intermediate pools to reach steady-state levels (10). Hearts were then switched to the ^{13}C-enriched substrate(s) at an identical concentration, and perfusion was continued for 30 min. Each heart was freeze-clamped at −73 °C, powdered, extracted into 3.6% ice-cold perchloric acid, neutralized to pH 7 with KOH, centrifuged, and freeze-dried. The powder was redissolved into 2 cc of 50% $^2H_2O/H_2O$ and filtered prior to examination by high resolution NMR.

NMR Spectroscopy—^{13}C NMR spectra were obtained at 75.45 MHz using a Nicolet NT 300 spectrometer with proton broad-band decoupling using the MLEV-16 sequence (26). All spectra (intact hearts and extracts) were collected using a 45° carbon pulse utilizing 8,192 points over a 16,000 Hz spectral width. Prior to Fourier transformation, the spectra were zero-filled (to improve digital resolution) and multiplied by an exponential function of 1–4 Hz (to improve the signal-to-noise ratio). The intact heart spectra were run in a 20-mm Bruker probe using a pulse repetition time of 1.5 s without a field lock. The field homogeneity was adjusted using the ^{23}Na-free induction decay. After switching to a ^{13}C-enriched substrate, ^{13}C spectra were collected in 5-min blocks (200 scans) until no further changes in the spectra were apparent (approximately 20 min). Steady-state spectra were then acquired over the next 40 min (1,600 scans). Spectra of the extracts (approximately 6,000 scans) were obtained in a Bruker 10-mm probe using a repetition time of 6.5 s. Since the longitudinal relaxation time (T_1) of the protonated glutamate carbons is approximately 2 s, no correction for partial saturation was necessary using these pulsing conditions. The sample temperature was maintained at 37 °C using a Nicolet variable temperature accessory.

Analysis of Spectra—The abbreviations C1, C2, C3, C4, and C5 refer to the respective carbons of glutamate. In a pool of glutamate molecules consisting of a mixture of ^{13}C isotopomers, each glutamate resonance consists of from 1 to 9 lines, depending upon the position of the carbon within the glutamate molecule and the labeling of the adjacent carbons (10–15). For example, Fig. 1 illustrates that the 5-line multiplet usually observed for glutamate C3 at 27.5 ppm is actually the sum of a singlet (carbons adjacent to the enriched C3 are unenriched), two equivalent doublets (C3 and either C2 or C4 are enriched), and a triplet (C2, C3, and C4 are all enriched). The doublets are equivalent because the coupling constant is nearly equal for C2–C3 and C3–C4. The relative areas of the individual components of the multiplet may be measured and, in this example, it may easily be determined that the singlet contributes 12%, the doublets 46%, and the triplet 42% to the total measured area of the multiplet. We designate these experimental measurements as C3S, C3D, and C3T, respectively. Since C3S + C3D + C3T = 1, any two measurements determine the third. The C2 and C4 resonance multiplets may be similarly resolved into their individual components. The C2 and C4 components differ in one respect from C3 in that the coupling constants between adjacent enriched carbons are not equal. Hence, the C2 resonance, for example, may contain two doublets, and these are designated C2D12 and C2D23 to indicate which of the carbons adjacent to C2 is also enriched.

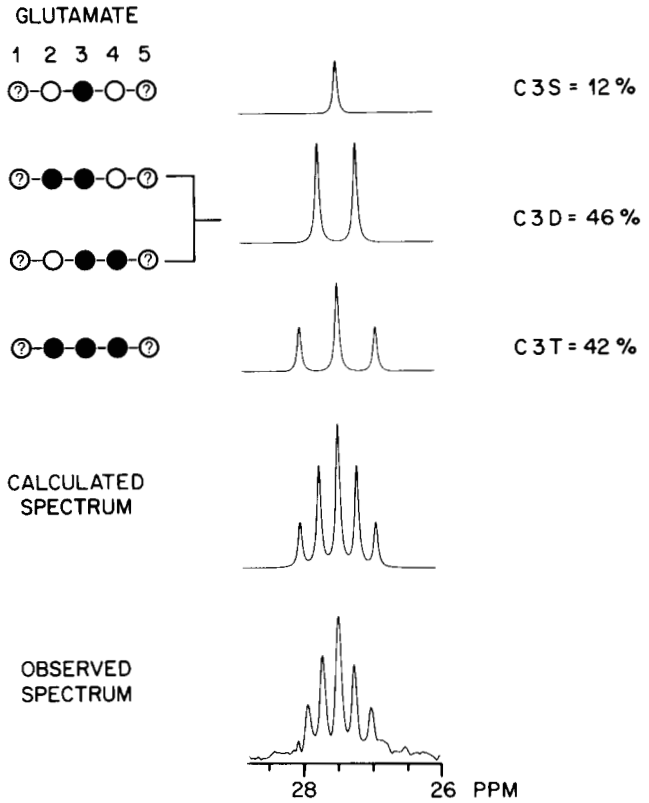

FIG. 1. **Diagram illustrating the singlet, doublet, and triplet components of a typical glutamate C3 resonance multiplet.** The observed multiplet is that of the C3 resonance from a heart perfused with 2.5 mM [2-^{13}C]acetate and 2.5 mM pyruvate (Fig. 2*c*). As shown, the contribution of the singlet (*C3S*), the doublet (*C3D*), and the triplet (*C3T*) to the total multiplet area is 12, 46, and 42%, respectively.

