Analysis of tricarboxylic acid cycle of the heart using ^{13}C isotope isomers

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MALLOY, CRAIG R., A. DEAN SHERRY, AND F. MARK H. JEFFREY. *Analysis of tricarboxylic acid cycle of the heart using ^{13}C isotope isomers.* Am. J. Physiol. 259 (Heart Circ. Physiol. 28): H987–H995, 1990.—^{13}C-nuclear magnetic resonance (NMR) spectroscopy provides a new approach to the analysis of metabolic pathways, because it detects an interaction between adjacent ^{13}C nuclei. Previous models of isotope distribution in the tricarboxylic acid cycle were designed for analysis of radioisotope data and did not consider the information provided by ^{13}C-^{13}C coupling. A mathematical model of the tricarboxylic acid cycle was developed that preserves all isotope isomer (isotopomer) information and yields simple relationships between ^{13}C-NMR spectra of glutamate and metabolic parameters under steady-state conditions. With the use of relative peak areas measured from the spectra of tissues supplied with ^{13}C-enriched substrate(s), the relative fluxes through both the oxidative (acetyl-CoA utilization) and nonoxidative (anaplerotic) pathways of the tricarboxylic acid cycle can be determined. Furthermore, with the judicious selection of ^{13}C-labeling patterns in a mixture of substrates, direct substrate competition experiments can be performed. The perchloric acid extracts of Langendorff and working rat hearts oxidizing ^{13}C-enriched fatty acids or carbohydrates are analyzed to illustrate this approach, and the importance of measuring the fractional enrichment of the available substrate is demonstrated. The technique can of course be used with all tissues, not just heart, and is applicable to the analysis of in vivo ^{13}C-NMR spectra.

myocardial metabolism; carbon isotope; nuclear magnetic resonance spectroscopy; tracer studies

THE ACTIVITY of a metabolic pathway feeding the tricarboxylic acid cycle may be determined by providing a substrate enriched in a carbon isotope and analyzing the distribution of the isotope in a tricarboxylic acid cycle intermediate. Since the first report by Strisower et al. (28) more than 35 years ago, numerous models have been developed that relate these measurements to relevant fluxes (5, 9–13, 23, 30–32). The use of ^{13}C-nuclear magnetic resonance (NMR) to measure enrichment in each site offers a substantial simplification compared with radionuclide tracer studies: isolation of a compound and carbon-by-carbon degradation is unnecessary (1, 7, 15, 20). ^{13}C-NMR also distinguishes among patterns of enrichment that are not detected by traditional radionuclide methods. This information, a consequence of a magnetic spin-spin interaction between adjacent ^{13}C nuclei, offers a powerful new approach to the study of intermediary metabolism.

Glutamate is easily detected in the ^{13}C-NMR spectrum of a heart oxidizing a ^{13}C-enriched substrate. To derive physiologically meaningful information from a single spectrum of glutamate, it was necessary to develop a mathematical model that incorporates the effects of ^{13}C-^{13}C coupling (18). This report emphasizes the practical aspects of this approach. The objectives are *1*) to review the relation between ^{13}C enrichment in glutamate and the ^{13}C-NMR spectrum, *2*) to present a model of the tricarboxylic acid cycle and the equations relevant to most experimental conditions, *3*) to demonstrate this analysis and the importance of measuring fractional enrichment of the labeled substrate, *4*) to estimate the influence of error on the calculated physiological variables, and *5*) to present the input-output matrix necessary to derive the simple expressions resulting from the model.

Glossary

C*i*F	Fraction of carbon in position *i* that is ^{13}C
C1S	Area of singlet/C-1 resonance area
C1D	Area of doublet/C-1 resonance area, where C1S + C1D = 1
C2S	Area of singlet/C-2 resonance area
C2D12	Area of doublet (C-1, C-2)/C-2 resonance area
C2D23	*Area of doublet (C-2, C-3)/C-2 resonance area*
C2Q	Area of quartet/C-2 resonance area, where C2S + C2D12 + C2D23 + C2Q = 1
C3S	Area of singlet/C-3 resonance area
C3D	Area of doublet/C-3 resonance area
C3T	Area of triplet/C-3 resonance area, where C3S + C3D + C3T = 1
C4S	Area of singlet/C-4 resonance area
C4D34	Area of doublet (C-3, C-4)/C-4 resonance area
C4D45	Area of doublet (C-4, C-5)/C-4 resonance area

C4Q	Area of quartet/C-4 resonance area, where C4S + C4D34 + C4D45 + C4Q = 1
C5S	Area of singlet/C-5 resonance area
C5D	Area of doublet/C-5 resonance area, where C5S + C5D = 1
F_{ai}	Fractional enrichment of a substrate for an anaplerotic reaction
F_{a0}	Fraction of anaplerotic substrate that is not enriched
F_{a1}	Fraction of anaplerotic substrate that yields oxaloacetate enriched in C-2 or C-3, where $F_{a0} + F_{a1} = 1$
F_{ci}	Fractional enrichment of a substrate for citrate synthase, acetyl-CoA, which provides one pathway for carbon entry into tricarboxylic acid cycle
F_{c0}	[[1,2-^{12}C]acetyl-CoA]/[acetyl-CoA]
F_{c1}	[[1-^{13}C]acetyl-CoA]/[acetyl-CoA]
F_{c2}	[[2-^{13}C]acetyl-CoA]/[acetyl-CoA]
F_{c3}	[[1,2-^{13}C]acetyl-CoA]/[acetyl-CoA], where $F_{c0} + F_{c1} + F_{c2} + F_{c3} = 1$
y	Ratio of flux through combined anaplerotic reactions (a) relative to citrate synthase (c) = a/c

DETECTION OF ^{13}C ENRICHMENT IN GLUTAMATE BY NMR SPECTROSCOPY

Each carbon in glutamate shows a separate resonance in the ^{13}C-NMR spectrum that is well resolved from the carbon resonances of other metabolites. In a tissue oxidizing a ^{13}C-enriched substrate, each glutamate resonance usually appears as a group of overlapping multiplets (singlets, doublets, triplets, etc.). Thus two types of information may be obtained: relative signal intensity from different carbon sites in a molecule and the relative intensity of the various multiplets within a resonance. Because both types of information are useful, the origin of the multiplets is introduced, followed by a brief description of the effects of experimental conditions in ^{13}C-enrichment measurements.

