Sources of Acetyl-CoA Entering the Tricarboxylic Acid Cycle As Determined by Analysis of Succinate ^{13}C Isotopomers†

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ABSTRACT: A new ^{13}C NMR technique for measuring substrate utilization by the citric acid cycle based on an analysis of succinate ^{13}C isotopomers is presented. The relative contribution of up to three different labeling patterns in acetyl-CoA entering the citric acid cycle may be determined under non-steady-state conditions. We present experimental data from perfused rat hearts subjected to a brief period of ischemia, where both succinate and glutamate resonances were observed in the ^{13}C spectrum. The contributions of labeled exogenous acetate and lactate and unlabeled sources to the acetyl-CoA pool were compared using this succinate analysis and a previously published glutamate analysis [Malloy *et al.* (1990) *Biochemistry 29*, 6756–6761], and the two methods give identical results. This indicates that the succinate and glutamate isotopomers originated from a common α-ketoglutarate pool, verifying that glutamate is in isotopomeric equilibrium with α-ketoglutarate under these conditions.

^{13}C NMR has been used to determine relative fluxes of labeled metabolites into the tricarboxylic acid (TCA) cycle in a wide variety of organisms and tissues (Dickinson *et al.*, 1983; Walker *et al.*, 1982; Malloy *et al.*, 1990a,b; Jans *et al.*, 1991). Spin–spin coupling between glutamate carbons in a mixture of ^{13}C isotopomers (called an isotopomer analysis) provides a convenient method for monitoring metabolite flux into the cycle (Malloy *et al.*, 1987, 1990a,b). An analysis of the glutamate C4 multiplet allows the relative contribution of singly- and doubly-labeled acetyl-CoA units into the TCA cycle to be determined rapidly and unambiguously (Malloy *et al.*, 1990b; Sherry *et al.*, 1992). In addition, the contribution of unlabeled acetyl-CoA units to the cycle can be determined from the analysis of C4 and C3 resonances of glutamate (Malloy *et al.*, 1987) or by analysis of the C3 multiplet during decoupling of the C2 carbon (Sherry *et al.*, 1992). Underlying these methods is the assumption that the distribution of ^{13}C in glutamate is identical to that in α-ketoglutarate.

In some instances, however, the glutamate concentration in tissue may not be as high as other metabolites, such as aspartate or succinate (Sherry *et al.*, 1985; Blackburn *et al.*, 1986; Mackenzie *et al.*, 1982; Kawanaka *et al.*, 1989; Rainey & Mackenzie, 1991), and it is important to extend the isotopomer technique to a variety of other metabolites that may be visible by NMR. This report demonstrates the use of succinate isotopomers to calculate the relative contributions of up to three different acetyl-CoA isotopomers to the TCA cycle in rat heart under both steady-state and non-steady-state conditions. Spectra are presented containing both succinate and glutamate resonances, enabling a direct comparison of the two analyses to test whether these two metabolites share a common pool of α-ketoglutarate.

EXPERIMENTAL PROCEDURES

Materials. Sodium [3-^{13}C]lactate, sodium [2-^{13}C]acetate, sodium [1,2-$^{13}C_2$]acetate, uniformly-labeled succinate, and sodium [1,2,3-$^{13}C_3$]propionate (all 99% enriched) were obtained from Cambridge Isotopes Laboratories, Woburn, MA.