Equations relating these multiplet areas to three groups of physiological variables, F_c, y, and F_a, are developed in the Appendix.[1] F_c refers to the fractional enrichment in ^{13}C of the acetyl-CoA pool which condenses with OAA to form citrate. F_{c0} indicates the fraction of the acetyl-CoA pool that is unlabeled. F_{c1}, F_{c2}, and F_{c3} refer to the fraction of acetyl-CoA that is labeled in C1, C2, or C1 and C2, respectively. By definition, $F_{c0} + F_{c1} + F_{c2} + F_{c3} = 1$. The second variable, y, relates the total flux through the anaplerotic reactions (a) to the total flux through citrate synthase (c), or $y = a/c$ (12, 16–18). Since the concentrations of the citric acid cycle intermediates are not changing in steady state, it was assumed that the pathways for removal of carbon skeletons are as active as those for entrance of carbon skeletons. The third group of variables refers to the ^{13}C labeling of carbon skeletons entering the citric acid cycle via the various anaplerotic pathways. F_{a0} indicates the fraction of anaplerotic substrate that is unlabeled and F_{a1} is the fraction of substrate that will yield either [2-^{13}C]oxaloacetate or [3-^{13}C]oxaloacetate in the first span of the citric acid cycle. By definition, $F_{a1} + F_{a0} = 1$. Although the equations presented in the Appendix are perfectly general and include each of these variables, it is possible to choose labeled sub-

[1] The "Appendix" is presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

strates which permit a considerable simplification of these equations. For example, [3-^{13}C]pyruvate and [2-^{13}C]acetate can only produce [2-^{13}C]acetyl-CoA; hence, F_{c1} and F_{c3} are zero and $F_{c0} + F_{c2} = 1$. Furthermore, we have chosen to limit our analysis to the protonated carbon resonances of glutamate (C2, C3, and C4) to avoid T_1 and nuclear Overhauser enhancement differences which may distort the individual multiplet components of the nonprotonated C1 and C5 resonances (10, 13, 14, 27). Within these constraints, the equations developed in the Appendix simplify to:

$$C2S = [2(y + 1)^2 - 2F_{c2}(y + 1) - F_{c2} + (F_{c2})^2]/(2(y + 1)^2) \quad (1)$$

$$C2D12 = F_{c0}F_{c2}/(2(y + 1)^2) \quad (2)$$

$$C2D23 = F_{c2}(2 + 2y - F_{c2})/(2(y + 1)^2) \quad (3)$$

$$C3S = F_{c0}(F_{c0} + y)/(y + 1) \quad (4)$$

$$C3T = (F_{c2})^2/(y + 1) \quad (5)$$

$$C4D34 = (yF_{a1} + F_{c2})/(2y + 1) \quad (6)$$

The relative areas of the multiplets were measured from the ^{13}C NMR spectrum by peak integration using the NMC software, by triangulation (peak height × width at half-height) and by plotting and weighing each resonance. There was no difference among the methods. Three independent variables (F_{c2}, y, and F_{a1}) were evaluated for each perfusion condition using ZZSXQ, a Levenberg-Marquardt algorithm from the International Mathematical and Statistical Library, Houston, Texas, run on a DEC-10 at The University of Texas Health Science Center, Dallas. The program permitted solutions with the following boundary conditions: $1 > F_{c2} > 0$, $F_{c0} + F_{c2} = 1$, $y > 0$, $1 > F_{a1} > 0$, and $F_{a1} + F_{a0} = 1$. The results for group 8 were obtained using the more general equations presented in the Appendix (since $F_{a3} > 0$). It should be noted that substrates containing any level of ^{13}C enrichment may be used in these experiments, but the final fractional enrichments (F_c and F_a) must be corrected if the level of ^{13}C enrichment is below 99%. All results are expressed as the mean ± standard deviation.

RESULTS

Factors Which Influence the ^{13}C NMR Spectrum of Glutamate—The observed proton decoupled ^{13}C NMR spectrum of glutamate from rat hearts is sensitive to the substrate(s) available to the heart and the position of ^{13}C enrichment within that substrate. Five spectra representing hearts perfused with different combinations of ^{13}C-enriched plus, in some cases, unenriched substrates, are compared in Fig. 2. The individual component areas required for solving Equations 1–6 were measured directly from the spectrum of each individual heart. These raw data may be found in the Appendix. The nonlinear least squares solutions of these data are summarized in Table I as the average ± S.D. for the four hearts for each substrate combination.

The results show that hearts perfused with pyruvate or pyruvate plus glucose and insulin (groups 1 and 2) derive about 85% of the acetyl-CoA entering the citric acid cycle from pyruvate in the perfusate. Under both conditions, the anaplerotic activity is 13–18% of oxidative flux. Hearts presented solely with acetate in the perfusate (group 3) derived 88% of acetyl-CoA from the available acetate. The remaining unlabeled acetyl-CoA was obtained from endogenous triglycerides or glycogen. The activity of the anaplerotic reactions was low (5 ± 1%), but significantly and reproducibly (12) greater than 0. This indicates that unenriched, endogenous substrates are also mobilized through the anaplerotic reactions during perfusion with acetate.

The addition of unlabeled propionate as a cosubstrate (group 4) resulted in activation of an anaplerotic pathway which was measured from the ^{13}C spectrum as a significant increase in y from 5% to approximately 30%. As in the hearts perfused solely with acetate, the measured enrichment of the anaplerotic substrate was not different from zero (12 ± 20%). The presence of [3-^{13}C]propionate (group 5) in addition to acetate does not significantly alter y (compare 29 ± 17% *versus* 40 ± 5%), but the ^{13}C enrichment of the anaplerotic pathway shows the expected increase. The observation that F_{a1} (63 ± 11%) is significantly less than 100% in this experiment suggests that propionate also stimulates the flux of

