

Improved estimation of anaplerosis in heart using ^{13}C NMR

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Cohen, David M., and Richard N. Bergman. Improved estimation of anaplerosis in heart using ^{13}C NMR. *Am. J. Physiol.* 273 (*Endocrinol. Metab.* 36): E1228–E1242, 1997.—Anaplerotic enzymes, such as pyruvate carboxylase or malic enzyme, catalyze reactions that fill up the pools of the citric acid cycle (CAC), thereby increasing the total mass of CAC intermediates. Relative anaplerosis (y) denotes the ratio of anaplerotic flux to the flux catalyzed by citrate synthase. We examine conventional methods [C. R. Malloy, A. D. Sherry, and F. M. H. Jeffrey. *J. Biol. Chem.* 263:6964–6971, 1988; C. R. Malloy, A. D. Sherry, and F. M. H. Jeffrey. *Am. J. Physiol.* 259 (*Heart Circ. Physiol.* 28): H987–H995, 1990] of measurement of y using ^{13}C -labeled precursors and analysis of [^{13}C]glutamate labeling by nuclear magnetic resonance (NMR) spectroscopy. Through mathematical analysis and computer simulation, we show that isotopic enrichment of the pool of pyruvate that is substrate for anaplerosis will severely decrease the accuracy of estimates of y made with conventional methods no matter how small the mass of the pool of pyruvate. Suppose that the recycling parameter R denotes the fraction of molecules of pyruvate that contain carbons derived from intermediates of the CAC. Each means of estimation of relative anaplerosis in the peer-reviewed literature assumes that $R = 0$, although this assumption has not been confirmed by experiment. We show that conventional formulas, using either fractional enrichments of carbons or isotopomer analysis, actually estimate at most $y \cdot (1 - R)$ instead of y during administration of [$2\text{-}^{13}\text{C}$]acetate and unlabeled pyruvate. Using a new formula for estimation of y , we recalculate values of y from the literature and find them $\sim 50\%$ too low. We assume that all anaplerosis is via pyruvate and that the difference in isotopic enrichment between cytosolic and mitochondrial malate is negligible.

Krebs cycle; citric acid cycle; tricarboxylic acid cycle; nuclear magnetic resonance spectroscopy; pyruvate; metabolism; isotopomer analysis; malate-aspartate shuttle

THE CITRIC ACID CYCLE (CAC) is the common pathway for oxidative metabolism of carbohydrate, protein, and fat. Maintenance of concentrations of intermediary substrates within the cycle is accomplished by means of reactions that do not belong to the CAC, e.g., reactions catalyzed by pyruvate carboxylase and by malic enzyme. Chemical reactions that add to (or remove from) the mass of pools of metabolic intermediates of the CAC are termed anaplerotic (or cataplerotic, respectively). The functional significance of changes in anaplerosis and cataplerosis is unclear. Anaplerotic reactions may

contribute to the maintenance of contractile function of the heart under normal conditions (1, 24) or during ischemia (10, 15, 33). Even under conditions in which the rates of anaplerosis and cataplerosis are nearly equal, resulting in little net flux, these reactions can still contribute to cellular regulation. For example, Newsholme and Stanley (19) presented evidence that so-called substrate cycles increase the sensitivity of net pathway flux to small changes in concentrations of metabolic substrates. In addition, it has been suggested that transitory increases in anaplerosis may increase the production of energy by the CAC by increasing the concentrations of substrates of enzymes that are operating near their Michaelis-Menten kinetic values (21).

In this report, we examine the assumptions of formulas for relative anaplerosis and demonstrate that conventional formulas, using isotopomer analysis (17) or fractional enrichments of carbons of glutamate (16), may underestimate its value.¹ We show that ignoring the isotopic enrichment of intracellular pyruvate during administration of [$3\text{-}^{13}\text{C}$]pyruvate or [$1\text{-}^{13}\text{C}$]glucose will cause underestimation of anaplerosis. Even if exogenous pyruvate is not isotopically labeled, it is possible that increases in the enrichment of mitochondrial oxaloacetate and malate (e.g., during metabolism of [$2\text{-}^{13}\text{C}$]acetate) will enhance the fractional enrichment of pyruvate via cataplerotic fluxes. We propose a new formula for estimation of relative anaplerosis during administration of isotopically labeled substrate and demonstrate that it is more accurate than currently available formulations.

Glossary

Fractional enrichment of C- i : number of molecules isotopically labeled at C- i divided by number of molecules in the entire metabolic pool

a1, a2: fractional enrichment of C-1 and C-2 of the acetyl moiety of acetyl-CoA (where carbon C-2 is the methyl carbon)

¹ We recognize that any model is founded on a set of assumptions and is only as accurate as the assumptions are valid. Our analysis points out the consequences of the isotopic labeling of the pyruvate that is substrate for anaplerosis and suggests that it be included in any formula for relative anaplerosis. Future experiments may demonstrate the insufficiency of our assumptions and require yet additional assumptions to be incorporated in a model that is used to estimate relative anaplerosis.

g1, g2, g3, g4, g5: fractional enrichment of carbons C-1, C-2, C-3, C-4, and C-5 of glutamate
 o1, o2, o3, o4: fractional enrichment of carbons C-1, C-2, C-3, and C-4 of oxaloacetate
 p1, p2, p3: fractional enrichment of carbons C-1, C-2, and C-3 of pyruvate
 p4: fractional enrichment of the pool of carbon dioxide that condenses with pyruvate in the anaplerotic reactions catalyzed by pyruvate carboxylase and by malic enzyme
 r: "reverse" flux catalyzed by fumarase
 y: ratio of anaplerotic flux to flux catalyzed by citrate synthase
 v_{TCA} : rate of flux catalyzed by α -ketoglutarate dehydrogenase complex
 R: recycling parameter
 PDH: pyruvate dehydrogenase complex
 OAA: oxaloacetate
 FE: fractional enrichment

These symbols do not refer to symbols used by other investigators in describing metabolic fluxes, fractional enrichments, or recycling of metabolic intermediates.