The interaction between adjacent ^{13}C nuclei, known as ^{13}C-^{13}C coupling, is observed as splitting of a single resonance into two or more resonances, depending on enrichment of adjacent carbon nuclei. This splitting, called a coupling constant (J), is ~34 Hz between protonated carbons and ~52 Hz between protonated and carboxyl carbons of glutamate (2). If ^{13}C is detected by NMR in the C-2 of glutamate at 55.2 parts per million (ppm), the presence of ^{13}C in either the C-1 or C-3 in the same molecule is evident by splitting of the C-2 resonance into a doublet. Because the coupling constants between C-1–C-2, and C-2–C-3 differ, the relative concentrations of four pools of glutamate molecules labeled in C-2 can be measured: C-1 and C-3 unlabeled, label in C-1 but not C-3, label in C-3 but not C-1, and label in both C-1 and C-3. Consequently, the C-2 resonance often appears as nine partially overlapping resonances. A maximum of five resonances are observed in C-3, because coupling between C-2–C-3 and C-3–C-4 cannot be distinguished. A ^{13}C-NMR spectrum of a rat heart extract is shown in Fig. 1, and the contribution of each pattern of ^{13}C labeling to the spectrum is shown in Fig. 2.

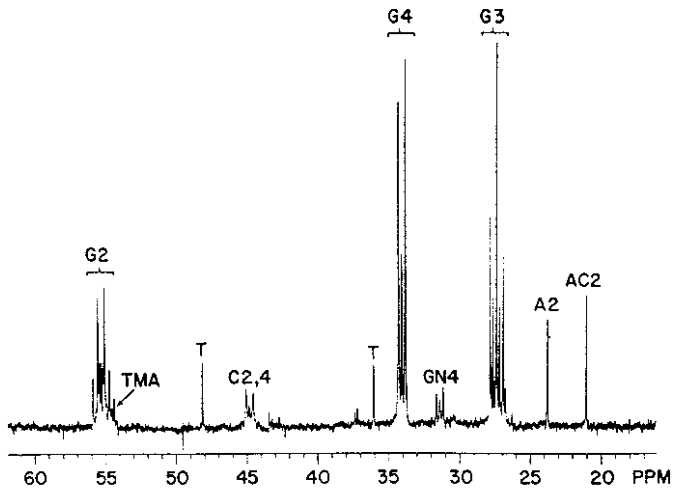

FIG. 1. Proton decoupled ^{13}C-nuclear magnetic resonance (NMR) spectrum of extract of a rat heart supplied with 2.5 mM [2-^{13}C]acetate. Resonance assignments: G, glutamate; TMA, trimethylamino resonance of carnitine (natural abundance); T, taurine (natural abundance); C, citrate; GN, glutamine; A, acetate; AC, acetylcarnitine. Multiplets of this spectrum are analyzed in Fig. 2. Carboxyl carbons are not shown.

The relative intensities of the carbon resonances in glutamate may not directly reflect the relative enrichment of ^{13}C at each site for two reasons (16). First, the glutamate protons must be irradiated to remove ^{13}C-^{1}H spin-spin coupling from the carbon spectrum, and one consequence of proton irradiation is an enhancement in intensity of the ^{13}C resonances. This phenomenon, called the nuclear Overhauser enhancement (NOE), is different for some of the glutamate ^{13}C resonances. For example, the NOE of carboxyl carbons is much smaller than for protonated carbons, and consequently their intensities cannot be directly compared without appropriate correction factors. Second, the spin-lattice relaxation times (T_1) of carboxyl carbons are generally much longer than those for protonated carbons, and there are small differences in T_1 among protonated carbons. As a result, rapid pulsing (which improves signal-to-noise ratios) may further alter relative signal intensities. Nevertheless, accurate relative enrichments may be measured if appropriate correction factors are applied.

The relative intensities of the multiplets within a single protonated carbon resonance are not influenced by these experimental factors, and hence the data are more convenient to determine and interpret, providing of course that resolution is adequate. The T_1s of carboxyl carbons, however, are sensitive to ^{13}C-^{13}C interactions, and their multiplet intensities may be distorted by T_1 and NOE effects (19). For these reasons, multiplet analyses have not been applied to the carboxyl carbons (C-1 and C-5) of glutamate (17, 18, 25).

ANALYSIS OF ISOTOPE DISTRIBUTION: A MODEL OF THE TRICARBOXYLIC ACID CYCLE

The maximum number of labeling patterns in a molecule with n carbons is 2^n. Glutamate potentially has 32 isotopomers. The relative concentration of these isotopomers produced by a heart utilizing ^{13}C-enriched sub-