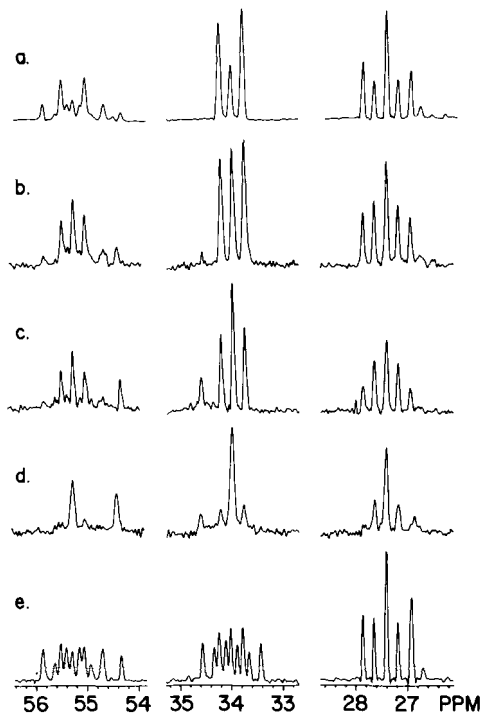

FIG. 2. **^{13}C NMR spectra of glutamate in heart extracts.** Spectra were obtained with the following substrates: *a*, 2.5 mM [2-^{13}C]acetate; *b*, 2.5 mM [2-^{13}C]acetate, and 1.0 mM [3-^{13}C]propionate; *c*, 2.5 mM [2-^{13}C]acetate and 2.5 mM pyruvate; *d*, 2.5 mM [3-^{13}C]pyruvate and 2.5 mM acetate; *e*, 2.5 mM [1,2-^{13}C]acetate and 2.5 mM [3-^{13}C]pyruvate. The spectra of the glutamate 2 carbon (*left column*), 4 carbon (*middle column*), and 3 carbon (*right column*) are expanded. Other compounds were also detected: the trimethylamino resonance of carnitine and acylcarnitine (natural abundance) at 54.3 ppm; the C2 and C3 of succinate at 34.6 ppm and the C3 of pyruvate at 26.9 ppm.

TABLE I

Physiological variables derived from ^{13}C spectra of glutamate in perfused rat hearts

Abbreviations: F_{c2}, fraction of acetyl-CoA labeled in C2; y, ratio anaplerotic to citrate synthase flux; F_{a1}, fractional enrichment of anaplerotic substrate.

Group	Substrate	F_{c2}	y	F_{a1}
1	[3-^{13}C]Pyruvate	84 ± 6%	18 ± 6%	30 ± 32%
2	[3-^{13}C]Pyruvate + glucose + insulin	86 ± 2%	13 ± 7%	24 ± 38%
3	[2-^{13}C]Acetate	88 ± 2%	5 ± 1%	18 ± 21%
4	[2-^{13}C]Acetate + propionate	77 ± 5%	29 ± 17%	12 ± 20%
5	[2-^{13}C]Acetate + [3-^{13}C]propionate	89 ± 3%	40 ± 5%	63 ± 11%
6	[2-^{13}C]Acetate + pyruvate	71 ± 4%	12 ± 5%	13 ± 23%
7	[3-^{13}C]Pyruvate + acetate	28 ± 8%	24 ± 22%	82 ± 15%[a]
8	[3-^{13}C]Pyruvate + [1,2-^{13}C]acetate	F_{c2} = 32 ± 7% F_{c3} = 63 ± 12%	21 ± 10%	25 ± 12%

[a] If the ratio of the total areas of C3 and C4 are also used in least squares fitting, this value is 33 ± 27%.

unenriched molecules through other undefined anaplerotic reactions.

Propionate also reduced the contribution of exogenous acetate to acetyl-CoA (compare $F_{c2} = 88 \pm 2\%$ in group 3 with $F_{c2} = 77 \pm 5\%$ in group 4). However, the source of unlabeled substrate (endogenous sources or unlabeled propionate) cannot be identified by the results of these two groups. In group 5, however, unlabeled propionate was replaced by [3-^{13}C] propionate, and under these conditions 89 ± 3% of the acetyl-CoA was labeled in C2. By comparison with group 4, approximately 12% of the acetyl-CoA must have become labeled as a consequence of providing [3-^{13}C]propionate in place of unlabeled propionate. Previous studies have shown that propionate is metabolized through several steps to succinyl-CoA in the heart (3, 6). This net addition of carbon to the citric acid cycle is balanced by disposal reactions which include decarboxylation of malate to pyruvate, which may undergo oxidation through the usual mechanisms. These results confirm that propionate provides a net source of carbon for oxidation. Since [3-^{13}C]propionate would be metabolized to equal amounts of [2-^{13}C]malate and [3-^{13}C]malate (because of scrambling through fumarate), the [2-^{13}C]acetyl-CoA derived from propionate represents only half of the total propionate which contributes to acetyl-CoA. Thus, propionate reduces the contribution of both exogenous acetate and endogenous unlabeled sources to acetyl-CoA. Under these perfusion conditions, 77% of the oxidized carbon is derived from acetate, and the remainder (2 × 12%) is derived from propionate via a disposal pathway of the citric acid cycle.

Competition of acetate with pyruvate was studied in groups 6–8. Hearts perfused with [2-^{13}C]acetate plus pyruvate (group 6) derive about 70% of acetyl-CoA from acetate; the remainder is derived from pyruvate in the perfusate or from other endogenous sources. Hearts perfused with [3-^{13}C]pyruvate and acetate derive about 30% of acetyl-CoA from pyruvate. These experiments indicate that nearly all of the oxidized substrate is derived from the perfusate and little from endogenous sources. The results from hearts perfused with [1,2-^{13}C] acetate plus [3-^{13}C]pyruvate confirm this. Since the concentration of the available substrates are the same in groups 6–8, the anaplerotic flux (y) should be the same. In spite of the differences in the ^{13}C spectra (Fig. 2, *c–e*), y was not significantly different among the groups (groups were compared using Newman-Keuls multiple comparison procedure (28)). For all three groups, y averages 0.19, consistent with earlier observations of significant anaplerotic flux measured by ^{14}C techniques (7, 21). Groups 7 and 8 indicate that a significant fraction of the anaplerotic substrate is derived from labeled pyruvate in the perfusate which is again consistent with previous reports (5, 7, 21). Thus, the ^{13}C analysis indicates that anaplerotic flux is active in hearts coperfused with pyruvate and acetate and that a significant fraction of the anaplerotic substrate is pyruvate from the perfusate.