METHODS

First we describe the model of the myocardial CAC and anaplerosis from pyruvate (Fig. 1). Next we describe in general terms our technique for deriving algebraic formulas related to flux rates at isotopic and metabolic steady state. Details of all derivations are given in APPENDIX A. Finally, we describe the methods by which we tested the accuracy of proposed formulas for relative anaplerosis y , the ratio of anaplerotic flux to the flux catalyzed by citrate synthase.

Biochemical Reactions and Flux Rates

The model of the CAC of myocytes used in our investigations (Fig. 1) is a slight modification of the model used by Chance et al. (3) for a Langendorff perfusion of the heart of fed rats. Malate and OAA are combined into a single pool because of the extremely small content of the latter compound (<0.7% of the malate pool) (3). We used flux rates and pool sizes of CAC intermediates estimated by Chance et al. (3) during administration of 5.0 mM glucose and 5.0 mM acetate (see Fig. 1 and APPENDIX A). We simulated the effect of [2- ^{13}C]acetate or [3- ^{13}C]pyruvate on the labeling of metabolites of the CAC. In order to change as few parameters as possible during simulated perfusion with [3- ^{13}C]pyruvate, the pool sizes were maintained at the same levels as during simulated perfusion with [2- ^{13}C]acetate.² We did not simulate alanine aminotransferase because alanine is believed to be at equilibrium with pyruvate (12, 13, 22) and its omission will not change the enrichment of pyruvate or other metabolites at steady state. The rate of flux catalyzed by the pyruvate dehydrogenase complex was set to approximately the value estimated (3) during administration of glucose and pyruvate, and to 10% of that value during acetate administration.

In the current model (unlike our earlier model described in Refs. 5 and 7), we include the reversibility of the chemical flux catalyzed by fumarase (*reaction e*, Fig. 1). Indeed, using radioisotopes, Nuutinen et al. (20) estimated that the ratio of

the flux (r) from malate to fumarate to the flux (v_{TCA}) catalyzed by α -ketoglutarate dehydrogenase complex in the perfused rat heart was 4.13 ($r/v_{\text{TCA}} = 4.13$). Except where noted, simulations presented in this report were run with the r/v_{TCA} value equal to 10, representing a compromise between the value measured in perfused rat heart and the infinitely large value assumed in the classic model (so-called "instant randomization of carbons of OAA") (35).³ In simulations with different values of relative anaplerosis (increasing y from 0.1 to 1 to 10), the value of r remains the same to simulate activation of the anaplerotic enzymes rather than increased concentrations of substrates, which would affect the value of r as well as the value of y .

We assume that anaplerotic fluxes are catalyzed predominantly by malic enzyme and, to a lesser extent, by pyruvate carboxylase (28). In perfused rat heart, with glucose as the sole energy source, anaplerosis is almost entirely (>93%) via production of malate and/or OAA, with significantly less (<8%) via metabolism of propionate formed from oxidation of endogenous amino acids (21). We assume that cataplerotic reactions use malate or OAA as substrate and are catalyzed by the same enzymes as the anaplerotic reactions (Fig. 2). At metabolic steady state, the masses of individual pools of intermediates of the CAC are not changing, and therefore the rate of anaplerosis equals cataplerosis ($v_{\text{ANA}} = v_{\text{CATA}}$).

Model of pyruvate. To invoke a minimal number of hypotheses in our demonstration of the effects of recycling of pyruvate on the accuracy of formulas for estimation of relative anaplerosis, we have adopted a simple model of pyruvate production and disposal in myocytes (Fig. 2). For the purpose of evaluating alternative algebraic formulas (see Table 1), we hypothesize a single pool of "pyruvate" that is substrate for anaplerosis and in which we include those metabolic pools the carbons of which have the same steady-state fractional enrichment as pyruvate, e.g., alanine and lactate (see Fig. 2).⁴ Cataplerotic reactions add molecules to this pool of pyruvate (see *Eqs. A1-A15*). We consider an influx ("pyrInflux") of pyruvate (e.g., from exogenous pyruvate or from phosphoenolpyruvate via pyruvate kinase) and an efflux ("pyrEfflux") out of this particular pool of pyruvate. Effects observed in this simple model can be created in more complicated models as well (e.g., Refs. 13 and 22).