Preparation and Analysis of Tissue Extracts. Male rats (300–350 g) of the Sprague–Dawley strain were allowed free access to water and food. Rats were anaesthetized by intraperitoneal injection of pentobarbital. Hearts were perfused using the Langendorff technique at a pressure of 100 cm of H_2O with standard Krebs–Henseleit medium ($[Ca^{2+}]$ = 1.25 mM), bubbled continuously with a 95%/5% O_2/CO_2 gas mixture. The spontaneous heart rate was 300–380 beats/min. The perfusate temperature was maintained at 37 °C, and the O_2 tension at the level of the aorta was >550 mmHg. For acetyl-CoA utilization studies, hearts were perfused initially with 10 mM unlabeled glucose during an initial stabilization period of 5–10 min, followed by the addition of sodium acetate to give a concentration of 0.25 mM and of sodium lactate to give a concentration of 0.25–2.0 mM. In some experiments, the acetate alone was labeled as [2-^{13}C]-acetate or [1,2-$^{13}C_2$]acetate, and in others, both acetate and lactate were labeled as [1,2-$^{13}C_2$]acetate and [3-^{13}C]lactate. For the propionate studies, 0.5 mM sodium [1,2,3-$^{13}C_3$]-propionate was added to an unlabeled lactate/acetate mixture. Following the addition of the label, the hearts were perfused for an additional 15 min. Total flow to the heart was then stopped for 5 min, while maintaining the temperature at 37 °C by submersing the perfusion chamber in the same water bath unit used to control the perfusate temperature. After this period, the hearts were immediately freeze-clamped using aluminum tongs cooled in liquid nitrogen. The freeze-clamped tissue was stored at this temperature prior to the extraction procedure.

Freeze-clamped tissue was ground to a fine powder at liquid nitrogen temperature, and 3.6% perchloric acid was added (2 mL/g of tissue) and thoroughly homogenized with the tissue.

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The mixture was allowed to thaw slowly on ice and was centrifuged for 10 min at 13000*g*. The supernatant was retained, and its pH adjusted to 7.0 with KOH. Potassium perchlorate was precipitated by a second centrifugation step using the same speed, and the clear supernatant was freeze-dried. The lyophilized extracts were redissolved in 800 μL of D_2O for NMR analyses.

NMR Spectroscopy. Proton-decoupled ^{13}C spectra were obtained with a 5-mm probe on a 9.4-T General Electric Omega spectrometer operating at 100.61 MHz for ^{13}C. The pulse width was 45°, and the spectral sweep width was 20 000 Hz with a 2-s delay between pulses. The free-induction decays were collected in 32K of memory using consecutive blocks of 1000–2000 scans. Broad-band proton decoupling was employed using the WALTZ-16 sequence. A line broadening of 0.5–1.0 Hz was applied before Fourier transformation to improve the signal-to-noise ratio. Proton spectra of extracts were obtained with a 5-mm probe on a 11.75-T General Electric GN-500 spectrometer operating at 500 MHz for ^1H. A 45° pulse angle and delay time of 2 s were used following a 5-s presaturation of water protons. The sweep width was ±5600 Hz, and the spectra were collected in 16K of memory using 8–24 transients. Single-frequency carbon decoupling was used to irradiate the succinate methylene carbon at 35 ppm, resulting in the collapse of the ^{13}C-coupled doublets into a single center resonance. The fractional enrichment of the methylene carbons was obtained by comparing the relative areas of the center singlets in the decoupled and non-decoupled spectra. ^{13}C spectra were analyzed with the ^{1}NMR curve-fitting program (Sun Microsystems) while ^1H spectra were analyzed by cutting and weighing. Substrate utilization ratios from glutamate resonances were analyzed using the non-steady-state method (Malloy *et al.* 1990b).

Regression analysis of the correlation between succinate and glutamate isotopomer analyses was performed with a PC-compatible statistics program (Statview II, Abacus Concepts Inc., Berkeley, CA).

RESULTS

^{13}C and ^1H Spectra of Labeled Succinate. Figure 1 shows the 33.7–35.5-ppm region of ^{13}C spectra of extracts of perfused rat heart made briefly ischemic during perfusion with [2-^{13}C]-acetate (top) or [1,2-$^{13}C_2$]acetate and 2 mM [3-^{13}C]lactate (bottom). After 5 min of global ischemia, the succinate and glutamate concentrations were sufficiently high for detection by ^{13}C NMR, compared to standard perfusion conditions where the glutamate resonances normally dominate the spectrum (Sherry *et al.*, 1985; Malloy *et al.*, 1987, 1988). The glutamate/succinate ratio in the ischemic heart as measured by the glutamate C4 and succinate methylene intensities ranged between 3 and 9. Multiplets centered around 34.9 ppm belong to the chemically identical methylene carbons of succinate while those centered around 34.2 ppm are from glutamate C4.