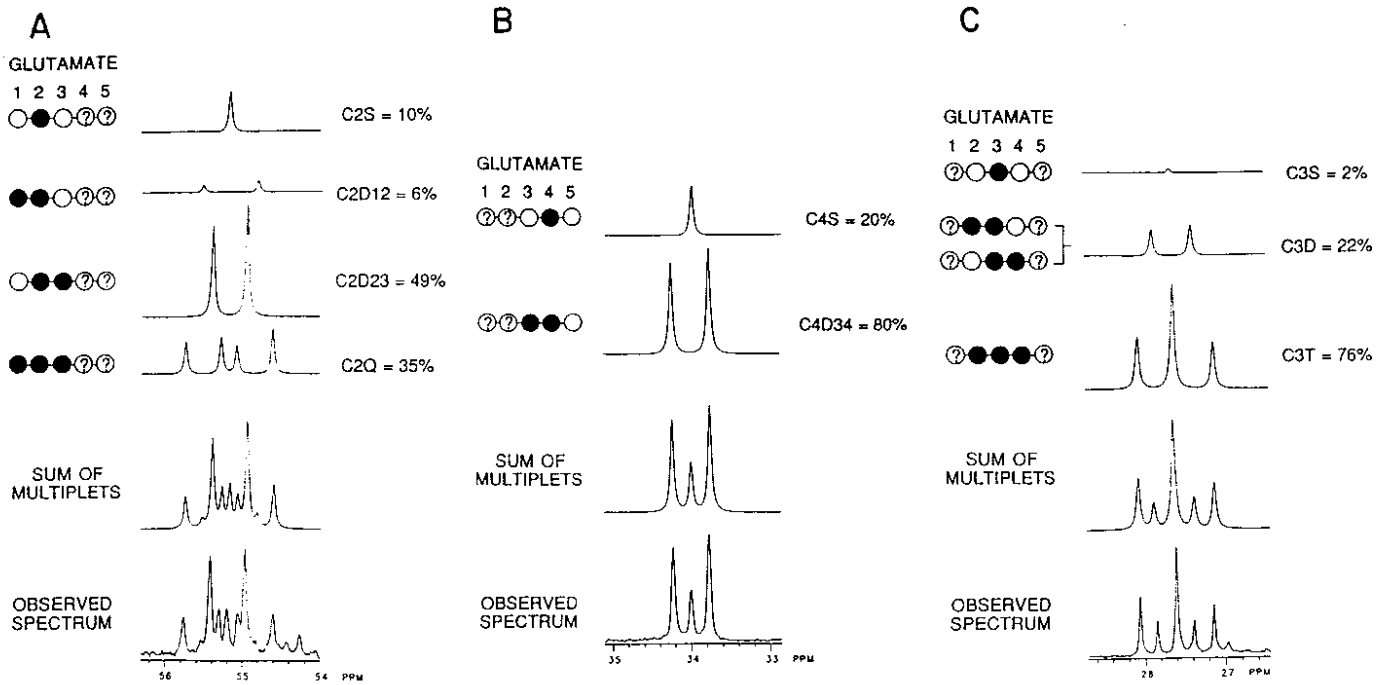

FIG. 2. Analysis of multiplets in protonated carbons of glutamate. C-2 resonance is composed of 9 lines resulting from 4 isotopomer pools (*A*). C-4 resonance is typically composed of 3 lines when acetyl-CoA is enriched in methyl carbon (*B*), and more complex spectra are observed if the carbonyl carbon is enriched as well. C-3 resonance is usually composed of 5 lines (*C*), because C-2, C-3 $\approx$ C-3, C-4, and doublet in C-3 due to coupling with C-2 cannot be distinguished from coupling with C-4. See *Glossary* for definitions of abbreviations.

strate depends on two factors, the site(s) of enrichment of the substrate and the activity of each pathway feeding the tricarboxylic acid cycle. Because the substrate enrichment site may be selected experimentally, the relative concentration of isotopomers can be analyzed to measure relative flow of ^{12}C and ^{13}C into the cycle. Although standard ^{13}C-NMR methods cannot directly detect each isotopomer, the relative concentrations of groups of isotopomers can be measured.

The objective of the model is to relate useful biochemical information to the relative concentration of these groups of isotopomers. Because the pathways for carbon flow in the tricarboxylic acid cycle are well established, the evolution of the ^{13}C-NMR spectrum is predictable. However, deriving quantitative information from time-dependent spectra is difficult, since the sizes of the various intermediate pools are unknown. Furthermore, because ^{13}C is a relatively insensitive NMR nucleus, it is difficult to obtain good spectra with adequate time resolution. For these reasons, the model was developed for steady-state conditions only. The mathematical details are summarized in Ref. 18 (the general case) and in the APPENDIX where the equations apply to simpler experimental conditions; the physiological variables are described below.

Most carbon enters the tricarboxylic acid cycle of the heart via acetyl-CoA with subsequent net oxidation to CO_2 and water. The entry of carbon via other pathways to form a tricarboxylic acid cycle intermediate (e.g., metabolism of propionate to succinyl-CoA or pyruvate to oxaloacetate) is termed anaplerosis (14, 21). Hassinen, Peuhkurinen, and colleagues (21, 29) found that flux through the anaplerotic reactions and simultaneous disposal reactions (which eliminate tricarboxylic acid cycle intermediates) occur continuously; their rates are sensitive to perfusion conditions and available substrate. Flux through both anaplerotic pathways and citrate synthase are included in the model.

The following variables are used to denote these fluxes: c, flux through citrate synthase, and a, the sum of fluxes feeding intermediates into the tricarboxylic acid cycle via pathways other than citrate synthase. Because steady state is assumed, the rate of disposal reactions eliminating intermediates from the cycle is assumed equal to a. The relative flux through anaplerotic and citrate synthase pathways (a/c) is defined as y to be consistent with earlier work (11, 28, 32). Typically, y is low (<10%) in the heart.

The fractional enrichment in ^{13}C of the pools feeding carbon into the tricarboxylic acid cycle are defined as follows. F_{c0} refers to the fraction of acetyl-CoA that is not labeled, F_{c1} indicates the fraction labeled in the carbonyl carbon, F_{c2} indicates the fraction labeled in the methyl carbon, and F_{c3} indicates labeling in both carbons. By definition, $F_{c0} + F_{c1} + F_{c2} + F_{c3} = 1$. Similarly, F_{a0} refers to the fraction of anaplerotic substrate not labeled, and F_{a1} indicates the fraction of anaplerotic substrate yielding oxaloacetate labeled in C-2 or C-3. The model assumes that the C-2 and C-3 (and the C-1 and C-4) of oxaloacetate are always equivalent. By definition, $F_{a0} + F_{a1} = 1$. If the labeled substrate cannot be converted into an anaplerotic substrates (as with acetate), then $F_{a0} = 1$.

