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Accelerated Publications

Contribution of Exogenous Substrates to Acetyl Coenzyme A: Measurement by ^{13}C NMR under Non-Steady-State Conditions[†]

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ABSTRACT: A method is presented for the rapid determination of substrate selection in a manner that is not restricted to conditions of metabolic and isotopic steady state. Competition between several substrates can be assessed directly and continuously in a single experiment, allowing the effect of interventions to be studied. It is shown that a single proton-decoupled ^{13}C NMR spectrum of glutamate provides a direct measure of the contribution of exogenous ^{13}C-labeled substrates to acetyl-CoA without measurement of oxygen consumption and that steady-state conditions need not apply. Two sets of experiments were performed: one in which a metabolic steady state but a non-steady-state ^{13}C distribution was achieved and another in which both metabolism and labeling were not at steady state. In the first group, isolated rat hearts were supplied with [1,2-^{13}C]acetate, [3-^{13}C]lactate, and unlabeled glucose. ^{13}C NMR spectra of extracts from hearts perfused under identical conditions for 5 or 30 min were compared. In spite of significant differences in the spectra, the measured contributions of acetate, lactate, and unlabeled sources to acetyl-CoA were the same. In the second set of experiments, the same group of labeled substrates was used in a regional ischemia model in isolated rabbit hearts to show regional differences in substrate utilization under both metabolic and isotopic non steady state. This sensitive probe of substrate selection was also demonstrated in intact hearts where excellent time resolution (3 min) of substrate selection was feasible. Since the basis of the method involves measurement of two resonance areas (C4 and C3) and the relative multiplet components of the carbon 4 resonance, simple *J*-modulated editing schemes allow substrate selection to be measured even when ^{13}C–^{13}C coupling cannot be resolved, as would be expected in vivo. The time resolution of these measurements may not be limited by technical contraints but by the rate of carbon flux in the citric acid cycle. Although this technique is demonstrated for the heart, it is applicable to all tissues.

The contribution of exogenous substrates to acetyl-CoA is an important aspect of myocardial energetics and has been an objective of numerous studies (Williamson & Krebs, 1961; Williamson, 1962; Neely et al., 1972; Mickle et al., 1986; Myears et al., 1987; Liedtke et al., 1988, Wyns et al., 1989). Measurement of the respiratory fuel requires an estimate of the rate of acetyl-CoA utilization, typically from oxygen consumption, and the rate of substrate utilization under steady-state conditions. The latter usually is measured by the rate of appearance of ^{14}CO$_2$ from a ^{14}C-enriched substrate, the rate of removal of substrate from the perfusion medium, or multiexponential analysis of ^{11}C time–activity curves. However, substrate and oxygen removal are difficult to

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measure under some important conditions, and steady state often cannot be assured. Further, since pyruvate may be metabolized either by pyruvate dehydrogenase or through a pyruvate carboxylation pathway, the appearance of $^{14}CO_2$ from ^{14}C-enriched pyruvate (or its precursors) indicates net substrate oxidation only if the carbon skeleton enters the citric acid cycle via pyruvate dehydrogenase (Peuhkurinen, 1980). Similarly, $^{14}CO_2$ release from fatty acids is an unreliable measure of this oxidation (Veerkamp et al., 1986). Thus, standard methods for assessing substrate competition may not be satisfactory for rapidly changing or spatially heterogeneous metabolic states or if more than one pathway is available for carbon flow into the citric acid cycle.

The analogous use of ^{13}C-enriched substrates to monitor intermediary metabolism is valuable since multiple enriched intermediates of the citric acid cycle may be detected by NMR spectroscopy (Cohen, 1983; London, 1988). Citric acid cycle flux may be determined if the fractional enrichments in intermediates are measured repeatedly after the addition of enriched substrate (Chance et al., 1983). The multiplets in a ^{13}C NMR spectrum may be analyzed to determine the relative rates of competing metabolic pathways or the label scrambling which leads to [1-^{13}C]acetyl-CoA from a precursor that primarily produces [2-^{13}C]acetyl-CoA (Walker et al., 1982; Walker & London, 1987). Other techniques that allow measurement of the source of acetyl-CoA from a single spectrum are limited to steady-state conditions (Malloy et al., 1988; Sherry et al., 1988). A method is now presented for measuring the contribution of labeled substrates to acetyl-CoA in the heart from a single NMR spectrum without oxygen consumption measurements or the assumption of metabolic or isotopic steady state. Although it is illustrated for the heart, the method is applicable to any tissue.