The measured fuel of oxidation in these hearts (groups 6–8) provides an interesting example of the flexibility of this analysis. The dramatic differences among the spectra of these groups (Fig. 2, *c–e*) are a consequence solely of the site of ^{13}C enrichment in the available substrates. The metabolic results are complementary. The results from groups 6–8 indicate that when both acetate and pyruvate are available to the heart, acetate is oxidized in preference to pyruvate and that a significant fraction of pyruvate entering the citric acid cycle does so via an anaplerotic mechanism. To confirm the latter observation, we perfused hearts under identical conditions with 2.5 mM unlabeled acetate and 2.5 mM [2-^{13}C]pyruvate. Thus, ^{13}C entering the citric acid cycle via citrate synthase will enrich only the C1 and C5 of glutamate (since [1-^{13}C] acetyl-CoA is derived from [2-^{13}C]pyruvate). However, if pyruvate undergoes carboxylation, the C2 and C3 of glutamate will become enriched, but the C4 of glutamate (at 34 ppm) cannot become enriched. As expected from the results of groups 6–8, Fig. 3 shows enrichment of glutamate C2 and C3 but not C4, thereby confirming pyruvate carboxylation under our perfusion conditions.

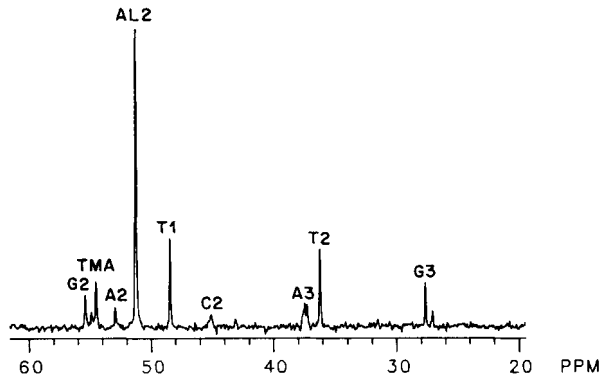

FIG. 3. **Direct detection of pyruvate carboxylation under steady-state conditions in the rat heart.** This spectrum was obtained from the extract of a heart perfused with 2.5 mM [2-^{13}C] pyruvate and 2.5 mM acetate. Resonance assignments: *A2*, aspartate C2; *A3*, aspartate C3; *C2*, citrate C2; *T1*, *T2*, taurine (natural abundance); *Al2*, alanine C2; *G2*, glutamate C2; *G3*, glutamate C3; and *TMA*, trimethylamino resonance of carnitine and acylcarnitine.

^{13}C Spectra of Intact Hearts—Although the results presented in Table I were derived from spectra of heart extracts, similar data may be obtained from spectra of intact, perfused hearts. Fig. 4 shows the expanded C4 and C3 resonances of hearts perfused with 2.5 mM [2-^{13}C]acetate alone (*lower panel*) or with a mixture of 2.5 mM [1,2-^{13}C]acetate plus 2.5 mM [3-^{13}C]pyruvate (*upper panel*). These intact heart spectra may be compared with the spectra of extracts shown in Fig. 2, *a* and *e*, respectively. The intact heart spectra illustrate that a 9-line C4 resonance may be detected but base-line resolution is probably not possible at this field. However, deconvolution methods may be applied to such signals to derive the physiological parameters of interest. In most simple cases, such as perfusion with singly labeled substrates (*i.e.* acetate or pyruvate), the relative multiplet intensities are easily obtained from the spectrum after a 30–40-min accumulation, and the physiological parameters derived from those spectra are within the error limits reported in Table I.

DISCUSSION

The detection of ^{13}C by NMR in intermediates during metabolism of ^{13}C-enriched compounds has major advantages over corresponding ^{14}C studies. As we have shown here, the relative concentration of ^{13}C isotopomers may be measured directly by NMR without purification and atom-by-atom degradation of the intermediate. This makes it possible to study intact tissue as it responds to physiological interventions.

Substrate Oxidation and Competition—Our NMR method shows that 88% of the total acetyl-CoA utilized by the heart during an acetate perfusion is derived directly from the exogeneous substrate. This agrees with ^{14}C tracer results which indicate that acetate provides between 85 and 89% of the total oxidized substrate in the perfused heart (8, 29, 30). Our method indicates that when pyruvate and glucose plus insulin were provided (group 2), most (86%) of the acetyl-CoA was derived from pyruvate. This observation differs from a previous report which found that hearts perfused with glucose