The variables used to describe the ^{13}C-NMR spectrum of glutamate refer to the areas of each multiplet relative

to total resonance area for that carbon (see *Glossary*). Although the mathematical relationships between the isotopomers and physiological variables are complex, the equations describing the glutamate multiplets at steady state are remarkably simple (Tables 1 and 2). These equations may be used to predict the steady-state glutamate spectrum under various conditions or to analyze the glutamate spectrum in terms of relative fluxes. Perfusion conditions may be selected that simplify the equations. For example, hearts supplied with [2-^{13}C]acetate show relatively low anaplerosis, and the equations that result if $y = 0$ approximately describe the glutamate spectrum (17, 24). Hearts supplied with [2-^{13}C]acetate and unlabeled propionate will have significant flux of unlabeled carbon into the tricarboxylic acid cycle and can be analyzed setting $F_{a1} = 0$ (25).

These equations will also prove useful for analysis of

TABLE 1. *Relation between ^{13}C-nuclear magnetic resonance (NMR) spectrum of glutamate and physiological variables when labeled substrate is metabolized via [2-^{13}C]acetyl-CoA*

	Fractional enrichment
C1F	$(yF_{a1} + F_{c2})/[(2y + 1)(2y + 2)]$
C2F	$(yF_{a1} + F_{c2})/(2y + 1)$
C3F	$(yF_{a1} + F_{c2})/(2y + 1)$
C4F	F_{c2}
C5F	0
	Multiplets
C1S	$(y + F_{c0})/(y + 1)$
C2S	$[2(y + 1)^2 - 2F_{c2}(y + 1) - F_{c2} + F_{c2}^2]/[2(y + 1)^2]$
C2D12	$F_{c0}F_{c2}/[2(y + 1)^2]$
C2D23	$F_{c2}(2 + 2y - F_{c2})/[2(y + 1)^2]$
C3S	$F_{c0}(F_{c0} + y)/(y + 1)$
C3T	$F_{c2}^2/(y + 1)$
C4D34	$(yF_{a1} + F_{c2})/(2y + 1)$
C5S	0

Under these conditions $F_{c0} + F_{c2} = 1$, and substrates of anaplerotic reactions are unlabeled or singly labeled in C-2 or C-3 ($F_{a0} + F_{a1} = 1$). See *Glossary* for definitions of abbreviations.

TABLE 2. *Relation between ^{13}C-NMR spectrum of glutamate and physiological parameters when labeled substrate is metabolized via [1,2-^{13}C]acetyl-CoA*

	Fractional enrichment
C1F	$F_{c3}/(2y + 1)$
C2F	$F_{c3}/(2y + 1)$
C3F	$F_{c3}/(2y + 1)$
C4F	F_{c3}
C5F	F_{c3}
	Multiplets
C1S	$(F_{c0} + y)/[2(y + 1)^2]$
C2S	$[F_{c0}(F_{c0} + y) - F_{c3}(1 + y)]/[2(y + 1)^2]$
C2D12	$(F_{c0} + 2y)(1 + y) + (F_{c0}F_{c3})/[2(y + 1)^2]$
C2D23	$F_{c3}(2y + 1 + F_{c0})/[2(y + 1)^2]$
C3S	$F_{c0}(F_{c0} + y)/(y + 1)$
C3T	$F_{c3}^2/(y + 1)$
C4D45	$(2y + 1 - F_{c3})/(2y + 1)$
C4Q	$F_{c3}/(2y + 1)$
C5S	0

Under these conditions $F_{c0} + F_{c3} = 1$, and substrates of anaplerotic reactions are unlabeled ($F_{a0} = 1$). See *Glossary* for definitions of abbreviations.

^{13}C-decoupled ^1H spectra of glutamate. The ^{13}C fractional enrichment at each protonated glutamate carbon (C2F, C3F, and C4F) may be obtained from a high-resolution ^1H spectrum of purified glutamate or from quantitative analysis of ^1H spectra from tissues with and without ^{13}C broad-band decoupling (4, 27). ^1H observation is attractive for in vivo applications because of its sensitivity.

This model differs from earlier models of isotope distribution in the tricarboxylic acid cycle. Strisower and colleagues (28, 32) derived expressions from the sum of an infinite convergent series. Katz and Grunnet (11) introduced the input-output analysis that is intuitively appealing and based on solutions of equations describing the relative fluxes of isotope into a unique carbon position; a comprehensive model was proposed by Goebel et al. (5). The analysis described here utilizes the input-output concept but considers each isotopomer (rather than each carbon site) as a unique state.

One approach to testing the validity of these equations is to simulate the reaction pathways in the tricarboxylic acid cycle (suggested previously, see Ref. 6) and to determine the predicted ^{13}C-NMR spectrum after many cycles. A computer simulation of the tricarboxylic acid cycle (available from the authors) gave the same spectra as those predicted by the equations in Tables 1 and 2. Earlier studies also reported identical expressions for C1F/C3F, C3F/C4F, and C4D34 as those in Table 1 (11, 26, 28, 31, 32).

EXAMPLES OF ISOTOPOMER ANALYSIS

This analysis is illustrated for hearts utilizing acetate, glucose, fatty acids, or fatty acids plus lactate. Hearts were perfused in the Langendorff or working mode as described previously, except that the free calcium was 1.25 mM (8, 18). The left atrial pressure was 12 cmH_2O, and the afterload was 60 cmH_2O for the working hearts and 70 cmH_2O for the Langendorff perfused hearts. Results from five perfusion conditions are presented: *1*) Langendorff perfusion, 2.5 mM [2-^{13}C]acetate (99% enriched) (Figs. 1 and 2); *2*) Langendorff perfusion, 10 mM [1-^{13}C]glucose (99% enriched) and insulin (5 U/l) (see Fig. 3*A*); *3*) working heart, 10 mM [1-^{13}C]glucose (56% enriched) and insulin (5 U/l) (see Fig. 3*B*); *4*) working heart, 0.35 mM [U-^{13}C]fatty acid (99% enriched, a mixture of palmitate, oleate, and linoleic and linolinic acid) [bound to 1% (wt/vol) bovine serum albumin] (see Fig. 4*A*); and *5*) working heart, 0.35 mM [U-^{13}C]fatty acid, as described above, plus 1 mM [3-^{13}C]lactate (see Fig. 4*B*). At the end of 30 min of perfusion, the hearts were freeze-clamped, and the frozen tissue was extracted in perchloric acid, neutralized with KOH, freeze-dried, and reconstituted in D_2O (18).