EXPERIMENTAL PROCEDURES

Heart Perfusions. Male Sprague-Dawley rats weighing 300–350 g were anesthetized in an ether atmosphere. Male New Zealand White rabbits weighing 2.5–3.0 kg were anesthetized with intramuscular acepromazine (0.7 mg/kg) and nembutal (25 μg/kg), with the assistance of technicians from the Animal Resources Center. Hearts were rapidly excised and placed briefly in 4 °C arrest medium. The aorta was immediately cannulated and perfused retrograde at a pressure of 70 cm of H_2O. The perfusate, a modified, phosphate-free Krebs–Henseleit medium, was gassed with 95% O_2–5% CO_2 and contained the following (all concentrations millimolar): NaCl, 119.2; KCl, 4.7; $CaCl_2$, 3.0; $MgSO_4$, 1.2; $NaHCO_3$, 25; EDTA, 0.5 (free calcium, 2.5). The perfusate contained 10 mM glucose during the initial nonrecirculating perfusion.

Hearts and perfusion media were maintained at 37 °C. Hearts were initially perfused for about 10 min without recirculation of the perfusate until a protocol was initiated. The recirculating volume of perfusate was 210 mL for the rat experiments and 1000 mL for the rabbit experiments. Preliminary studies showed no significant change in the relative concentrations of substrates after 30 min of perfusion. The following ^{13}C-enriched substrates were used: [3-^{13}C]lactate (99%, Isotec), [2-^{13}C]acetate (99%, MSD Isotopes), and [1,2-^{13}C]acetate (99%, MSD Isotopes).

Experimental Protocols. Five groups were studied; rat hearts were used in groups 1–3, and rabbit hearts were used in groups 4 and 5. Hearts in group 1 were switched to a recirculating system with the following substrates (all concentrations millimolar): acetate, 0.25; lactate, 1.0; glucose, 10. After 20 min of recirculation, the perfusate was changed to one containing [1,2-^{13}C]acetate, [3-^{13}C]lactate, and glucose at the same concentrations. At the end of 30 min the heart was freeze clamped. Hearts in group 2 were treated identically with those in group 1 except that the perfusion period with labeled substrates was 5 min, followed by freeze clamping. Hearts in group 3 were studied directly in the NMR spectrometer; the substrates were 3 mM [2-^{13}C]acetate and 10 mM glucose.

Rabbit hearts (groups 4 and 5) were used as a model of regional ischemia. The initial perfusate contained 10 mM glucose, 1.0 mM lactate, and 0.5 mM acetate, and the hearts were allowed to stabilize for 15 min. Hearts in group 4 were switched to perfusate containing [1,2-^{13}C]acetate, [3-^{13}C]lactate, and glucose at the same concentrations; perfusion was continued for 30 min followed by freeze clamping. After stabilization, regional ischemia was produced in group 5 by occluding the left anterior descending coronary artery with an encircling suture for 30 min. Just prior to reperfusion methylene blue was added to the perfusate to stain the normally perfused myocardium. After reperfusion for 5 min, the perfusate was changed to one in which the unlabeled lactate and acetate were replaced by [1,2-^{13}C]acetate and [3-^{13}C]lactate in the same concentration; perfusion was continued for 7 min. Ischemic and normally perfused myocardium (identified visually by methylene blue stain) were freeze clamped. Typically, perfusion was preserved in the posterior septum, posterior wall, and variable portions of the lateral wall.

Hearts that were freeze clamped were extracted in perchloric acid, neutralized with KOH, freeze-dried, and dissolved in D_2O for NMR study (Sherry et al., 1990).