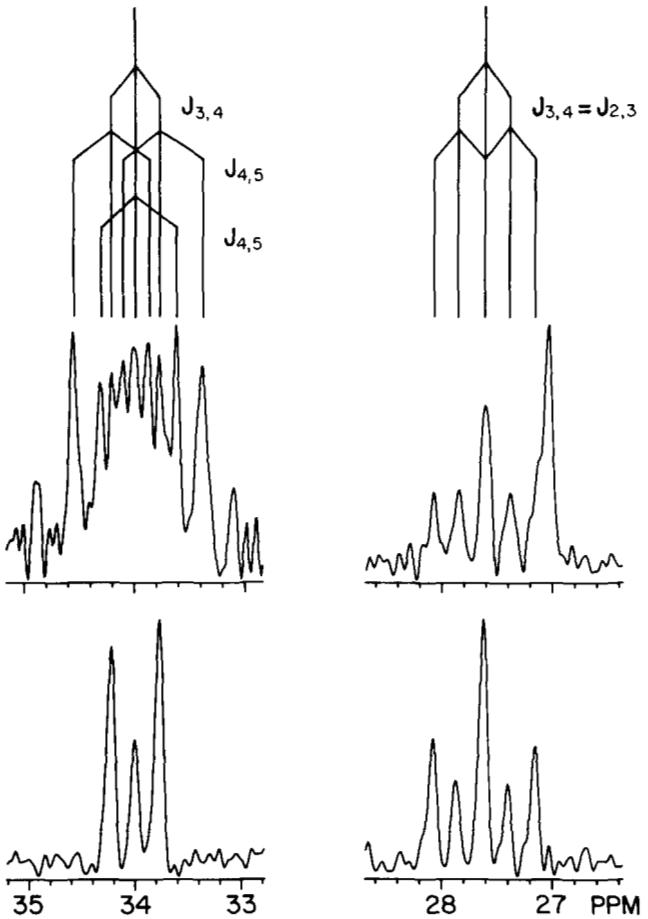

FIG. 4. **Glutamate C4 (34 ppm) and C3 (27.6 ppm) resonances from an intact heart perfused with 2.5 mM [2-^{13}C] acetate (*bottom*, 1200 scans, 30 min) or 2.5 mM [1,2-^{13}C] acetate plus 2.5 mM [3-^{13}C]pyruvate (*top*, 1600 scans, 40 min).** The methyl resonance of [3-^{13}C]pyruvate is detected at 26.9 ppm in the *upper spectrum*.

and 0.2 mM pyruvate only oxidize glucose (31). However, our results agree with Williamson's who used 10 mM pyruvate in competition with glucose and insulin (32). The concentration of pyruvate in the perfusing medium is obviously important since pyruvate inhibits pyruvate dehydrogenase kinase which, in turn, modulates pyruvate dehydrogenase activity (33).

Pyruvate reduced the contribution of exogenous acetate to the acetyl-CoA pool from 88% to between 65 and 70%. This measurement was obtained directly from hearts perfused with labeled acetate (groups 6 and 8) and indirectly from hearts perfused with labeled pyruvate and unlabeled acetate. In hearts presented with acetate or pyruvate as the sole exogenous substrate, about 12–16% of acetyl-CoA is derived from endogenous sources. When both acetate and pyruvate are available, less than 5% of acetyl-CoA is derived from endogenous sources (groups 6–8). We cannot identify the endogenous sources in these experiments, but since glucose does not compete significantly with pyruvate, it is likely that this source is triglycerides.

Propionate also reduces the contribution of acetate to acetyl-CoA. Previous studies have established that pyruvate may be generated from citric acid cycle intermediates derived from propionate (6). It is, therefore, not entirely unexpected that propionate reduces the net amount of acetyl-CoA derived from acetate, particularly in view of the known maximal activation of pyruvate dehydrogenase in the rat heart by propionate (29, 34).

Flux through Anaplerotic Pathways—Hassinen and colleagues (5–7, 21) have shown that there is constant flux through reactions which generate and eliminate citric acid cycle intermediates in the perfused heart even under steady-state conditions. The reactions which generate citric acid cycle intermediates were termed anaplerotic by Kornberg (35) over 20 years ago. Three sources of carbon skeletons for the citric acid cycle are relevant to the perfusion conditions of this report: carboxylation of propionate, carboxylation of pyruvate, and proteolysis followed by metabolism of certain amino acids through the citric acid cycle. Although propionate is a nonphysiological substrate, propionate carboxylation is quite active, presumably to provide for metabolism of the terminal carbons of longer chain odd-carbon fatty acids and of certain amino acids derived from breakdown of endogenous protein. The steady-state activity of this pathway may be measured by ^{13}C NMR spectroscopy even without presenting a labeled substrate which enters the citric acid cycle via this pathway (group 4). This study confirms earlier reports that propionate is readily metabolized by the heart (6, 29).

A pathway for pyruvate carboxylation in heart muscle has been established, although the activity of pyruvate carboxylase is much less in heart than in the gluconeogenic tissues (36). Nevertheless, pyruvate carboxylation plays an important role in cardiac metabolism (5–7). In a previous study using ^{14}C tracer techniques, the flux of pyruvate through carboxylating mechanisms was about 8% of the oxidative flux in hearts perfused with 10 mM glucose plus 0.2 mM pyruvate (21). Our measurement of 13 ± 7% (group 2) includes pyruvate carboxylation plus other sources of citric acid cycle skeletons (see below). The ^{13}C results are significant in this regard because entry of pyruvate-derived carbon through citrate synthase or through a carboxylation pathway may be distinguished (compare groups 1 and 7).

The steady-state anaplerotic flux of all hearts perfused with pyruvate was independent of cosubstrate (glucose plus insulin, acetate, or no other substrate; groups 1, 2, 6, 7, and 8) and averaged about 15% of the total oxidative flux. The source of carbon skeletons for anaplerosis, however, depended upon the cosubstrate. In both groups of hearts perfused with labeled pyruvate plus acetate (groups 7 and 8), a significant fraction of the carbon skeletons entering the citric acid cycle were derived from pyruvate in the perfusion medium. In contrast, the hearts perfused with pyruvate or pyruvate plus glucose and insulin (groups 1 and 2) did not derive a significant fraction of the substrate for anaplerotic reactions from pyruvate in the perfusate. These observations are quite consistent with the known stimulation of pyruvate carboxylase by acetyl-CoA which increases in the heart during perfusion with acetate (5). The observation that a significant fraction of pyruvate entering the citric acid cycle does so by a carboxylation pathway when acetate is present (groups 7 and 8) is very important when analyzing results of metabolic balance studies or release of $^{14}CO_2$ during metabolism of a ^{14}C-labeled precursor of pyruvate. Specifically, as noted by others previously (7), the procedure of calculating pyruvate dehydrogenase flux from the yield of $^{14}CO_2$ from [1-^{14}C]pyruvate may not be justified. Similarly, the assumption that all pyruvate removed from the perfusion medium by the heart is oxidized is probably not justified.