Proton-decoupled ^{13}C-NMR spectra of the extracts were obtained in a GN-500 NMR spectrometer operating at 125.7 MHz for ^{13}C. Under these conditions a signal from ^{13}C (natural abundance) in glutamate could not be detected when unlabeled substrate was present in control experiments. Various methods of measuring resonance areas are possible; operator-guided curve fitting was used in these examples. Multiplet resonance areas from C-2, C-3, and C-4 (6 or 8 independent measurements) were

used to solve the necessary equations for the physiological variables using standard algorithms for nonlinear least-squares fitting.

The variance in the parameter estimates from numerical solution of these equations may be determined by two methods. One approach involves generating the so-called Hessian matrix (the matrix of second cross partial derivatives), which on inversion gives the variance-covariance matrix. This is appropriate only when certain assumptions are valid (22). Another approach involves the use of Monte Carlo simultation. In this case, a synthetic spectrum is generated from the parameters obtained after nonlinear least-squares fitting to a data set. With the use of this spectrum, several simulated spectra are generated by the addition of noise. This noise is normalized to have a mean of zero and a standard deviation equal to that calculated from the difference between the experimental data and the synthetic spectrum. Each simulated spectrum is fitted, and the results used to estimate the standard deviation for every parameter.

Both approaches have been applied in this study and give reasonably similar results. Because fewer assumptions are required in the use of the Monte Carlo simulation, the standard deviations given in this report are a result of this procedure.

Analysis of the multiplets in the protonated carbons of glutamate is shown in Fig. 2 for a heart utilizing [2-^{13}C]acetate (Langendorff perfusion). In this example, $F_{c2} = 0.89 \pm 0.01$, $y = 0.05 \pm 0.01$, and $F_{a1} = 0.0 \pm 0.0$.

The variables describing fractional enrichment, such as F_{c2}, refer to enrichment in an intermediate pool after metabolism of the labeled precursor. Interpretation of F_{c2} in terms of exogenous substrate contribution to the tricarboxylic acid cycle requires measurement of the fractional enrichment in ^{13}C of the substrate supplied to the heart. For example, in a heart supplied with 1 mM [2-^{13}C]acetate and 1 mM unlabeled acetate, this analysis showed a fractional enrichment of the acetyl-CoA pool (F_{c2}) of ~0.44 (data not shown). Because the exogenous acetate contained equal fractions of labeled and unlabeled material, the contribution of exogenous acetate to acetyl-CoA was therefore 0.88, with the remaining 12% derived from endogenous sources. This example illustrates that ^{13}C enrichment of the available substrate need not be 99%.

Analysis of the oxidation of [1-^{13}C]glucose further illustrates the importance of determining fractional enrichment of the substrate available to the heart. Because of the small volume of the Langendorff apparatus, it was possible to use perfusate containing 99% enriched [1-^{13}C]glucose (Fig. 3*A*). To conserve labeled substrate in the larger volume of the working heart apparatus, [1-^{13}C]glucose was added to perfusate already containing unenriched glucose only after adequate cardiac output was ensured, yielding a final enrichment of 56% (Fig. 3*B*). Least-squares fitting of the multiplet measurements from both the Langendorff heart and the working heart yielded $F_{c2} = 0.24$. To correct for the production of two pyruvate molecules (1 unlabeled) from each glucose molecule and the relatively low enrichment in the C-1 of glucose in the working heart experiment, the following relation was used: F_{c2}(corrected) = F_{c2}(observed) × 2/substrate enrichment. Thus the actual contribution of exogenous glucose to acetyl-CoA was 0.48 in the case of the Langendorff heart and 0.86 in the working heart. The remaining acetyl-CoA was derived from endogenous sources, either glycogen or lipids.

Quantitation of fatty acid oxidation is more relevant to conditions in vivo than oxidation of the substrates already described. [U-^{13}C]fatty acids undergo β-oxidation to [1,2-^{13}C]acetyl-CoA. In this case, the equations in Table 1 cannot be used (since $F_{c2} = 0$), but the equations in Table 2 apply. These equations were used to analyze the spectra in Fig. 4*A*. Once again six independent multiplet areas from C-2, C-3, and C-4 were used to determine $F_{c3} = 0.79 \pm 0.01$ and $y = 0.04 \pm 0.01$ using nonlinear least-squares methods. As may be expected (because