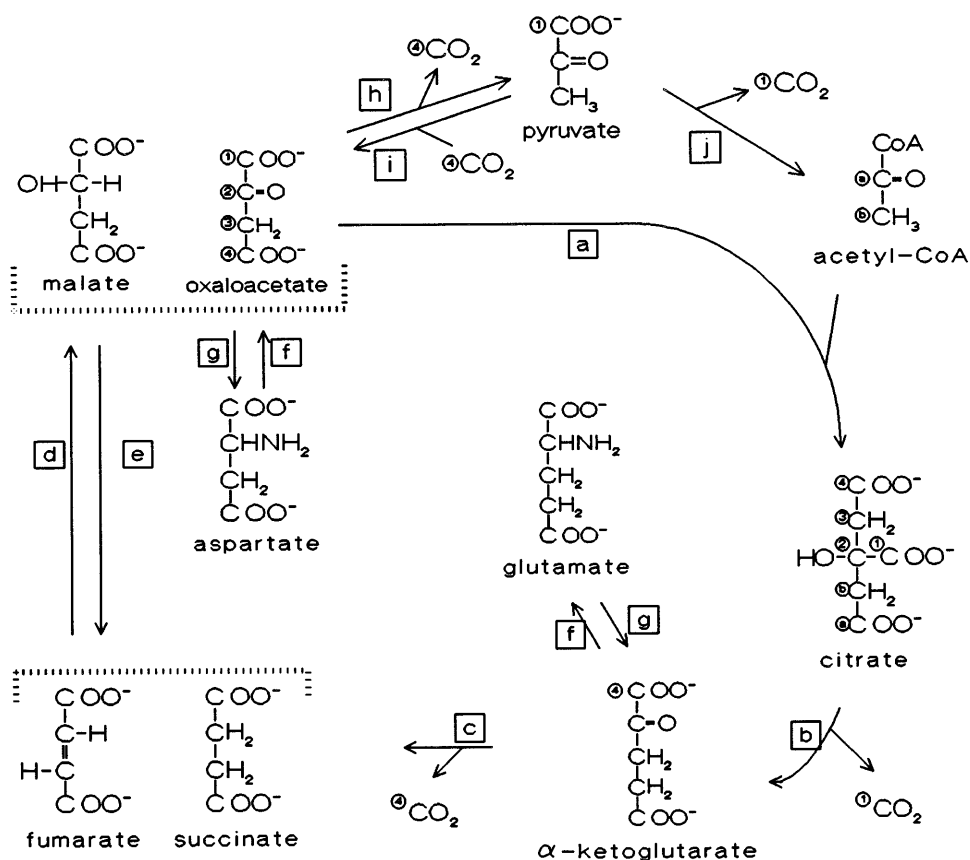
It is convenient to have a parameter that indicates whether the pyruvate pool is more or less composed of newly arrived molecules (from pyrInflux in Fig. 2) or molecules that came from the CAC (via cataplerosis, v_{CATA}). We define the recycling parameter R as the fraction of substrate for anaplerosis that contains one or more carbons exported via cataplerosis. This definition of R does not depend on a particular model of pyruvate metabolism. For the purposes of simulating a specific model of metabolism with a single anaplerotic pool

³ Increasing r/v_{TCA} in the model increases (decreases) the accuracy of *Eq. A19* during administration of [3- ^{13}C]pyruvate ([2- ^{13}C]acetate, respectively) and has no effect on the accuracy of *Eq. A18* during administration of either [3- ^{13}C]pyruvate or [2- ^{13}C]acetate (data not shown).

⁴ Our assumption of equal FEs of carbons of pyruvate, lactate, and alanine has been found to be valid in heart extracts for experiments in vivo in the dog heart during infusion of [3- ^{13}C]lactate (12). However, experiments in the in vivo dog heart (12), as well as in the isolated perfused rabbit heart (13), found that administration of [3- ^{13}C]pyruvate caused the fractional enrichment of C-3 of alanine to equal the fractional enrichment of C-3 of intracellular pyruvate, but to exceed the fractional enrichment of C-3 of intracellular lactate. Therefore, our hypothetical 1-pool model of pyruvate metabolism may be realistic during administration of [3- ^{13}C]lactate but not during administration of [3- ^{13}C]pyruvate.

² Note that efflux of AcCoA during simulated administration of [3- ^{13}C]pyruvate is 10 times greater than that during simulated administration of [2- ^{13}C]acetate (Table 2). The former simulations were redone at zero efflux of AcCoA (Table 4).

Fig. 1. Schematic of citric acid cycle and associated reactions in myocytes, showing transfer of carbons. Chemical fluxes are denoted by letters in boxes: *a*, citrate synthase; *b*, aconitase and isocitrate dehydrogenase; *c*, α -ketoglutarate dehydrogenase complex and succinyl-CoA synthetase; *d*, fumarase; *e*, fumarase (reverse); *f*, aspartate aminotransferase; *g*, aspartate aminotransferase (reverse); *h*, malic enzyme and pyruvate carboxylase (cataplerosis); *i*, malic enzyme and pyruvate carboxylase (reverse, anaplerosis); and *j*, pyruvate dehydrogenase complex. Fumarate and succinate are combined into 1 pool, as are malate and OAA. Reversible fluxes (catalyzed by fumarase, aspartate aminotransferase, malic enzyme, and pyruvate carboxylase) have been separated into "forward" and "reverse" fluxes. Rate of reverse flux of fumarase (*e*) equals *r* in Fig. 6 and in *Eqs. A7-A14*. Chemical structures have been drawn so that canonical numbering of carbons proceeds from *top to bottom* (top carbon is C-1) in each compound. To facilitate tracing the fate of individual carbons, carbons of OAA are numbered 1-4, and carbons of the acetyl moiety of acetyl-CoA are labeled *a* and *b*. On compounds other than OAA and acetyl-CoA, numerals and letters represent the locations to which the corresponding carbons of OAA and acetyl-CoA, respectively, are transferred by actions of enzymes of citric acid cycle.



and ignoring mitochondrial/cytosolic compartmentation (for the present), the formula for *R* at metabolic steady state is $R = v_{\text{CATA}} / (\text{pyrInflux} + v_{\text{CATA}})$.

Suppose pyruvate and $[2-^{13}\text{C}]$ acetate are provided exogenously. In the extreme case of zero influx of pyruvate from external sources ($R = 1$), at isotopic steady state the enrichment of pyruvate would equal that of OAA, and flux catalyzed by pyruvate dehydrogenase complex would equal zero (see Fig. 2). As we show in APPENDIX A (see *Eq. A28*), anaplerotic flux can become considerably labeled because (for example) molecules of OAA doubly labeled at C-2 and C-3 are produced by the chemical reactions of the CAC. In the other extreme, if the influx into pyruvate is extremely large compared with v_{CATA} ($R \approx 0$), then the dilution of the pyruvate pool by unlabeled molecules of pyruvate would render it effectively unlabeled.