NMR Methods. Proton-decoupled ^{13}C NMR spectra were obtained at 125.7 MHz with a GN 500 spectrometer. Tissue extract samples were studied in a dual ^1H–^{13}C 5-mm probe under high-resolution conditions with WALTZ decoupling, a 45° observe pulse, 16384 data points, 8000 scans, and a pulse delay of 6 s. Intact hearts were studied in an 18-mm tube with WALTZ decoupling, a 30° observe pulse, 16384 data points, and a delay between pulses of 1.3 s. In the intact heart experiments, field homogeneity was adjusted by use of the free induction decay of the ^{23}Na signal; typical ^{23}Na line widths were 17–22 Hz.

Analysis of ^{13}C NMR Spectra. A steady-state analysis was applied to the hearts supplied with labeled substrates for at least 30 min (Malloy et al., 1988, 1990). This analysis requires measurement of the relative areas of the singlet, doublet, triplet, and/or quartet components of the glutamate C2, C3, and C4 resonances (C1, C2, C3, C4, and C5 refer to carbons 1, 2, 3, 4, and 5, respectively). The non-steady-state analysis requires measurement of the total ^{13}C enrichment in glutamate C4 versus glutamate C3 (see below). The only oxaloacetate carbon position that is relevant to this analysis is the carbonyl carbon, C2, since this carbon becomes C3 of glutamate after a half turn of the citric acid cycle. C4 and C3 of oxaloacetate become respectively C1 and C2 of glutamate and are not involved in the analysis. C1 of oxaloacetate is lost as CO_2 at the isocitrate dehydrogenase step. Because relative enrichments in the acetyl-CoA pool are being examined, the absolute enrichment in the C2 of oxaloacetate need not be known. The analysis assumes rapid exchange between α-ketoglutarate and glutamate (Velick & Vavra, 1962).

The simplicity of the technique is demonstrated in Figure 1. The chance that an oxaloacetate molecule labeled in the 2-carbon will condense with a given acetyl-CoA isotopomer equals the relative concentration of that isotopomer in the acetyl-CoA pool. Three different acetyl-CoA isotopomers may

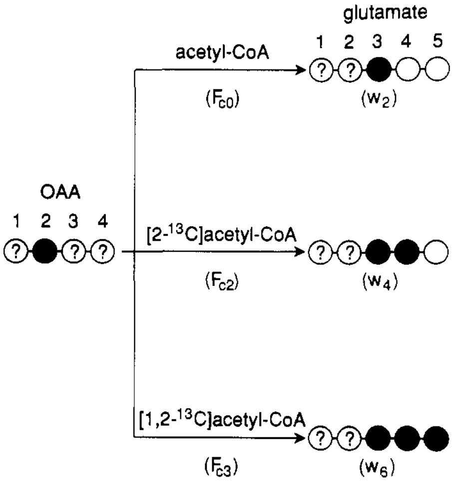

FIGURE 1: Summary of non-steady-state analysis. Oxaloacetate may condense with one of three acetyl-CoA isotopomers to produce various glutamate isotopomers. Symbols: open circles, ^{12}C; filled circles, ^{13}C; circled ?, labeling not relevant. F_{c0}, F_{c2}, and F_{c3} indicate the relative concentration of that acetyl-CoA isotopomer; w_2, w_4, and w_6 indicate the relative concentrations of groups of glutamate isotopomers as defined in the text.

occur under our perfusion conditions: unlabeled acetyl-CoA, [2-^{13}C]acetyl-CoA, and [1,2-^{13}C]acetyl-CoA, and their relative concentrations are defined as F_{c0}, F_{c2}, and F_{c3}, respectively. Although glutamate has 32 possible isotopomers, only 24 may occur under these conditions (since there is no pathway for generation of [1-^{13}C]acetyl-CoA). Variables that indicate the relative concentrations of glutamate isotopomers are

$$x_1 = [[1,2,3,4,5\text{-}^{12}\mathrm{C}]\mathrm{glutamate}]/[\mathrm{glutamate}]$$

$$x_2 = [[1\text{-}^{13}\mathrm{C}]\mathrm{glutamate}]/[\mathrm{glutamate}]$$

$$x_3 = [[2\text{-}^{13}\mathrm{C}]\mathrm{glutamate}]/[\mathrm{glutamate}]$$

$$x_4 = [[1,2\text{-}^{13}\mathrm{C}]\mathrm{glutamate}]/[\mathrm{glutamate}]$$

$$x_5 = [[3\text{-}^{13}\mathrm{C}]\mathrm{glutamate}]/[\mathrm{glutamate}]$$

$$x_6 = [[1,3\text{-}^{13}\mathrm{C}]\mathrm{glutamate}]/[\mathrm{glutamate}]$$

$$x_7 = [[2,3\text{-}^{13}\mathrm{C}]\mathrm{glutamate}]/[\mathrm{glutamate}]$$

$$x_8 = [[1,2,3\text{-}^{13}\mathrm{C}]\mathrm{glutamate}]/[\mathrm{glutamate}]$$