The third pathway for generation of citric acid cycle intermediates under these conditions is metabolism of endogenous proteins. Since amino acids were not added to the perfusing medium, ammonia generation from protein metabolism is anticipated. The total anaplerosis due to amino acid degradation under steady-state conditions in hearts perfused with

glucose is about 2% of the oxidative flux (37). During perfusion with acetate in this study, the low level of anaplerotic activity (about 5% of the oxidative flux) probably includes metabolism of endogenous amino acids.

The Isotopomer Analysis Approach—Chance *et al.* (10) previously reported a sophisticated model of the citric acid cycle in heart to analyze the time-dependent evolution of ^{13}C enrichment in glutamate. This elegant approach requires that ^{13}C fractional enrichments be measured in each glutamate carbon at several different times during the first 20–30 min after presenting a ^{13}C-enriched substrate to the heart. Signal-to-noise limitations for ^{13}C spectroscopy currently do not permit this time resolution for a single heart. However, as we have shown here, the relative rates of several important pathways in the citric acid cycle may be measured directly by analysis of multiplets in the glutamate spectrum on a single heart after reaching a steady-state ^{13}C distribution. Although the information available from this single spectrum is limited compared to the more complex kinetic model, it offers the best possibility of deriving important metabolic information in future *in vivo* studies. The validity of this method may be judged by criteria previously developed for assessing kinetic models of metabolism (38).

First, is the model based on reasonable assumptions? Well-established pathways for carbon flow previously used for analysis of ^{14}C radiotracer studies formed the basis of our model (16–18, 39). Therefore, to the extent that ^{13}C and ^{14}C results may be compared, the equations derived from earlier models should be identical to ours. After converting the notation to that appropriate for ^{13}C NMR studies, three equations may be derived from the earlier ^{14}C studies: C1/C3 $= 1/(2 + 2y)$, C3/C4 $= 1/(2y + 1)$, and C4D34 $= F_{c2}/(2y + 1)$ (16–18). Katz (18, 19) has validated these measurements using a variety of techniques in the liver. Walsh and Koshland (15), in a ^{13}C study, noted that C3/C4 = 1 and C1/C3 = ½ when the carbon enters the citric acid cycle solely through citrate synthase, *i.e.* $y = 0$. In another ^{13}C NMR study, Sherry *et al.* (11) concluded that the C3D34 $= F_{c2}$. In every case, the equations presented in the Appendix reduce to the previously derived equations under appropriate simplifying conditions. As in ^{14}C studies, we assumed that kinetic isotope effects are small (40, 41).

Second, how reliable are the experimental results? The quality of each spectrum is determined by two factors: the absolute concentration of glutamate in exchange with citric acid cycle intermediate pools and the total amount of ^{13}C entering those pools. Thus, the results are likely to be most accurate when the glutamate concentration and the enrichment are both high, *e.g.* during perfusion with [2-^{13}C]acetate. Of the three variables reported in Table I, the measurement of the fractional enrichment of anaplerotic substrate (F_{a1}) is likely to be least accurate since most carbon entering the citric acid cycle is derived from the oxidized substrate. This was confirmed by the data presented in Table I. Equations 1–6 indicate that the measurement of the three physiological variables is possible using only three observations, *e.g.* C4D34, C3S, and C3T. Although this approach is feasible in selected circumstances, more consistent results were obtained with a least squares fitting of all possible multiplet/total ratios from the C2, C3, and C4 resonances.

In summary, the approach presented here is a straightforward and easily performed method for deriving metabolic information from a single ^{13}C NMR spectrum of the heart obtained under steady-state conditions. The method may be applied to a wide variety of substrates under various physiological conditions and with singly or doubly ^{13}C-enriched precursors. It offers the advantage of simplicity over the more complex kinetic models (10) and hence could prove useful for future *in vivo* NMR studies of the myocardium.

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Appendix to

Evaluation of Carbon Flux and Substrate Selection Through Alternate Pathways Involving the Citric Acid Cycle of the Heart by C-13 NMR Spectroscopy

by

Craig R. Malloy, A. Dean Sherry, and F. Mark H. Jeffrey

The relative concentrations of glutamate ^{13}C isotopomers and the corresponding ^{13}C NMR spectrum may be related to various physiological parameters using a model of carbon flow in the citric acid cycle, which includes all standard cycle reactions except those involving the glyoxalate pathway. The model incorporates the following assumptions.

1. The citric acid cycle is in steady-state: the concentrations of the citric acid cycle intermediates and their exchanging pools are constant, the fractional enrichment at each carbon of the substrates and citric acid cycle intermediates is not changing, and the rate of disposal reactions equals the rate of anaplerotic reactions (16-19).
2. All ^{13}C entering oxaloacetate has been randomized between C1 and C4 and between C2 and C3 (11-13).
3. The fraction of labeled CO_2 involved in carboxylation of pyruvate is negligible (14,19,38).
4. The relative concentration of glutamate isotopomers is identical to the relative concentrations of α-ketoglutarate isotopomers.

These assumptions have been discussed in models developed to analyze both ^{13}C and ^{14}C tracer data from liver and perfused heart studies (10,14,15,18,19,38). Natural abundance ^{13}C (1.1%) is ignored in the development of these equations and has a negligible effect on the physiological parameters measured from the spectrum.