Our investigation of the effect of "recycling" of the anaplerotic pool of pyruvate is necessarily speculative, because the disposition of pyruvate into kinetically discernible metabolic pools is not well understood.⁵ We illustrate the effects (see RESULTS) that partial sequestration of intracellular pyruvate and consequent recycling of labeled carbons would have on the accuracy of classic formulas for estimation of relative anaplerosis (γ). It is important to note that our formulas (see *Eqs. A1-A15*) that do not involve the recycling parameter *R* do not depend on a particular model of intracellular pyruvate compartmentation but solely on the fractional enrichment of

C-2 and C-3 of the pool of pyruvate that is substrate for anaplerosis.

Derivation of Algebraic Formulas

We begin with the metabolic pathways shown in Figs. 1 and 2, from which details of the transfer of carbons from reactants to products were obtained (Fig. 3). To simplify the presentation of mathematical material, we have gathered all the derivations of formulas into APPENDIX A. Next we illustrate our method for deriving formulas relating rates of metabolic flux to fractional enrichments of carbons of pools of metabolic intermediates by use of the principle of conservation of mass. For example, assume that compounds A and B are converted to compound C at the rate of v_A and v_B moles of C per second, respectively (Fig. 4). Let v_C equal the rate of conversion of C to other compounds, which might even include A or B. If no compounds other than A or B are converted to C, then at metabolic steady state we can equate the rate of production of C with the rate of disposal of C by means of the equation $v_A + v_B = v_C$.

Suppose that examination of the chemical reactions shows that carbon position C-1 of A and carbon position C-2 of B are transferred to carbon position C-3 of compound C. (Note that A and B compete for the same carbon position in compound C.) Let a source of labeled carbon (i.e., ^{13}C) be introduced, resulting in the isotopic labeling of compounds A, B, and C. At metabolic and isotopic steady state, compounds A, B, and C will maintain constant concentrations and constant fractional enrichments of individual carbons. Let the fractional enrichments of C-1 of A, C-2 of B, and C-3 of C be denoted f_A ,

⁵ Several studies have found evidence that the pool of pyruvate that is used as substrate for anaplerosis is distinct from the pool of pyruvate that communicates with tissue lactate or with extracellular pyruvate (13, 22, 35).

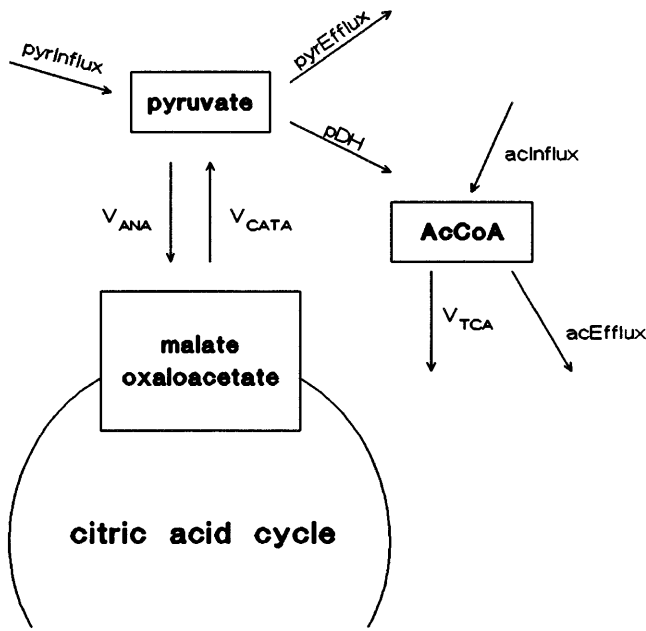


Fig. 2. Schematic of fluxes into and out of acetyl-CoA (AcCoA) and pool of pyruvate that is substrate for anaplerotic reactions. Box labeled "pyruvate" includes those pools of metabolites (e.g., alanine) having the same fractional enrichment of corresponding carbons as pyruvate at isotopic steady state. At metabolic steady state anaplerosis equals cataplerosis ($v_{CATA} = v_{ANA}$). Not shown: chemical fluxes between malate and fumarate, between OAA and citrate, and between OAA and aspartate. Fluxes: pyrInflux, influx of pyruvate from precursor pool; pyrEfflux, loss of pyruvate via other pathways; pDH, flux catalyzed by pyruvate dehydrogenase complex; v_{ANA} , anaplerotic flux from pyruvate; v_{CATA} , cataplerotic flux into pyruvate; acInflux, influx into acetyl-CoA (e.g., from β -oxidation of fatty acids); acEfflux, efflux from acetyl-CoA (e.g., catalyzed by carnitine acetyltransferase). Recycling parameter R is fraction of pool of pyruvate that is substrate for anaplerosis containing carbons exported from citric acid cycle (via cataplerosis). In this diagram, formula for R is $R = v_{CATA}/(v_{CATA} + \text{pyrInflux})$.

f_B , and f_C , respectively. Then the rate of conversion of labeled C-1 of A to C-3 of C equals $f_A \cdot v_A$, and the rate of conversion of labeled C-2 of B to C-3 of C equals $f_B \cdot v_B$. Equating the rate of production of labeled C-3 of C with the rate of removal of label from C-3 of compound C, we obtain the following equation

$$f_A \cdot v_A + f_B \cdot v_B = f_C \cdot v_C$$

All of the formulas in APPENDIX A are derived with this principle.