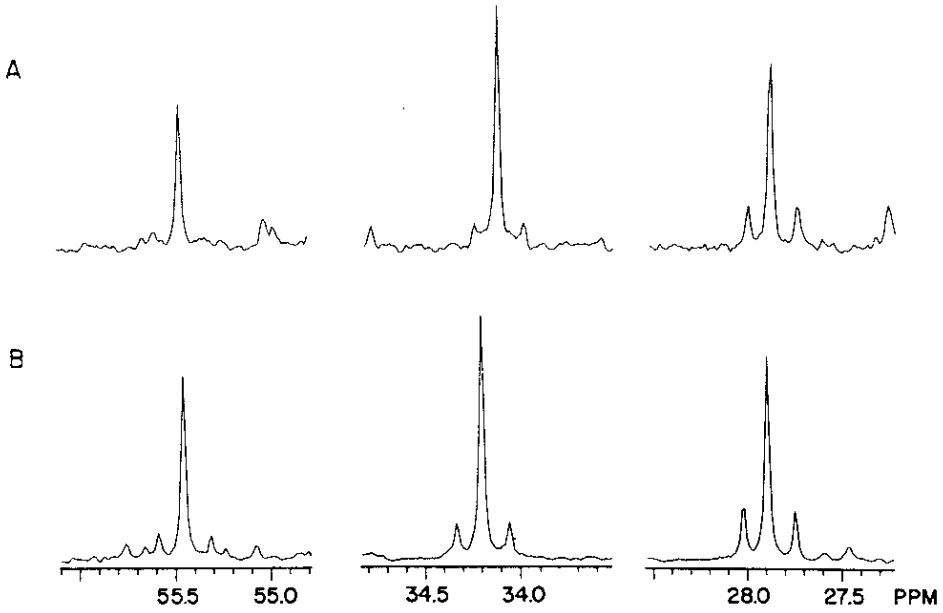

FIG. 3. Proton-decoupled ^{13}C-NMR spectra of glutamate in extract of rat hearts supplied with glucose in Langendorff heart (*A*) and working heart (*B*).

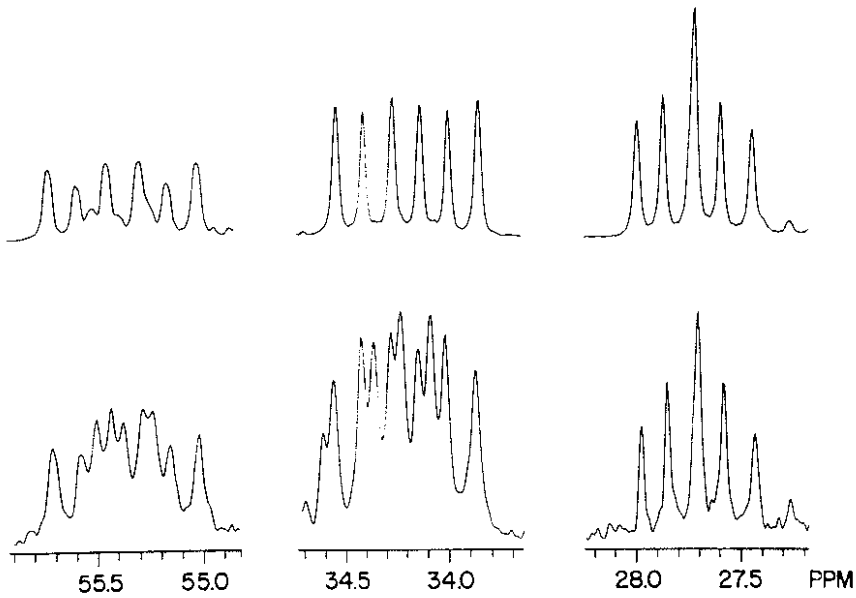

FIG. 4. Proton-decoupled ^{13}C-NMR spectra of glutamate in extracts of rat hearts. Substrate was long-chain fatty acids (*A*) or long-chain fatty acids plus lactate (*B*).

anaplerotic substrates cannot be derived from [1,2-^{13}C]-acetyl-CoA), F_{a1} was found to be 0.

Oxidation of physiologically relevant mixtures of ^{13}C-enriched compounds may also be analyzed quantitatively. When both [3-^{13}C]lactate and [U-^{13}C]fatty acids are available to the heart (*condition 5*, Fig. 4*B*), analysis using the equations reported in the appendix yielded $F_{c2} = 0.17 \pm 0.02$, $F_{c3} = 0.68 \pm 0.04$, $y = 0.06 \pm 0.04$, and $F_{a1} = 0.00 \pm 0.00$. Hence 17% of acetyl-CoA was derived from lactate in the perfusate, 68% was derived from fatty acids in the perfusate, and anaplerotic flux was low, only 6% of citrate synthase flux. Enrichment of the anaplerotic substrates were not significantly different from 0.

SENSITIVITY ANALYSIS

Because the signal-to-noise ratio of the ^{13}C-NMR spectrum is limited, it is important to determine the effect of noise in the spectrum on the variables returned from the least-squares fitting routine. Furthermore, under most conditions, sufficient information is contained in the multiplets of C-3 and C-4 to determine these variables. This is particularly important, since the detection of multiplets in the resonance from C-2 (which contains 9 lines) will be difficult in vivo. A simple sensitivity analysis was performed to estimate the effects of noise on the accuracy of the parameters determined by two different methods of data analysis.

A ^{13}C-NMR spectrum was calculated assuming $F_{c2} = 0.7$, $y = 0.1$, and $F_{a1} = 0.2$. Random numbers from a table were converted to a Gaussian distribution centered at 0 using the Box-Muller function (3). With the use of these numbers, error was added to each of the variables describing the ^{13}C spectrum (e.g., C3S, C4D34, etc.). Fifty spectra were generated at three levels of error such that the standard deviation of the mean value for each variable was 1, 3, or 5%. Each simulated spectrum was analyzed by nonlinear least-squares fitting (numerical analysis) of the six equations describing the protonated carbons or by analytical solution of the equations for C3S, C3T, and C4D34 in Table 1. The former represents conditions typical of the analysis of extracts from perfusions with substrates enriched at a single site; the latter represents the data that may be obtained from an equivalent experiment in vivo. The calculated values for F_{c2}, y, and F_{a1} at each level of error and by both methods of analysis are shown in Table 3. This analysis and our earlier observations (18) suggest that the fractional enrichment of the acetyl-CoA pool (F_{c2}) is determined with good accuracy by both methods. Measurement of y and F_{a1} at the higher levels of error is better using all available information from C-2, C-3, and C-4 (numerical analysis). The poor measurement of F_{a1} is not unexpected, since it has little influence on the glutamate spectrum under these conditions (F_{c2} is high).

LIMITATIONS OF ISOTOPOMER ANALYSIS

This analysis provides a simple and direct measurement of relative fluxes into the tricarboxylic acid cycle. Unlike other tracer methods it is well suited to measurement of utilization of two different substrates in a single experiment. However, flux through a pathway is not quantified. If oxygen consumption or some other index of citrate synthesis is measured, then absolute fluxes through anaplerotic pathways or pathways feeding acetyl-CoA may be determined.