Similarly, x_9–x_{16} are identical with the first group, except that carbon 4 (but not carbon 5) is enriched. Enrichment in carbon 5 but not carbon 4 is represented by x_{17}–x_{24}, which in this study are zero. Finally, x_{25}–x_{32} refer to the same pattern of labeling in carbons 1, 2, and 3, plus labeling in both carbons 4 and 5. By definition, $F_{c0} + F_{c2} + F_{c3} = 1$, and $x_1 + x_2 + ... + x_{32} = 1$. Six groups of glutamate isotopomers may be defined as

$$w_1 = x_1 + x_2 + x_3 + x_4 \text{ (not labeled in C3, C4, or C5)}$$

$$w_2 = x_5 + x_6 + x_7 + x_8 \text{ (labeled in C3 but not in C4 or C5)}$$

$$w_3 = x_9 + x_{10} + x_{11} + x_{12} \text{ (not labeled in C3 or C5, labeled in C4)}$$

$$w_4 = x_{13} + x_{14} + x_{15} + x_{16} \text{ (labeled in C3 and C4, not labeled in C5)}$$

$$w_5 = x_{25} + x_{26} + x_{27} + x_{28} \text{ (not labeled in C3, labeled in C4 and C5)}$$

$$w_6 = x_{29} + x_{30} + x_{31} + x_{32} \text{ (labeled in C3, C4, and C5)}$$

Table I: Influence of Perfusion Time on Components of the Glutamate Multiplets in ^{13}C NMR Spectra from Hearts Utilizing Glucose, [3-^{13}C]Lactate, and [1,2-^{13}C]Acetate[a]

NMR measurements	perfusion time 5 min	 30 min
carbon 3		
C3S	0.35 ± 0.12	0.14 ± 0.04
C3D	0.57 ± 0.06	0.34 ± 0.06
C3T	0.08 ± 0.06	0.52 ± 0.10
carbon 4		
C4S	0.31 ± 0.04	0.16 ± 0.02
C4D34	0.07 ± 0.02	0.28 ± 0.02
C4D45	0.52 ± 0.07	0.21 ± 0.03
C4Q	0.10 ± 0.02	0.35 ± 0.03
C4/C3	4.48 ± 0.60	1.18 ± 0.09

[a] Data are the means ± standard deviations. C3S, C3D, and C3T refer to the area of the singlet, doublet, and triplet, respectively, in the 3-carbon resonance relative to the total area of that resonance. The variables describing the 4-carbon resonance are defined in the text.

Table II: Steady-State and Non-Steady-State Analysis of Carbon Flow into the Citric Acid Cycle after Brief (5 min) or Prolonged (30 min) Perfusion with Labeled Substrates[a]

perfusion time (min)	source of acetyl-CoA unlabeled (F_{c0})	 lactate (F_{c2})	 acetate (F_{c3})
5 (n = 3)	0.28 ± 0.09	0.30 ± 0.07	0.42 ± 0.02
30 (n = 4, non-steady-state analysis)	0.24 ± 0.11	0.34 ± 0.05	0.42 ± 0.06
30 (steady-state analysis)	0.26 ± 0.09	0.32 ± 0.04	0.42 ± 0.05

[a] Data are means ± standard deviations.

The area of the 4-carbon resonance relative to the 3-carbon resonance is defined as $\mathrm{C4/C3} = (w_3 + w_4 + w_5 + w_6)/(w_2 + w_4 + w_6)$. The area of the components of the 4-carbon multiplet are defined relative to the total area of the resonance (Malloy et al., 1988): singlet, C4S; doublet due to J_{34}, C4D34 $= w_4/(w_3 + w_4 + w_5 + w_6)$; doublet due to J_{45}, C4D45; doublet of doublets (quartet), C4Q $= w_6/(w_3 + w_4 + w_5 + w_6)$.