In the formulas presented below, it is assumed that each variable representing fractional enrichment of a compound refers to a single, well-mixed pool and excludes any molecules that are metabolically inactive (i.e., that do not exchange carbons with constituent metabolic pools of the CAC). In practice, this assumption may not hold. In APPENDIX B, we derive formulas to correct for a pool of glutamate that is metabolically inactive (we assume all acetyl-CoA is metabolically active).

Testing Algebraic Formulas

We tested the accuracy of formulas for relative anaplerosis (Table 1) by simulating the CAC in two ways: 1) as a system of ordinary differential equations to be solved by use of MLAB (2) and 2) by means of a computer simulation of a syntactic model (5). The dependent variables in the differential equations written for MLAB (see APPENDIX A) were the fractional enrichments of individual carbons of the metabolic intermedi-

Table 1. Algebraic formulas for relative anaplerosis

Equation No.	Formula
A10	$y = \frac{g4 - g2}{g2 + g3 - p2 - p3}$
A18	$\frac{g2}{g4} = \frac{1}{1 + 2 \cdot y} \quad \text{Therefore } y = \frac{g4}{2 \cdot g2} - \frac{1}{2}$
A19	$\frac{g1}{g3} = \frac{1}{2 + 2 \cdot y} \quad \text{Therefore } y = \frac{g3}{2 \cdot g1} - 1$
A22	$y = \frac{g4 \cdot g4}{C3T} - 1$
A25	$y = \frac{g3 \cdot (g4 \cdot g4 - C3T)}{g3 \cdot C3T - g4 \cdot p23}$
A26	$y = \left(\frac{g4 \cdot g4}{C3T} - 1 \right) \cdot \frac{1}{1 - R}$

Formulas involving solely fractional enrichment of carbons are our formula (Eq. A10) and classical formulas (Eqs. A18 and A19). Formulas involving the C-3 triplet resonance of the glutamate ¹³C nuclear magnetic resonance (NMR) spectrum are Eq. A22 from Malloy et al. (17) and Eqs. A25 and A26 (derived by us). See APPENDIX A for derivation of equations and assignment of numbers to equations. y , Relative anaplerosis; g_i , p_i , fractional enrichment of C- i of glutamate and pyruvate, respectively; p23, fraction of pyruvate molecules doubly labeled at C-2 and C-3; C3T, fraction of C-3 resonance of glutamate that is triplet (see Eq. A23); R , recycling parameter for pyruvate (see METHODS).

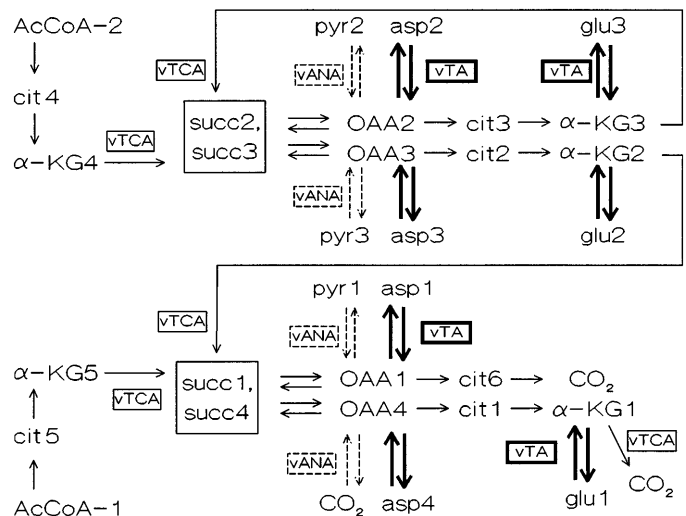


Fig. 3. Illustration of transfer of carbons within pools of citric acid cycle and between those pools and aspartate, pyruvate, and glutamate. Carbon positions C- i within molecules are denoted by the following symbols: AcCoA- i , acetyl-CoA; α -KG i , α -ketoglutarate; cit i , citrate; succ i , succinate; pyr i , pyruvate; asp i , aspartate; glu i , glutamate; OAA i , OAA. As in Fig. 1, fumarate and succinate are combined into a single pool, as are malate and OAA. Carbon exchange catalyzed by aspartate aminotransferase between C-4 (C-5) of α -ketoglutarate and C-4 (C-5) of glutamate is not shown. Alanine aminotransferase does not alter fractional enrichments of carbons of glutamate or α -KG and is therefore not included. v_{TCA} , Rate of flux through citric acid cycle; v_{ANA} , rate of anaplerotic flux; v_{TA} , rate of flux catalyzed by aspartate aminotransferase.

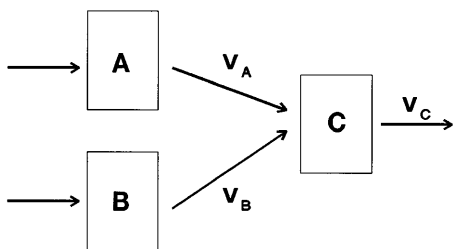


Fig. 4. Illustration of method for deriving equations for flux rates (see text). Metabolic intermediates A and B are independently converted to compound C at rates v_A and v_B (mol/min), respectively. The sum of rates of removal of compound C is v_C (mol/min), including chemical reactions and transport of molecules out of this metabolic pool of C. It is assumed that no other chemical intermediates (besides A and B) are converted directly to C.

ates (Fig. 3). Because several of the formulas to be tested rely on knowledge of positional isotopomers, we also employed a computer simulation of a syntactic model of these reactions. (A positional isotopomer of a compound is an isomer that is determined by the positions of isotopes within the compound, e.g., $[1,2-^{13}\text{C}]$ glutamate and $[1,3-^{13}\text{C}]$ glutamate are positional isotopomers of glutamate.) Use of MLAB to obtain the abundance of positional isotopomers of glutamate would have required solving more than 180 ordinary differential equations simultaneously. We therefore did not use MLAB to test the accuracy of Eqs. A22, A25, or A26 but used a computer simulation of a syntactic model instead. From these simulations, we were able to independently confirm the results obtained with differential equations.