This analysis is most accurate if the level of enrich-

TABLE 3. *Effect of noise in ^{13}C-NMR spectra on accuracy of variables measured by isotopomer analysis*

Method	Error, %	F_{c2}	y	F_{a1}
	0	0.700	0.100	0.200
Numerical	1	0.700±0.002	0.100±0.003	0.190±0.036
	3	0.700±0.005	0.099±0.008	0.170±0.107
	5	0.701±0.008	0.101±0.012	0.170±0.151
Analytical	1	0.697±0.014	0.092±0.046	0.192±0.170
	3	0.699±0.024	0.099±0.086	0.243±0.245
	5	0.703±0.030	0.114±0.112	0.275±0.283

Values are means ± SD; $n = 50$. Two different methods of analysis were used: numerical (nonlinear least-squares fitting) and analytical (solutions of equations for C3S, C3T, and C4D34 in Table 1).

ment of acetyl-CoA is relatively high (F_{c2} or $F_{c3} > \sim 0.25$). Although 100% enrichment of a substrate is not essential, the enrichment must be measured.

Although analysis of the glutamate spectrum was emphasized, this approach is applicable to spectra of intermediates or other metabolites in exchange with the tricarboxylic acid cycle intermediates. For example, because the ^{13}C-NMR spectrum of the C-2 or C-3 of malate or aspartate are equivalent to the C-2 of glutamate, the spectra of these metabolites may be analyzed if glutamate cannot be detected.

APPENDIX

Assumptions of Model

This model is based on standard assumptions about carbon flow in the citric acid cycle: *1*) carbon flows into the tricarboxylic acid cycle via acetyl-CoA or the anaplerotic pathways, *2*) the concentrations and fractional enrichment of tricarboxylic acid cycle intermediates and the exchanging pools are constant, *3*) all ^{13}C entering oxaloacetate has been randomized between C-1 and C-4 and between C-2 and C-3, *4*) flux through the combined anaplerotic reactions equals the flux through disposal reactions, *5*) the entry of $^{13}CO_2$ into the tricarboxylic acid cycle is negligible, and *6*) contribution of natural abundance ^{13}C to the NMR signal is negligible.

Definition of Isotopomers

The 32 possible isotopomers of glutamate may be divided into four groups that are distinguished by the labeling pattern in C-4 and C-5. The first group (x_1–x_8) are those isotopomers not labeled in C-4 or C-5

x_1 = {[1,2,3,4,5-^{12}C]glutamate]/[glutamate]
x_2 = {[1-^{13}C]glutamate]/[glutamate]
x_3 = [[2-^{13}C]glutamate]/[glutamate]
x_4 = [[1,2-^{13}C]glutamate]/[glutamate]
x_5 = [[3-^{13}C]glutamate]/[glutamate]
x_6 = [[1,3-^{13}C]glutamate]/[glutamate]
x_7 = [[2,3-^{13}C]glutamate]/[glutamate]
x_8 = [[1,2,3-^{13}C]glutamate]/[glutamate]

The second group (x_9–x_{16}) is identical to the first, except for labeling in C-4 but not C-5. Enrichment in C-5 but not C-4 is represented in x_{17}–x_{24}. Finally, x_{25}–x_{32} refers to the same labeling in C-1–C-3 plus labeling of both C-4 and C-5 (18). By definition, $x_1 + x_2 + \ldots + x_{32} = 1$.

Input-Output Matrix

The probability that any isotopomer of α-ketoglutarate will be replaced by the same or a different isotopomer can be written as the product of probabilities of a pathway at a metabolic branch point. For example, if the input isotopomer is α-{1,2,3-^{13}C]ketoglutarate (see Ref. 18), then the probability that its output will be α-[1,2,4,5-^{13}C]ketoglutarate is $(1/2)[1/(1+y)]F_{c3}$, which includes three terms: scrambling in fumarate/succinate, the effects of anaplerotic and disposal reactions, and the effects of labeling of acetyl-CoA.

The input-output matrix is generated by writing for each of the 32 possible isotopomers the probability that each isotopomer will yield a given product. Let $z = F_{c0}/(2 + 2y)$. The variables denoting fluxes and fractional enrichment are defined in the text. This yields a 32 × 32 matrix that simplifies to

$$
\begin{aligned}
x_1 &= (x_1 + x_1 + x_2 + x_2 + x_3 + x_4 + x_{17} + x_{18})z \\
&\quad + F_{c0}F_{a0}y/(1 + y) \\
x_2 &= (x_3 + x_4 + x_{17} + x_{18} + x_{18} + x_{19} + x_{20} + x_{20})z \\
x_3 &= (x_5 + x_6 + x_9 + x_{10} + x_{11} + x_{12} + x_{21} + x_{22})z \\
&\quad + F_{c0}F_{a1}y/(2 + 2y) \\
x_4 &= (x_7 + x_8 + x_{23} + x_{24} + x_{25} + x_{26} + x_{27} + x_{28})z \\
x_5 &= (x_5 + x_6 + x_7 + x_8 + x_9 + x_{10} + x_{17} + x_{26})z \\
&\quad + F_{c0}F_{a1}y/(2 + 2y) \\
x_6 &= (x_{11} + x_{12} + x_{21} + x_{22} + x_{23} + x_{24} + x_{27} + x_{28})z \\
x_7 &= (x_{13} + x_{13} + x_{14} + x_{14} + x_{15} + x_{16} + x_{29} + x_{30})z \\
x_8 &= (x_{15} + x_{16} + x_{29} + x_{30} + x_{31} + x_{31} + x_{32} + x_{32})z
\end{aligned}
$$