As illustrated in Figure 1, F_{c0}, F_{c2}, and F_{c3} are quantitatively related to w_2, w_4, and w_6, respectively. The chance that an oxaloacetate molecule labeled in C2 will condense with unlabeled acetyl-CoA equals F_{c0}. Therefore, $F_{c0}/(F_{c0} + F_{c2} + F_{c3}) = w_2/(w_2 + w_4 + w_6)$. Similar relations may be derived for F_{c2} and F_{c3}. By combining these relationships, one obtains quite simply

$$(\mathrm{C4D34})(\mathrm{C4/C3}) = w_4/(w_2 + w_4 + w_6) = F_{c2}/(F_{c0} + F_{c2} + F_{c3}) = F_{c2} \quad (1)$$

$$(\mathrm{C4Q})(\mathrm{C4/C3}) = w_6/(w_2 + w_4 + w_6) = F_{c3}/(F_{c0} + F_{c2} + F_{c3}) = F_{c3} \quad (2)$$

Therefore, by measuring two C4 multiplet components and the C4/C3 ratio, one can obtain values for F_{c2} and F_{c3} and by difference F_{c0} under any non-steady-state circumstances. This analysis was applied to all hearts in this study.

RESULTS AND DISCUSSION

Representative proton-decoupled ^{13}C NMR spectra of extracts from hearts supplied for 5 or 30 min with [1,2-^{13}C]acetate, [3-^{13}C]lactate, and glucose are shown in Figure 2, and the multiplet measurements are summarized in Table I. Since the metabolic state of the hearts and the concentration of substrates did not change when ^{13}C was introduced, the differences reflect only different stages in the distribution of isotope in the citric acid cycle. Solution of eqs 1 and 2 (applied to both groups of hearts) and the steady-state analysis (applied to hearts perfused for 30 min) were used to measure the

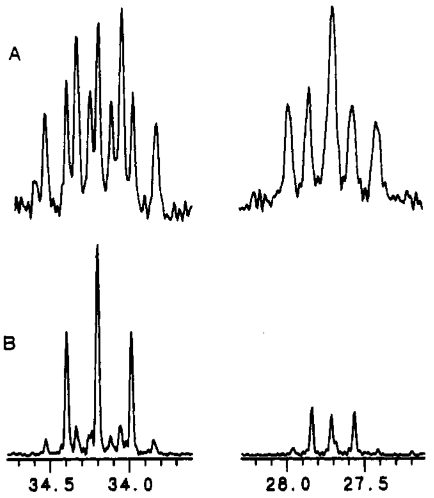

FIGURE 2: Proton-decoupled ^{13}C NMR spectra of the protonated carbons of glutamate from rat hearts perfused for different periods with ^{13}C-enriched substrates. The left column is the C4 resonance, and the right column is the C3 resonance. The spectrum in the upper panel (A) was from a heart supplied for 30 min with [3-^{13}C]lactate, [1,2-^{13}C]acetate, and unlabeled glucose. The spectrum in the lower panel (B) was from a heart supplied for 5 min with the same substrates.

contribution of acetate, lactate, and unlabeled sources to acetyl-CoA. The results, presented in Table II, show that the non-steady-state and steady-state analyses yield the same results for hearts metabolizing labeled substrate for 30 min. More importantly, in spite of the dramatic differences in the spectra, the measured contributions of exogenous substrates to acetyl-CoA after 5 min of perfusion were also the same. This measurement of substrate selection can be extended easily to conditions where other methods are difficult to apply, e.g., for serial measurements in the intact heart during rapid changes in metabolic state or in regionally ischemic, reperfused myocardium.