There are 32 possible ^{13}C glutamate (or α-ketoglutarate) isotopomers. The concentration of each isotopomer relative to the total exchanging glutamate pool is defined as:

x01 = [[1,2,3,4,5-^{12}C]glutamate]/[glutamate]
x02 = [[1-^{13}C]glutamate]/[glutamate]
x03 = [[2-^{13}C]glutamate]/[glutamate]
x04 = [[1,2-^{13}C]glutamate]/[glutamate]
x05 = [[3-^{13}C]glutamate]/[glutamate]
x06 = [[1,3-^{13}C]glutamate]/[glutamate]
x07 = [[2,3-^{13}C]glutamate]/[glutamate]
x08 = [[1,2,3-^{13}C]glutamate]/[glutamate]
x09 = [[4-^{13}C]glutamate]/[glutamate]
x10 = [[1,4-^{13}C]glutamate]/[glutamate]
x11 = [[2,4-^{13}C]glutamate]/[glutamate]
x12 = [[1,2,4-^{13}C]glutamate]/[glutamate]
x13 = [[3,4-^{13}C]glutamate]/[glutamate]
x14 = [[1,3,4-^{13}C]glutamate]/[glutamate]
x15 = [[2,3,4-^{13}C]glutamate]/[glutamate]
x16 = [[1,2,3,4-^{13}C]glutamate]/[glutamate]
x17 = [[5-^{13}C]glutamate]/[glutamate]
x18 = [[1,5-^{13}C]glutamate]/[glutamate]
x19 = [[2,5-^{13}C]glutamate]/[glutamate]
x20 = [[1,2,5-^{13}C]glutamate]/[glutamate]
x21 = [[3,5-^{13}C]glutamate]/[glutamate]
x22 = [[1,3,5-^{13}C]glutamate]/[glutamate]
x23 = [[2,3,5-^{13}C]glutamate]/[glutamate]
x24 = [[1,2,3,5-^{13}C]glutamate]/[glutamate]
x25 = [[4,5-^{13}C]glutamate]/[glutamate]
x26 = [[1,4,5-^{13}C]glutamate]/[glutamate]
x27 = [[2,4,5-^{13}C]glutamate]/[glutamate]
x28 = [[1,2,4,5-^{13}C]glutamate]/[glutamate]
x29 = [[3,4,5-^{13}C]glutamate]/[glutamate]
x30 = [[1,3,4,5-^{13}C]glutamate]/[glutamate]
x31 = [[2,3,4,5-^{13}C]glutamate]/[glutamate]
x32 = [[1,2,3,4,5-^{13}C]glutamate]/[glutamate]

By definition, x01 + x02 + ... + x32 = 1

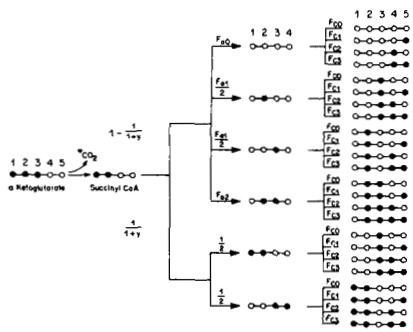
The fractional ^{13}C enrichment at each carbon (relative to total exchanging glutamate) is given by:

C1F=x02+x04+x06+x08+x10+x12+x14+x16+x18+x20+x22+x24+x26+x28+x30+x32
C2F=x03+x04+x07+x08+x11+x12+x15+x16+x19+x20+x23+x24+x27+x28+x31+x32
C3F=x05+x06+x07+x08+x13+x14+x15+x16+x21+x22+x23+x24+x29+x30+x31+x32
C4F=x09+x10+x11+x12+x13+x14+x15+x16+x25+x26+x27+x28+x29+x30+x31+x32
C5F=x17+x18+x19+x20+x21+x22+x23+x24+x25+x26+x27+x28+x29+x30+x31+x32

The relative area of each glutamate in a ^{13}C NMR spectrum multiplet is given by:

C1S=singlet/total=(x02+x06+x10+x14+x18+x22+x26+x30)/C1F
C1D=doublet/total=(x04+x08+x12+x16+x20+x24+x28+x32)/C1F
C2S=singlet/total=(x03+x11+x19+x27)/C2F
C2D12=doublet/total=(x04+x12+x20+x28)/C2F
C2D23=doublet/total=(x07+x15+x23+x31)/C2F
C2Q=quartet/total=(x08+x16+x24+x32)/C2F
C3S=singlet/total=(x05+x06+x21+x22)/C3F
C3D=doublet/total=(x07+x08+x13+x14+x23+x24+x29+x30)/C3F
C3T=triplet/total=(x15+x16+x31+x32)/C3F
C4S=singlet/total=(x09+x10+x11+x12)/C4F
C4D34=doublet/total=(x13+x14+x15+x16)/C4F
C4D45=doublet/total=(x26+x27+x28+x29)/C4F
C4Q=quartet/total=(x29+x30+x31+x32)/C4F
C5S=singlet/total=(x17+x18+x19+x20+x21+x22+x23+x24)/C5F
C5D=doublet/total=(x25+x26+x27+x28+x29+x39+x31+x32)/C5F

The steady-state isotopomer distribution was determined by the input-output method (18,19). The alternative pathways available to an α-ketoglutarate input molecule define the probability of a given output isotopomer after one turn of the citric acid cycle. As an example, the pathways available to [1,2,3-^{13}C] α-ketoglutarate are shown below.