The syntactic approach provides a convenient means of predicting the time-dependent changes in concentrations of positional isotopomers of metabolic intermediates (5–7). It is a stochastic simulation of chemical kinetics that relies on a rule-based description of the transfer of atoms among chemical intermediates of metabolic pathways (5, 7). Instead of describing the changes over time with differential equations, we specify the transfer of carbons from reactants to products by use of syntactic rules. The time course of changes in the simulation is determined by a Monte Carlo simulation algorithm (6). Flux rates are determined at any time by the product of concentrations of substrates and a first-order rate constant. Because of its stochastic nature, the pool sizes fluctuate somewhat around the steady-state levels, just as one would expect in the cell. Because the molecules in each metabolic pool are simulated as if a miniature replica of the CAC existed inside the computer, one can examine the concentration of positional isotopomers of any pool at any time. This allows for the testing of mathematical relationships among flux rates, pool sizes, isotopomer abundances, and fractional enrichments.

RESULTS

Accuracy of Eq. A18

As predicted from theoretical analysis of its relative error with the assumption of instant equilibration of OAA and fumarate (see APPENDIX A, Eq. A32), Eq. A18 consistently underestimates the true value of relative anaplerosis y (Tables 2, 3, and 4). During administration of $[2-^{13}\text{C}]$ acetate, the accuracy in Eq. A18 increases with increasing dilution of pyruvate by molecules of exogenous pyruvate (i.e., decreasing R). In fact, computer simulation (Tables 2 and 3) and mathematical analysis (Eq. A20) indicate that the value obtained from Eq. A18

equals $(1 - R) \cdot y$, instead of y , during administration of $[2-^{13}\text{C}]$ acetate and unlabeled pyruvate.

If the exogenously administered substrate was $[3-^{13}\text{C}]$ pyruvate, estimates of relative anaplerosis by use of Eq. A18 gave values indistinguishable from zero (slightly negative) for all values of y and all values of recycling parameter R (Table 2). Simulations in which exogenous pyruvate was 50% unlabeled and 50% $[3-^{13}\text{C}]$ pyruvate gave exactly the same estimates for y by use of Eq. A18, but the fractional enrichments of each carbon of glutamate and pyruvate were reduced by 50% (data not shown). Increased enrichment of acetyl-CoA from the decreased rate of acetyl-CoA turnover improved the estimate of y by use of Eq. A18; the estimated value of y increased to the range of positive values (Table 4). The increased accuracy (underestimation by 61, 81, and 98% for $y = 0.1, 1, \text{ and } 10$, respectively, and $R = 0.1$) can be explained by the increase in enrichment of carbons of glutamate relative to pyruvate (see Eq. A32). The value of y estimated by Eq. A18 will be negative whenever the fractional enrichment of C-2 of glutamate exceeds the fractional enrichment of C-4 of glutamate, or, equivalently, whenever the sum of fractional enrichments of C-2 and C-3 of pyruvate (i.e., $p_2 + p_3$) exceeds that of glutamate C-2 plus C-3 (i.e., $g_2 + g_3$). The accuracy of Eq. A18 increases as $(p_2 + p_3)/(g_2 + g_3)$ decreases (see Eq. A32).

Accuracy of Eq. A19

Equation A19, a formula for measuring relative anaplerosis from fractional enrichments of glutamate C-1 and C-3 (Table 1), underestimates the value of y . As the dilution of the pyruvate pool by exogenous pyruvate increases (i.e., decreasing R), the ratio of fractional enrichments of C-1 of pyruvate to C-1 of glutamate decreases (Table 2), thereby decreasing the magnitude of the relative error in y incurred by use of Eq. A19 (see Eq. A33).

For administration of $[2-^{13}\text{C}]$ acetate, the accuracy of Eq. A19 improves with decreasing recycling R (Tables 2 and 3). During administration of exogenous $[3-^{13}\text{C}]$ pyruvate at $R = 0.1$, Eq. A19 underestimated relative anaplerosis (y) by 68% when $y = 0.1$, 40% when $y = 1$, and by 70% when $y = 10$ (Tables 2 and 3). At values of recycling parameter R of 0.5 or 0.9, Eq. A19 underestimated y by ~65%. Computer simulations in which exogenous pyruvate was 50% unlabeled and 50% $[3-^{13}\text{C}]$ pyruvate gave exactly the same estimates of y by use of Eq. A19, but the fractional enrichment of each carbon of glutamate and pyruvate was reduced by 50% (data not shown). Increasing the enrichment of acetyl-CoA by decreasing its turnover rate decreased the accuracy of Eq. A19 for $y = 0.1$ but had no effect for higher values of y (Table 4).