A proton spectrum (Figure 2, top) of the heart extract used for the experiment in Figure 1 (bottom) showed a resolvable succinate singlet at 2.4 ppm and one of the ^{13}C-coupled doublets (J_{CH} = 126 Hz), the other being obscured by overlapping resonances. The assignment of the doublets was confirmed by obtaining a spectrum with single-frequency carbon decoupling of the succinate methylene carbons at 35 ppm (Figure 2, bottom). In the ^{13}C-decoupled spectrum, the doublets were absent, and the intensity of the center singlet was equivalent to the sum of the singlet and doublets in the undecoupled spectrum. Thus, the ^1H spectrum provides a direct measurement of the ^{13}C fractional enrichment of the succinate methylene carbon pool.

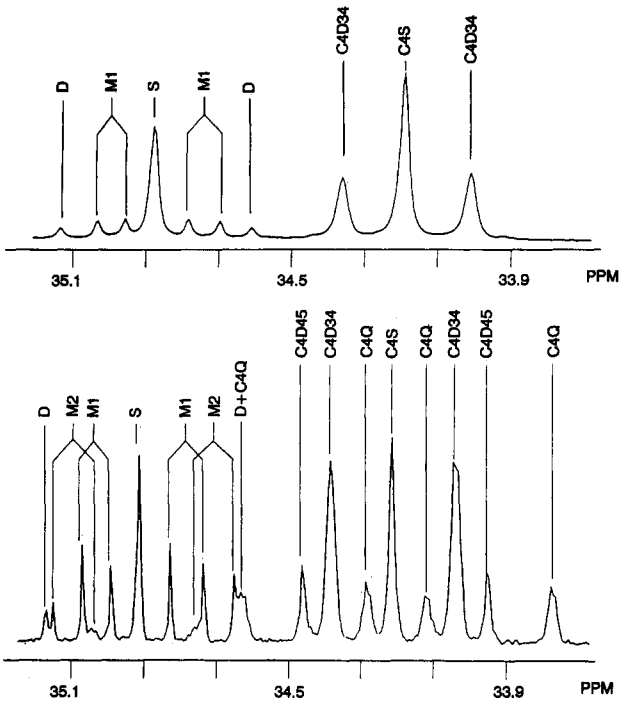

FIGURE 1: ^{13}C NMR extract spectra of glutamate C4 and succinate methylene multiplets from a heart perfused with [2-^{13}C]acetate (top) and a mixture of [1,2-$^{13}C_2$]acetate and [3-^{13}C]lactate (bottom). Abbreviations: D, succinate doublet from [1,2-$^{13}C_2$]succinate; M2, succinate multiplet from [U-$^{13}C_4$]succinate; M1, succinate multiplet from [1,2,2-$^{13}C_3$]succinate; S, succinate singlet arising from [2-^{13}C]- and [2,2′-$^{13}C_2$]succinate; C4Q, glutamate quartet; C4D45, glutamate doublet due to J_{45}; C4D34, glutamate doublet due to J_{34}; C4S, glutamate singlet.