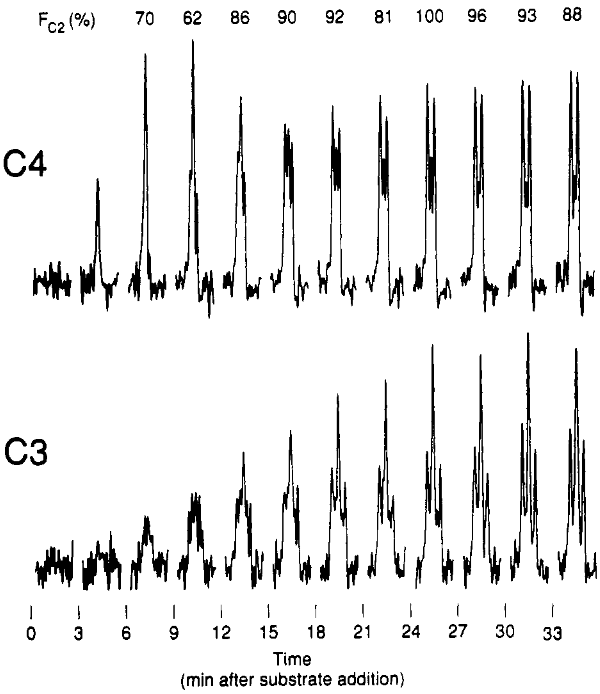

FIGURE 3: Evolution of the ^{13}C NMR spectrum in normal rat myocardium after addition of [2-^{13}C]acetate. The 4-carbon resonance (34.2 ppm, upper panel) and the 3-carbon resonance (27.8 ppm, lower panel) are shown. Each spectrum was acquired during the time shown on the abscissa after addition of 3.0 mM [2-^{13}C]acetate. The contribution of exogenous acetate to acetyl-CoA was calculated from each spectrum (F_{c2}).

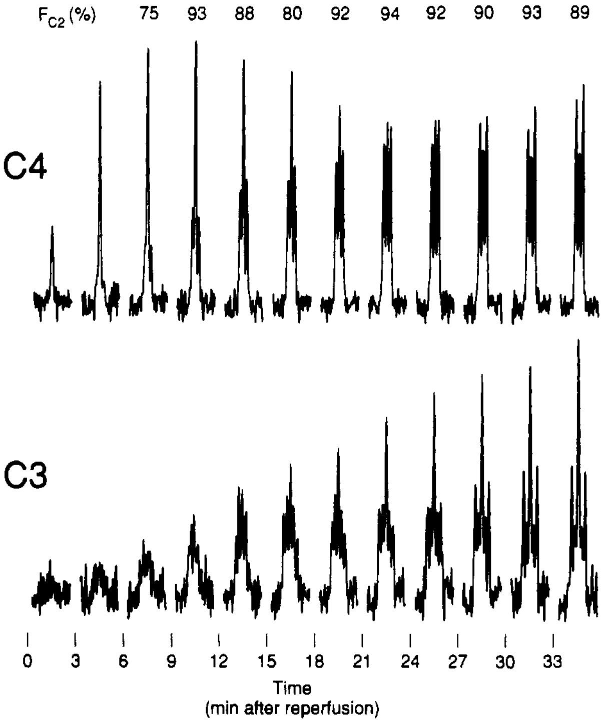

FIGURE 4: Evolution of the ^{13}C NMR spectrum in postischemic rat myocardium after reperfusion and addition of [2-^{13}C]acetate. The 4-carbon resonance (34.2 ppm, upper panel) and the 3-carbon resonance (27.8 ppm, lower panel) are shown. The contribution of exogenous acetate to acetyl-CoA was calculated from each spectrum (F_{c2}).