Accuracy of Eq. A10

We propose a new formula (Eq. A10) for estimation of relative anaplerosis. Equation A10 is completely accurate when the CAC is simulated by solving a system of differential equations numerically during administration of either $[2-^{13}\text{C}]$ acetate or $[3-^{13}\text{C}]$ pyruvate (Table

Table 2. Estimates of relative anaplerosis (y) and values of fractional enrichments of carbons C- i of glutamate and pyruvate for different values of y and of recycling parameter R

R	Estimate of y / Actual Value of y			Fractional Enrichments								
				Glutamate					Pyruvate			
	<i>Eq. A10</i>	<i>Eq. A18</i>	<i>Eq. A19</i>	g1	g2	g3	g4	g5	p1	p2	p3	
Administered substrate: $[2-^{13}\text{C}]$ acetate												
$y=0.1$												
0.90		NF										
0.50	1.0000	0.5000	0.3192	0.4184	0.8636	0.8636	0.9499	0.0380	0.2102	0.4318	0.4318	
0.10	1.0000	0.9000	0.8593	0.3585	0.7786	0.7786	0.9188	0.006860	0.03588	0.07786	0.07786	
$y=1$												
0.90	1.0000	0.1000	0.4775	0.2754	0.8137	0.8137	0.9764	0.06452	0.2679	0.7323	0.7323	
0.50	1.0000	0.5000	0.7074	0.1365	0.4662	0.4662	0.9324	0.02054	0.07123	0.2331	0.2331	
0.10	1.0000	0.9000	0.9409	0.08416	0.3267	0.3267	0.9148	0.002879	0.0084871	0.03267	0.03267	
$y=10$												
0.90	1.0000	0.1000	0.5717	0.02324	0.3122	0.3122	0.9367	0.02475	0.02476	0.28100	0.28100	
0.50	1.0000	0.5000	0.8039	0.0046	0.0832	0.0832	0.9156	0.00366	0.003021	0.0416	0.0416	
0.10	1.0000	0.9000	0.9656	0.00225	0.04801	0.04801	0.9123	0.00042	0.000237	0.00480	0.00480	
Administered substrate: $[3-^{13}\text{C}]$ pyruvate												
$y=0.1$												
0.90		NF										
0.50		NF										
0.09	0.9996	-0.05846	0.3210	0.2255	0.4737	0.4655	0.4681	0.02059	0.02031	0.04190	0.9526	
$y=1$												
0.90		NF										
0.50	1.0000	-0.09633	0.3146	0.1499	0.4375	0.3940	0.3532	0.09680	0.07818	0.1970	0.7187	
0.10	1.0000	-0.04411	0.6020	0.1364	0.5127	0.4371	0.4675	0.02148	0.01376	0.04371	0.9513	
$y=10$												
0.90	1.0000	-0.0223	0.3331	0.04150	0.4428	0.3594	0.2450	0.1590	0.06536	0.3235	0.4985	
0.50	1.0000	-0.01831	0.3250	0.0377	0.6331	0.3206	0.4012	0.0787	0.02475	0.1602	0.8165	
0.10	1.0000	-0.01648	0.2885	0.0337	0.7120	0.2620	0.4772	0.0128	0.00354	0.0262	0.9712	

Rates of flux (see Fig. 2), $v_{\text{TCA}} = 8.28$; $v_{\text{TA}} = 22.87$; acetyl-CoA (AcCoA) influx = 8.28; $r = 82.8$ (units are $\mu\text{mol} \cdot \text{g dry wt}^{-1} \cdot \text{min}^{-1}$). Efflux from pool of AcCoA as well as flux catalyzed by pyruvate dehydrogenase complex are 0.8 and $8.0 \mu\text{mol} \cdot \text{g dry wt}^{-1} \cdot \text{min}^{-1}$ for perfusions with $[2-^{13}\text{C}]$ acetate and $[3-^{13}\text{C}]$ pyruvate, respectively. Rate of anaplerosis (v_{ANA}) equals $y \cdot v_{\text{TCA}}$ for a given value of y . Results shown were obtained from solution of differential equations (see APPENDIX A) by use of MLAB. Assumptions include no recycling of isotopically labeled CO_2 and zero natural abundance of ^{13}C . Simulated elapsed time was 80 min. Cases "not feasible" are impossible at current rates of flux catalyzed by α -ketoglutarate dehydrogenase complex and by pyruvate dehydrogenase complex (see APPENDIX A). To have a feasible simulation, the value of R was 0.09 for $y = 0.1$ with $[3-^{13}\text{C}]$ pyruvate. Simulated influx of $[2-^{13}\text{C}]$ acetate and of $[3-^{13}\text{C}]$ pyruvate is 100% enriched. NF, not feasible.

2). Computer simulations in which exogenous pyruvate was 50% unlabeled and 50% $[3-^{13}\text{C}]$ pyruvate gave exactly the same estimates of y by use of *Eq. A19*, but the fractional enrichment of each carbon of glutamate and pyruvate was reduced by 50% (data not shown). During administration of $[3-^{13}\text{C}]$ pyruvate, increasing the enrichment of acetyl-CoA by decreasing its turnover rate likewise resulted in zero error in estimation of y by use of *Eq. A10* (Table 4). The relative error in the estimate of y obtained from *Eq. A10* is <0.0005 under all conditions tested.

Equation A10 may be inaccurate when applied to the output of a simulation of a syntactic model (Table 3). If y equals ~ 0.1 and $[2-^{13}\text{C}]$ acetate is administered, *Eq. A10* overestimates the true value by 17% for $R = 0.5$ and underestimates the true value by 3% for $R = 0.1$ (Table 3). If y equals 0.1 and $[3-^{13}\text{C}]$ pyruvate is administered, then *Eq. A10* estimates y as 0.21 (overestimation by 114%) when $R = 0.1$. When the value of relative anaplerosis is ~ 1.0 , the estimate is within 10% of the correct value for all values of R and with application of either $[2-^{13}\text{C}]$ acetate or $[3-^{13}\text{C}]$ pyruvate (Table 3). If the relative anaplerosis is increased to ~ 10.0 , the magnitude of the relative error is $<10\%$ during administration of $[2-^{13}\text{C}]$ acetate but increases to 25% (40%) during

administration of $[3-^{13}\text{C}]$ pyruvate with R equal to 52% (90%), respectively. Nevertheless, with a single exception ($y = 0.1$, $R = 0.1$, $[3-^{13}\text{C}]$ pyruvate), *Eq. A10* exhibits better accuracy than *Eqs. A19* or *A18* for all cases simulated with the syntactic model (Table 3).