$$
\begin{aligned}
x_9 &= x_1F_{c2}/F_{c0} \\
x_{10} &= x_2F_{c2}/F_{c0} \\
x_{11} &= x_3F_{c2}/F_{c0} \\
x_{12} &= x_4F_{c2}/F_{c0} \\
x_{13} &= x_5F_{c2}/F_{c0} \\
x_{14} &= x_6F_{c2}/F_{c0} \\
x_{15} &= x_7F_{c2}/F_{c0} \\
x_{16} &= x_8F_{c2}/F_{c0}
\end{aligned}
$$

$$
\begin{aligned}
x_{17} &= x_1F_{c1}/F_{c0} \\
x_{18} &= x_2F_{c1}/F_{c0} \\
x_{19} &= x_3F_{c1}/F_{c0} \\
x_{20} &= x_4F_{c1}/F_{c0} \\
x_{21} &= x_5F_{c1}/F_{c0} \\
x_{22} &= x_6F_{c1}/F_{c0} \\
x_{23} &= x_7F_{c1}/F_{c0} \\
x_{24} &= x_8F_{c1}/F_{c0}
\end{aligned}
$$

$$
\begin{aligned}
x_{25} &= x_1F_{c3}/F_{c0} \\
x_{26} &= x_2F_{c3}/F_{c0} \\
x_{27} &= x_3F_{c3}/F_{c0} \\
x_{28} &= x_4F_{c3}/F_{c0} \\
x_{29} &= x_5F_{c3}/F_{c0} \\
x_{30} &= x_6F_{c3}/F_{c0} \\
x_{31} &= x_7F_{c3}/F_{c0} \\
x_{32} &= x_8F_{c3}/F_{c0}
\end{aligned}
$$

Calculation of Isotopomers and Relation to ^{13}C-NMR Spectrum

After substitution, the eight equations in eight unknowns (x_1–x_8) can be solved to yield the relative concentration of each isotopomer in terms of the physiological variables. However, the observed ^{13}C-NMR spectrum measures groups of isotopomers. The next step, therefore, is to relate the groups of isotopomers (detected by ^{13}C-NMR) to the physiological variables.

The fractional ^{13}C enrichment at each carbon relative to total exchanging glutamate pool is determined by summing the isotopomers labeled in that position. For example, $C4F = x_9 + x_{10} + x_{11} + x_{12} + x_{13} + x_{14} + x_{15} + x_{16} + x_{25} + x_{26} + x_{27} + x_{28} + x_{29} + x_{30} + x_{31} + x_{32}$. The contribution of a pool of isotopomers to the observed ^{13}C spectrum is calculated by dividing the sum of relevant isotopomers by the fractional enrichment of that carbon. For example, the area of the doublet in C-4 due to coupling between C-3 and C-4, relative to the total area of C-4, is $(x_{13} + x_{14} + x_{15} + x_{16})/C4F$. Similar calculations apply to other glutamate multiplets (18). In the following equations, it is assumed that some ^{13}C enters the tricarboxylic acid cycle as the methyl carbon of acetyl-CoA (i.e., $F_{c2} + F_{c3} > 0$) and that $F_{c1} = 0$. The general solutions (which simplify to Tables 1 and 2 with appropriate boundary conditions) are

Fractional enrichment

$$C1F = (F_{c3} + 2F_{c3}y + w)/[(2y + 1)(2y + 2)]$$

$$C2F = w/(2y + 1)$$

$$C3F = w/(2y + 1)$$

$$C4F = F_{c2} + F_{c3}$$

$$C5F = F_{c3}$$

Multiplets

$$C1S = w(F_{c0} + y)/[(y + 1)(F_{c3} + 2F_{c3}y + w)]$$

$$C2S = (F_{c2} + yF_{a1})(2y + 1)/[w(2y + 2)] + [(F_{c0} + y)F_{c0} - F_{c2}(y + 1)]/[2(y + 1)^2]$$

$$C2D12 = F_{c3}(2y + 1)/[w(2y + 2)] + (F_{c0}F_{c2} + F_{c0}F_{c3})/[2(y + 1)^2] - F_{c3}/(2 + 2y)$$

$$C2D23 = [F_{c2}(y + 1) + (F_{c2} + F_{c3})(F_{c0} + y)]/[2(y + 1)^2]$$

$$C2Q = [F_{c3}(y + 1) + (F_{c2} + F_{c3})^2]/[2(y + 1)^2]$$

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

$$C3T = (F_{c2} + F_{c3})^2/(y + 1)$$

$$C4S = F_{c2}(2y + 1 - w)/[(2y + 1)(F_{c2} + F_{c3})]$$

$$C4D34 = wF_{c2}/[(2y + 1)(F_{c2} + F_{c3})]$$

$$C4D45 = F_{c3}(2y + 1 - w)/[(2y + 1)(F_{c2} + F_{c3})]$$

$$C4Q = wF_{c3}/[(2y + 1)(F_{c2} + F_{c3})]$$

$$C5S = 0$$

where $w = F_{c2} + F_{c3} + yF_{a1}$. These equations would be appropriate for analysis of the spectrum from a heart supplied with [1,2-^{13}C]acetate plus [3-^{13}C]pyruvate. However, if the substrate for an anaplerotic reaction is enriched in two sites (e.g., [2,3-^{13}C]propionate), or if [1-^{13}C]acetyl-CoA is present, then the more general equations in (18) must be used. It is of interest that $C4D45 + C4Q = F_{c3}/(F_{c2} + F_{c3})$ and $C4S + C4D34 = F_{c2}/(F_{c2} + F_{c3})$. Therefore relative substrate utilization between two different labeled substrates may be measured simply by analyzing the C-4 multiplet.

The computer simulation of the tricarboxylic acid cycle was initially written by C. R. Malloy and F. M. H. Jeffrey; later development was by Arun Rajagopal. The computer simulation can be obtained by sending a formatted 5¼″ floppy disk with an addressed mailing envelope. We thank Dr. James Willerson for continued support of these experiments, Hilary Srere for assisting with the experiments on fatty acids, and Debbie Shuttlesworth for excellent secretarial assistance.

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