Previous observations have suggested that the rate of acetate clearance in the heart is sensitive to the oxygen consumption rate and that fatty acid utilization is slowed in reperfused myocardium (Brown et al., 1987; Schwaiger et al., 1985). We measured the contribution of exogenous acetate to acetyl-CoA in normal and postischemic myocardium in hearts supplied with [2-^{13}C]acetate (Figures 3 and 4). After stable heart function was established in the magnet, the perfusate was switched to one containing 3 mM [2-^{13}C]acetate and 10 mM glucose. Proton-decoupled ^{13}C NMR spectra were acquired every 3 min. Normoxic hearts (Figure 3) showed rapid incorporation of ^{13}C into glutamate, and steady state was reached after about 20–25 min. Since ^{13}C–^{13}C homonuclear coupling was resolved easily, the contribution of acetate to acetyl-CoA (F_{c2}) could be measured after enrichment in the 3-carbon was detected. (Only eq 1 applies since [1,2-^{13}C]acetyl-CoA cannot be generated under these conditions and $F_{c3} = 0$.) The same procedure was repeated in hearts that were ischemic (no coronary flow) for 11 min, followed by reperfusion. As shown in Figure 4, steady state was not attained until after about 35 min (complete data not shown). The steady-state isotopomer analysis of the final ^{13}C NMR spectra showed that most of the acetyl-CoA was derived from exogenous acetate in both the normally perfused myocardium (0.95 ± 0.03) and the reperfused myocardium (0.96 ± 0.03). Thus, the fraction of respiratory fuel contributed by acetate throughout reperfusion was not altered by ischemia. These experiments also demonstrated that detection of ^{13}C–^{13}C coupling in spectra of intact hearts with excellent signal-to-noise and temporal resolution is feasible and that the non-steady-state analysis is suitable for quickly monitoring substrate selection in intact tissues as they respond to interventions.

Since this method does not require isotopic or metabolic steady state, it is also valuable for the study of regional var-

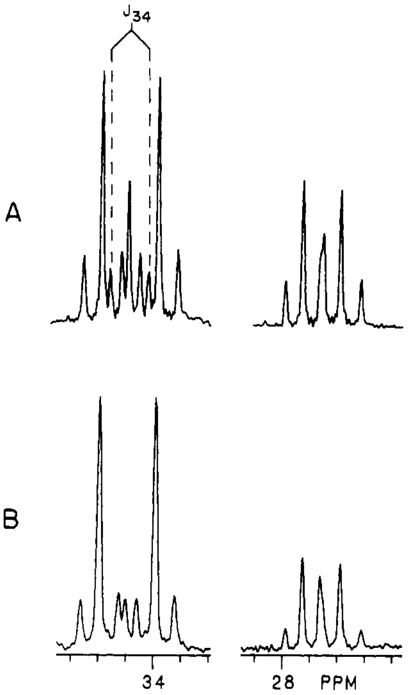

FIGURE 5: Proton-decoupled ^{13}C NMR spectra of tissue extracts from different regions of a single rabbit heart showing the glutamate C4 (left column) and C3 (right column) resonances. The substrates in the perfusate were [1,2-^{13}C]acetate, [3-^{13}C]lactate, and glucose. Spectra were acquired from normally perfused (A) and ischemic, reperfused (B) myocardium. The decrease in the contribution of the doublet due to J_{34} indicates, from eq 1, a decrease in the contribution of lactate to acetyl-CoA.

iations in substrate utilization during recovery from ischemia. Rabbit hearts were supplied with acetate, lactate, and glucose as described above. In the normoxic myocardium (group 4, $n = 3$) acetyl-CoA was derived from acetate (0.59 ± 0.05), lactate (0.32 ± 0.06), and unlabeled sources (0.09 ± 0.02), either exogenous glucose or endogenous stores. Substrate utilization was highly variable in myocardium reperfused for 12 min (group 5). In the example shown in Figure 5 acetyl-CoA in the normal myocardium was derived from acetate (48%), lactate (16%), and unlabeled sources (32%). In the same heart acetyl-CoA in reperfused myocardium was derived from acetate (73%), lactate (9%), and unlabeled sources (18%).

^{13}C NMR may be used to monitor the metabolism of labeled compounds in experimental animals and humans (Laughlin et al., 1988; Shulman et al., 1990). However, the study of substrate utilization in vivo is likely to be limited by two factors. First, the expense of highly enriched substrates will make it difficult to maintain a constant concentration in the blood for the time required to attain steady state. The method presented here allows quantitation of substrate selection even after brief exposure to ^{13}C-enriched compounds. Second, the isotopomer analysis, as demonstrated previously, has only been applied when B_0 homogeneity was sufficient to allow resolution of ^{13}C–^{13}C scalar coupling. This condition may be difficult to establish in vivo.