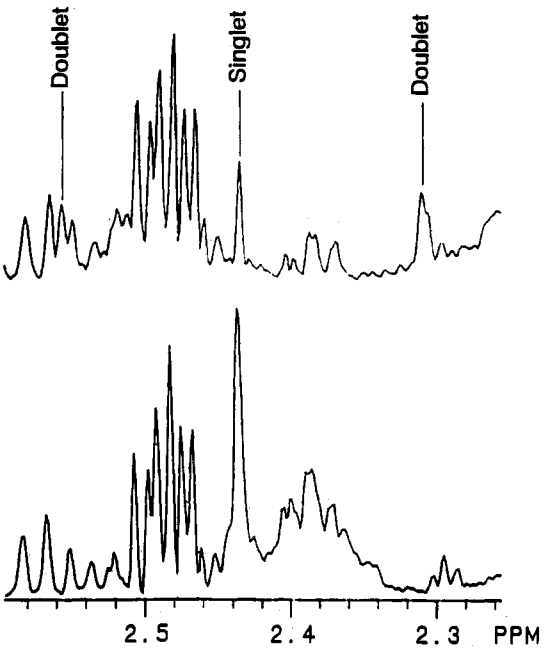

FIGURE 2: Proton spectra of extract featuring the succinate methylene region without (top) and with (bottom) single-frequency ^{13}C-decoupling. Spectra consist of 24 scans with resolution enhancement (double multiplication, 0.4 Hz) applied before Fourier transform to better resolve the doublets. Integration of the center singlet was performed on spectra processed without this apodization procedure.

Assignments of the Succinate ^{13}C Multiplets. The succinate methylene multiplet can be decomposed to several distinct isotopomer groups (Figure 3). Although the succinate

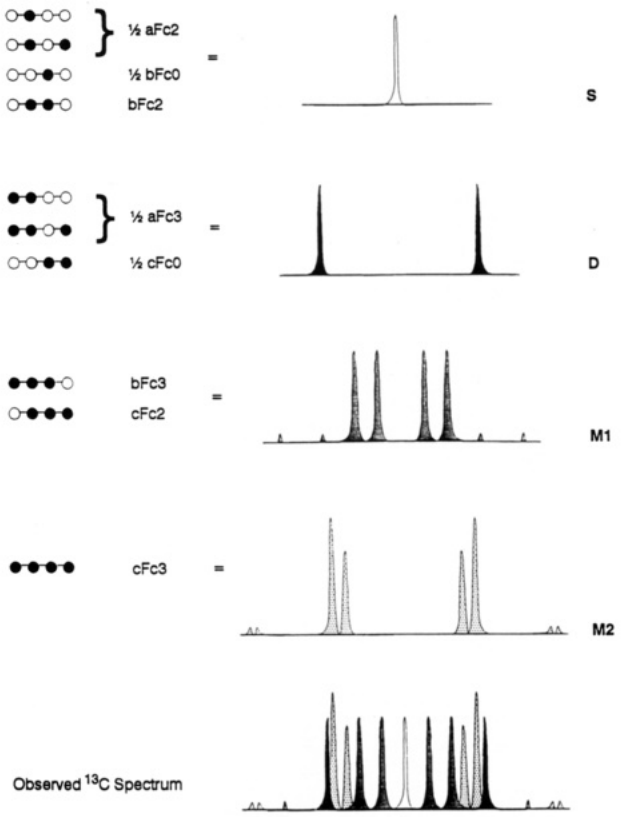

FIGURE 3: Relationship of the isotopomer groups to the succinate ^{13}C spectra. M1 refers to the [1,2,2′-$^{13}C_3$]succinate multiplet, and M2 refers to the uniformly-labeled succinate multiplet.

methylene spectrum at 4.7 T has been reported previously (Mackenzie *et al.*, 1982), the possibility of overlap of this resonance with those of other metabolite resonances in extracts necessitated explicit assignment of the spectrum at the higher field. The M2 component was assigned by obtaining a spectrum of commercially available uniformly-labeled succinate, while the remaining components were assigned in spectra of perfused hearts supplied with uniformly-labeled propionate. On entry into the citric acid cycle via propoinyl-CoA carboxylase, [1,2,2-$^{13}C_3$]succinate (M1) is initially generated, with [1,2-$^{13}C_2$]succinate (D) and [2-^{13}C]-succinate (S) formed on successive turns of the cycle. Uniformly-labeled succinate cannot be produced in this experiment. Figure 4 shows a spectrum of an extract obtained from a perfused heart where the sole source of the label was [1,2,3-$^{13}C_3$]propionate. Given the known chemical shifts of the singlet (S) and doublet (D), the resonances due to [1,2,2′-$^{13}C_3$]succinate (M1) could be assigned by the difference.