Depending upon perfusion conditions, the symmetric 4 carbon molecule [1,2-^{13}C]succinyl-CoA, may proceed to oxaloacetate (lower branch) or it may be lost to disposal reactions (upper branch). Note that the sum of the alternative pathways in each branch is 1. If the [1,2-^{13}C]succinate (or other equivalent 4 carbon intermediate) is disposed of, it will be replaced, through some anaplerotic reaction, by a molecule which may be unlabeled, labeled in the 2 position, the 3 position or both 2 and 3 depending upon the ^{13}C enrichment of the anaplerotic substrate. Finally, each possible oxaloacetate isotopomer may condense with unlabeled acetyl-CoA or acetyl-CoA labeled in C1, C2 or C1 and C2. Similar flow diagrams may be constructed for each of the 32 possible isotopomers. Since the system is at steady-state, mass is conserved, and the concentration of a given input isotopomer must equal the sum of all isotopomer concentrations times the probability that each isotopomer will yield that isotopomer of interest after one turn of the cycle. This step produces 32 equations in 32 unknowns which may be solved to relate the relative concentrations of each isotopomer to the physiological variables defined above.

Although the concentration of a single isotopomer cannot be measured from the ^{13}C spectrum, the relative concentration of various groups can be directly measured, thereby relating the ^{13}C spectrum to physiological variables.

The solutions for the most general case, including four possible labeling patterns in acetyl-CoA, three possible labeling patterns in the anaplerotic substrates, and the relative activity of the anaplerotic versus oxidative pathways are:

C1 resonance

C1F=1-(((g+h)a)/(2F_{c0}))-((a(F_{c0}+F_{c2})/2)-a+1)
C1S=(j-(aF_{c0}^{i}/2)-d-e)/(C1F.F_{c0})
C1D=1-C1S

C2 resonance

C2F=(g+i)/F_{c0}
C2S=((aF_{c2}(g+h)/2)+(a(F_{c0}+F_{c1})g/2)+e)/(g+i)
C2D12=(g/(g+i))-C2S
C2D23=((aF_{c2}+aF_{c3}/2)g+(aF_{c2}^{i}/2)+f)/(g+i)
C2Q=1-C2S-C2D12-C2D23

C3 resonance

C3F=(g+i)/F_{c0}
C3S=g(F_{c0}+F_{c1})/(g+i)
C3T=i(F_{c2}+F_{c3})/(g+i)
C3D=1-C3S-C3T

C4 resonance

C4F=F_{c2}+F_{c3}
C4S=(F_{c2}(g+h))/(F_{c0}(F_{c2}+F_{c3}))
C4Q=(F_{c3}(g+i))/(F_{c0}(F_{c2}+F_{c3}))
C4D34=(F_{c2}(g+i))/(F_{c0}(F_{c2}+F_{c3}))
C4D45=(F_{c3}(g+h))/(F_{c0}(F_{c2}+F_{c3}))

C5 resonance

C5F=F_{c1}+F_{c3}
C5S=F_{c1}/C5F
C5D=1-C5S

where:

a = 1/(1+y)
b = (F_{c0}+F_{c1})a
c = (F_{c2}+F_{c3})a
d = $F_{a0}$$F_{c0}$(1-a)
e = $F_{a1}$$F_{c0}$(1-a)/2
f = $F_{a2}$$F_{c0}$(1-a)
g = ((1-b)(1-c)F_{c0}-d(1-c)-f(1-b))/(2-a)
h = (bg+d)/(1-b)
i = (cg+F)/(1-c)
j = (g+h)(1-(aF_{c0}))-(a(1-F_{c0})h/2)

The raw data for the relative component areas of the glutamate C2, C3, and C4 multiplets for the eight groups of hearts presented in the manuscript is summarized below (the value in parenthesis is the standard deviation (n=4)).

		Glutamate Resonance										
		C2				C3			C4			
group	Available substrate	S	D12	D23	Q	S	D	T	S	D34	D45	Q
1	[3-^{13}C]pyruvate	0.22 (0.03)	0.08 (0.03)	0.43 (0.09)	0.27	0.02 (0.05)	0.35	0.63 (0.09)	0.34	0.66 (0.06)	0	0
2	[3-^{13}C]pyrvate +glucose+insulin	0.19 (0.05)	0.06 (0.01)	0.45 (0.02)	0.30	0.02 (0.01)	0.31	0.67 (0.04)	0.28	0.72 (0.02)	0	0
3	[2-^{13}C]acetate	0.11 (0.01)	0.04 (0.01)	0.52 (0.03)	0.33	0.03 (0.01)	0.25	0.72 (0.02)	0.19	0.81 (0.01)	0	0
4	[2-^{13}C]acetate +propionate	0.31 (0.01)	0.08 (0.02)	0.42 (0.02)	0.19	0.08 (0.03)	0.41	0.51 (0.04)	0.47	0.53 (0.04)	0	0
5	[2-^{13}C]acetate +[3-^{13}C]propionate	0.34 (0.03)	0.04 (0.01)	0.44 (0.02)	0.18	0.03 (0.03)	0.40	0.57 (0.05)	0.36	0.64 (0.03)	0	0
6	[2-^{13}C]acetate +pyruvate	0.29 (0.02)	0.09 (0.01)	0.41 (0.02)	0.21	0.12 (0.03)	0.46	0.42 (0.02)	0.43	0.57 (0.04)	0	0
7	[3-^{13}C]pyruvate +acetate	0.71 (0.06)	0	0.29 (0.06)	0	0.61 (0.09)	0.39	0	0.69	0.31 (0.06)	0	0
8	[1,2-^{13}C]acetate +[3-^{13}]pyruvate	0.10 (0.01)	0.15 (0.02)	0.24 (0.02)	0.51	0.01 (0.06)	0.31	0.68 (0.11)	0.17 (0.03)	0.24 (0.04)	0.18 (0.03)	0.41