Since the evolution of a spin system under the influence of scalar coupling is not altered by B_0 inhomogeneity, a *J*-modulated spin echo may be used to select the phase of each multiplet in a resonance (LeCocq & Lallemand, 1981; Patt & Shoolery, 1982). Therefore, even when lines are broad due to B_0 inhomogeneity, the contribution of each multiplet may be measured. The pulse sequence 90°–τ–180°–τ–acquire (where $\tau = 1/2J$) was used to create a 180° phase difference between the doublet ($J_{34} = 34$ Hz) and the other lines in the C3 and C4 resonance under conditions producing well-resolved or poorly resolved resonances. This is demonstrated in Figure 6. As shown, two fully relaxed spectra may be accumulated (preferably over the same time period in separate regions of computer memory), by use of a single pulse and a second using the spin-echo sequence. The C4/C3 ratio is obtained from the first spectrum (6A), and C4D34 is obtained from the difference between the two spectra. [C4D34 = $(A - B)/2A$ = $0.5 - B/2A$, where A is the area of the C4 resonance after a standard scan and B is the area with a spin echo.]

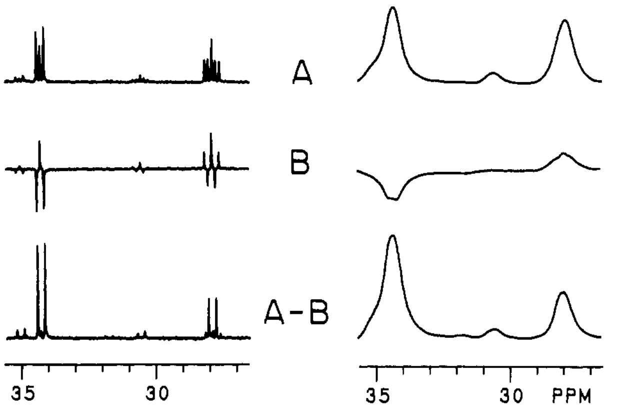

FIGURE 6: Proton-decoupled ^{13}C NMR spectra acquired with the *J*-modulated spin echo described in the text. The difference between spectra A (one-pulse experiment) and B (spin echo) is shown in the bottom panel (A–B). Good-resolution (left column) and poor-resolution (right column) spectra are shown for both sequences.

This analysis may be extended to more complex substrate combinations and metabolic conditions. [1-^{13}C]Acetyl-CoA may be generated from a labeled compound provided to the tissue such as [2-^{13}C]lactate or by ^{13}C flowing from oxaloacetate to phosphoenolpyruvate, pyruvate, and ultimately acetyl-CoA. Under either circumstance the resulting glutamate may become enriched in C5 but not in C4. This analysis (eqs 1 and 2) would remain valid, but the fraction of acetyl-CoA that is unlabeled (F_{c0}) could not be distinguished from the fraction of acetyl-CoA enriched in C1 (defined as F_{c1} in earlier studies; Malloy et al., 1990). However, the ratio F_{c1}/F_{c3} may be determined directly from the multiplets in the ^{13}C NMR resonance of the C5 of glutamate: F_{c1}/F_{c3} = C5S/C5D, where C5S and C5D refer to the areas of the singlet and doublet. In this case, appropriate experimental conditions or corrections must be used to quantify the ^{13}C enrichment in glutamate C5 because of the effects of ^{13}C–^{13}C dipolar interaction on T_1 (Moreland & Carroll, 1974; Walker et al., 1982). Hence, the relative concentrations of all four acetyl-CoA isotopomers may be determined in a single experiment.

In summary, this convenient measurement of the contribution a substrate makes to acetyl-CoA does not require determination of citric acid cycle flux, and it is free of the usual requirement of either assuming steady-state conditions or obtaining multiple measurements over time. It does require that the fractional enrichment in ^{13}C of the available substrate be known (Malloy et al., 1990), but it is not influenced by anaplerotic pathways of ^{12}C or ^{13}C entry into the citric acid cycle. Its simplicity lends itself to in vivo studies where substrate oxidation is used as an index of tissue viability.

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Registry No. Acetyl-CoA, 72-89-9; oxaloacetic acid, 328-42-7; glutamic acid, 56-86-0; lactic acid, 50-21-5; acetic acid, 64-19-7; glucose, 50-99-7.

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