Small outlying resonances situated symmetrically about M1 and M2 multiplets could be detected in some experimental spectra (see Figure 4). The experimental areas of the outlying resonances were found to be somewhat smaller compared to simulated spectra using coupling constants previously reported for succinate isotopomers (Mackenzie *et al.*, 1982).

Derivation of Equations Which Relate ^{13}C Succinate Isotopomers to Intensities of Multiplets in ^{13}C Spectrum. All succinate isotopomers are assumed to have been derived from the corresponding α-ketoglutarate isotopomers by the loss of the C1 carbon. The parent α-ketoglutarate isotopomers were divided into three sets labeled **a**, **b**, and **c**, based on the labeling permutations in carbons 1, 2, and 3 (see Figure 5). The first set, **a**, consists of all α-ketoglutarate isotopomers where C3 is never labeled; the second set, **b**, consists of isotopomers where C2 is never labeled; and the third group, **c**, consists of all isotopomers where either C2 and C3 are labeled, or C1, C2, and C3 are labeled.

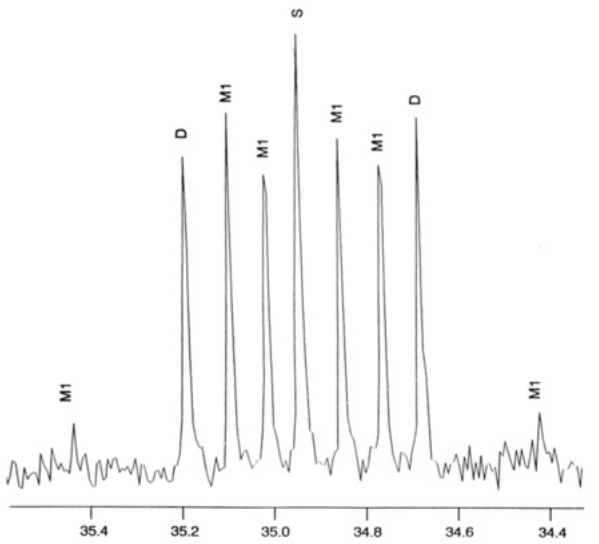

FIGURE 4: ^{13}C NMR extract spectrum of the succinate methylene multiplet from a heart freeze-clamped after perfusion with 10 mM unlabeled glucose, 1 mM unlabeled lactate, 0.25 mM unlabeled acetate, and 0.5 mM [1,2,3-$^{13}C_3$] propionate. This spectrum represents 5000 acquisitions with 0.2 Hz of exponential multiplication applied to the free-induction decays before Fourier transformation.

	X	Glutamate α-Ketoglutarate 1 2 3 4 5	Succinate 1 2 3 4	Methylene ^{13}C Spectrum	Methylene ^{1}H Spectrum
	1	○○○○○	○○○○		
aF_{C0}	2	●○○○○	○○○○		S
	3	○●○○○	●○○○		
	4	●●○○○	●○○○		
bF_{C0}	5	○○●○○	○●○○	S	S+D
	6	●○●○○	○●○○		
cF_{C0}	7	○●●○○	●●○○	D	S+D
	8	●●●○○	●●○○		
	9	○○○●○	○○●○		
aF_{C2}	10	●○○●○	○○●○	S	S+D
	11	○●○●○	●○●○		
	12	●●○●○	●○●○		
bF_{C2}	13	○○●●○	○●●○	S	D
	14	●○●●○	○●●○		
cF_{C2}	15	○●●●○	●●●○	M1	D
	16	●●●●○	●●●○		
	17	○○○○●	○○○●		
aF_{C1}	18	●○○○●	○○○●		S
	19	○●○○●	●○○●		
	20	●●○○●	●○○●		
bF_{C1}	21	○○●○●	○●○●	S	S+D
	22	●○●○●	○●○●		
cF_{C1}	23	○●●○●	●●○●	D	S+D
	24	●●●○●	●●○●		
	25	○○○●●	○○●●		
aF_{C3}	26	●○○●●	○○●●	D	S+D
	27	○●○●●	●○●●		
	28	●●○●●	●○●●		
bF_{C3}	29	○○●●●	○●●●	M1	D
	30	●○●●●	○●●●		
cF_{C3}	31	○●●●●	●●●●	M2	D
	32	●●●●●	●●●●		

FIGURE 5: Grouping of succinate isotopomers based on the permutation of singly-labeled, doubly-labeled, and unlabeled acetyl-CoA units of α-ketoglutarate C4 and C5, with all combinations of labeling in α-ketoglutarate C1, C2, and C3. Loss of the α-ketoglutarate C1 carbon generates the corresponding succinate isotopomer. The α-ketoglutarate isotopomer assignments (× 1 – × 32) are identical to those used in previous studies (Malloy *et al.*, 1988).

Carbons C4 and C5 of α-ketoglutarate are derived directly from acetyl-CoA on *each* turn of the TCA cycle. This pool potentially contains four kinds of acetyl-CoA units; an unlabeled fraction (F_{c0}), a C1-labeled fraction (F_{c1}), a C2-labeled fraction (F_{c2}), and a doubly-labeled fraction (F_{c3}). All 32 α-ketoglutarate isotopomers can be expressed as a combination of **a**, **b**, and **c** and the four acetyl-CoA fractions. All succinate isotopomers generated from α-ketoglutarate can also be represented by these expressions. The magnetically equivalent isotopomers (for example, 15,16 and 29,30 in Figure 5) are simply expressed as the sum of the parent α-ketoglutarate terms (in this example, $\mathbf{b}F_{c3} + \mathbf{c}F_{c2}$). The individual components of the succinate spectrum and the ^{13}C fractional enrichment (as measured by the ^1H spectrum) can be represented by the following simple equations.

S·C2F = (singlet area/total multiplet area) (C2 fractional enrichment)

$$= 1/2\mathbf{a}(F_{c2}) + \mathbf{b}(F_{c2}) + 1/2\mathbf{b}(F_{c0}) \quad (1)$$

D·C2F = (doublet area/total multiplet area) (C2 fractional enrichment)

$$= 1/2\mathbf{c}(F_{c0}) + 1/2\mathbf{a}(F_{c3}) \quad (2)$$

M1·C2F = (122′ area/total multiplet area) (C2 fractional enrichment)

$$= \mathbf{c}(F_{c2}) + \mathbf{b}(F_{c3}) \quad (3)$$

M2·C2F = (unlabeled succinate area/total multiplet area) (C2 fractional enrichment)

$$= \mathbf{c}(F_{c3}) \quad (4)$$

C2F = C2 fractional enrichment

= (^1H doublet area/total succinate proton area)

$$= 1 - 1/2(F_{c0}) - 1/2\mathbf{a} \quad (5)$$

These equations completely describe all the possible labeling permutations in the methylene carbons of succinate, where mixtures of substrates which can only generate C1-enriched, C2-enriched, C1 and C2-enriched, or unlabeled acetyl-CoA units. In experiments where only two types of enriched acetyl-CoA can be generated (for example, during perfusion with [2-^{13}C]acetate as the sole labeled substrate only those equations containing F_{c2} and F_{c0} are relevant), the equations simplify further due to the omission of the terms containing F_{c1} and F_{c3}. In this case, the F_{c2}/F_{c0} ratio is simply given by

$$F_{c2}/F_{c0} = \text{M1}/(2\cdot\text{D}) \quad (6)$$

When F_{c3} and F_{c0} are the only acetyl-CoA species possible, terms containing F_{c1} and F_{c2} are absent from the equations and the ratio of F_{c3}/F_{c0} is given by

$$F_{c3}/F_{c0} = \text{M1}/(2\cdot\text{S}) \quad (7)$$

Note that these determinations do not require the separate measurement of C2F by ^1H NMR since this term cancels out in both expressions.

For measuring the contribution from three different sources of acetyl-CoA, the succinate methylene carbon and proton resonances provide four simultaneous equations that are related to the experimental measurements S·C2F, D·C2F, M1·C2F, and M2·C2F (eqs 1–4). Further, by definition, $F_{c0} + F_{c2} + F_{c3} = 1$, and $\mathbf{a} + \mathbf{b} + \mathbf{c} = 1$. These six equations with six unknowns were solved for the observed ^{13}C data using a PC-compatible mathematical software program (Mathcad, Mathsoft Inc., Cambridge, MA) with a tolerance level of 0.05 for all the data. The tolerance level is the maximum allowed value for root of the sum of squares of the difference between the observed and the calculated variables.

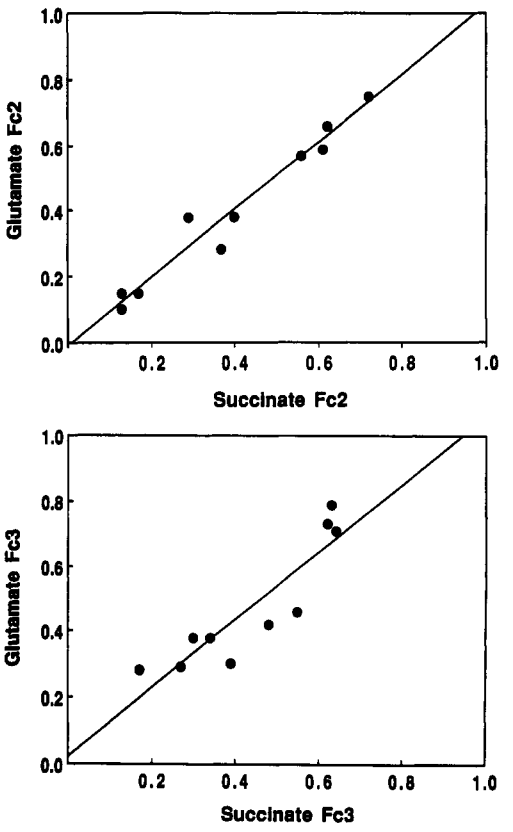

FIGURE 6: Comparison of glutamate and succinate analyses of the fraction of C2-labeled acetyl-CoA (F_{c2}) (top) and doubly-labeled acetyl-CoA (F_{c3}) (bottom). Regression analysis for the top graph gave a slope of 1.04, intercept of −0.01, and a coefficient of determination (r^2) of 0.96, while for the bottom graph, the slope was 1.03, the intercept was 0.02, and r^2 was 0.80.

The presence of a resolved glutamate C4 multiplet adjacent to the succinate multiplet in each of the ^{13}C spectra also allowed an independent measurement of substrate utilization as reported by the glutamate pool (Malloy *et al.*, 1990b). The physiological variables (F_{c2} and F_{c3}) obtained from the succinate analysis are plotted versus those obtained from the glutamate spectrum in Figure 6. The good agreement of these data indicate that the glutamate and succinate pools are in equilibrium with the same pool of α-ketoglutarate during ischemic episodes in perfused heart.

DISCUSSION

A significant feature of the succinate analysis is that the relative utilization of acetyl-CoA units may be obtained from one resonance, unlike in the glutamate analysis where information from both the C4 and C3 multiplets must be used (Malloy *et al.*, 1987; Sherry *et al.*, 1992). Like the direct glutamate C4 analysis presented earlier (Sherry *et al.*, 1992), the succinate analysis presented here is not affected by the flux of either labeled or unlabeled carbons through the citric acid cycle pools via an anaplerotic reaction or by non-steady-state isotopomeric conditions. The reasons for this may be explained by examining Figure 5. If, for example, anaplerosis was high and the anaplerotic substrate was unlabeled, the amount of unlabeled oxaloacetate entering the citrate synthase reaction would be high, and hence the glutamate isotopomers numbered 1, 9, 17, and 25 in Figure 5 would be high. This

would be reflected experimentally as a lower than expected C2F because of the high **a** terms. It would not, however, influence the values of F_{c0}, F_{c1}, F_{c2}, and F_{c3} because these are determined solely by the distribution of ^{13}C in the glutamate C4 and C5 positions. A similar argument can be made for a system not at isotopomeric steady-state.

The only assumptions made in the analysis presented is that the succinate pool has turned over at least once during the experiment, so that the contribution of unlabeled succinate methylenes reflects the contributions of unlabeled acetyl-CoA units and not the endogenous succinate pool present before label incorporation. The presence of substantial amounts of uniformly-labeled and [1,2,2′-$^{13}C_3$]succinate isotopomers, which can only be formed by multiple turns of the TCA cycle, provides strong support for this assumption.

In some of the ischemic hearts, nearly all of the acetyl-CoA units were derived from the labeled acetate with only minor amounts from other sources. This preferential utilization of acetate over the glycolytic substrates, lactate and glucose, is consistent with the known inactivation of pyruvate dehydrogenase that occurs during ischemia (Patel & Olson, 1984). This has been detected previously in ^{13}C NMR spectra of extracts of ischemic and reperfused rat hearts (Sherry *et al.*, 1992). While succinate accumulation was observed in all our experiments, increased utilization of acetate relative to the other available substrates was only observed in a few. This suggests that succinate accumulation precedes inactivation of the pyruvate dehydrogenase complex during the early stages of ischemia in perfused heart.

It has been suggested that a significant portion of the succinate pool under ischemic conditions is generated by a reductive pathway that converts aspartate and oxaloacetate to succinate (Owen & Hochachka, 1974; Taegtmeyer, 1978; Sandborn *et al.*, 1979). This is believed to be coupled to α-ketoglutarate oxidation, providing a mechanism for maintaining the mitochondrial NAD/NADH ratio in the absence of oxygen (Owen & Hochachka, 1974). In this scheme, the four-carbon pools (aspartate, oxaloacetate, malate, etc.) and the five-carbon pools (glutamate and α-ketoglutarate) would contribute equally to the pool of succinate generated during ischemia. Our results indicate that a close homology exists between the glutamate and succinate isotopomer pools, suggesting that the succinate carbons predominantly originated from α-ketoglutarate with no significant contributions from other pools, or that the aspartate/oxaloacetate isotopomeric composition exactly reflects the α-ketoglutarate/glutamate isotopomeric composition. Other studies of this reductive pathway suggest that the contribution of reductive TCA cycle reactions to the succinate pool in glucose perfused hearts during ischemia is only significant in the presence of high exogenous levels of fumarate and malate (Wiesener *et al.*, 1988).

Finally, the homology between the glutamate and succinate isotopomer pools under conditions where both the pool sizes and the utilization fraction of the competing labeled substrates are rapidly changing (Neely *et al.*, 1972; Kobayashi & Neely, 1983; Sherry *et al.*, 1992) strongly suggests that these pools are in isotopomeric equilibrium. This implies that the common intermediate, α-ketoglutarate, must also have the same isotopomeric composition, supporting the long-held view that glutamate analysis provides an accurate indication of label distributions in the carbons of α-ketoglutarate.

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