The source of inaccuracy of *Eq. A10* is as follows. As the denominator in this formula for y approaches zero (see fractional enrichments in Table 2), experimental error and noise decrease the precision of the estimate. The simulation of the syntactic model adds noise to the system both by reason of the stochasticity of the algorithm and because of the finite size of the simulated metabolic pools (5, 6). For larger R (less dilution by exogenous pyruvate), the difference in enrichment of C-2 (C-3) of pyruvate and C-3 (C-2) of glutamate approaches zero, leading to a numerically unstable calculation (division by a very small number).

Accuracy of *Eqs. A22, A25, and A26*

Malloy et al. (16, 17) derived formulas to estimate relative anaplerosis from the multiplets of the ^{13}C NMR spectrum of glutamate. We selected one of their formulas (*Eq. A22*) for the triplet resonance (C3T) of C-3 of glutamate and compared its accuracy with the classical

Table 3. Estimation of y by use of syntactic simulation (5) to obtain steady-state fractional enrichments of carbons and of positional isotopomers

R	p23	C3T	Actual y	Estimate of y /Actual Value of y					
				Eq. A10	Eq. A18	Eq. A19	Eq. A22	Eq. A25	Eq. A26
Administered substrate: $[2-^{13}\text{C}]$ acetate									
$y \sim 0.1$									
0.90	NF								
0.49	0.3985	0.8586	0.0882	1.1773	0.5795	0.3864	0.5924	1.2105	1.1515
0.10	0.0657	0.7728	0.1028	0.9725	0.8803	0.7801	0.8787	0.9769	0.9746
$y \sim 1$									
0.904	0.6420	0.8675	1.0024	1.0734	0.0976	0.4883	0.0993	0.8651	1.0354
0.48	0.1190	0.5793	0.8880	0.9432	0.5574	0.7973	0.5655	0.9559	1.0978
0.10	0.0122	0.4445	1.0785	0.9039	0.8337	0.8793	0.8125	0.8798	0.9055
$y \sim 10$									
0.90	0.1390	0.4400	9.0515	0.9299	0.1098	0.6299	0.1102	2.3526	1.0689
0.51	0.0041	0.1444	10.6477	1.0374	0.4755	0.7530	0.4510	0.6554	0.9143
0.11	0.0000	0.0791	10.6858	0.9411	0.8584	1.1780	0.8882	0.8882	0.9939
Administered substrate: $[3-^{13}\text{C}]$ pyruvate									
$y \sim 0.1$									
0.90	NF								
0.50	NF								
0.09	0.0150	0.2023	0.0984	2.1444	-0.1289	0.2992	0.6444	0.6960	0.7066
$y \sim 1$									
0.90	NF								
0.50	0.0468	0.0846	1.0154	0.9184	-0.0946	0.3260	0.4617	0.9142	0.9188
0.10	0.0123	0.1144	0.9611	1.0258	-0.0486	0.6337	0.9358	1.0573	1.0385
$y \sim 10$									
0.90	0.0337	0.0303	10.6540	0.6041	-0.0210	0.3218	0.0928	0.3882	0.9089
0.52	0.0095	0.0277	10.3623	1.2589	-0.0176	0.3301	0.4704	0.8246	0.9750
0.11	0.0000	0.0238	10.7875	0.9886	-0.0152	0.2604	0.7978	0.7978	0.8934

Simulated conditions were approximately the same as in Table 2. Values of R and y differ between Tables 2 and 3, because of stochastic nature of algorithm for simulation of chemical kinetics and finite pool sizes in syntactic simulation (5, 6). For each simulation, rescaling factor equals 4×10^{-4} and total elapsed time = 80 min. p23, Fraction of molecules of pyruvate that are isotopically labeled at both C-2 and C-3; C3T, fraction of ^{13}C NMR resonance of glutamate C-3 that is a triplet (see Eq. A23).

formulas and with our formulas for anaplerosis. To evaluate the accuracy of formulas that rely on C3T, we simulated each of the conditions in Table 2, using a syntactic model (5, 6) of the CAC and anaplerosis (see Table 3). During administration of either $[2-^{13}\text{C}]$ acetate or $[3-^{13}\text{C}]$ pyruvate, Eq. A22 underestimated the value of relative anaplerosis, with the magnitude of the error increasing with increasing recycling (R) of pyruvate. In

fact, computer simulations as well as mathematical analysis (Table 3, Eq. A26) confirm that the value estimated by Eq. A22 equals $(1 - R) \cdot y$ instead of y .

Equation A25 was consistently more accurate than Eq. A22 yet showed considerable error when $y = 0.1$ and $y = 10$ (Table 3). We traced the source of the error to the numerical instability of the formula, in which the denominator became almost zero. This can be seen by

Table 4. Estimates of y and values of fractional enrichments of carbons C- i of glutamate and pyruvate for different values of y and R

R	Estimate of y / Actual Value of y			Fractional Enrichments								
				Glutamate					Pyruvate			
	Eq. A10	Eq. A18	Eq. A19	g1	g2	g3	g4	g5	p1	p2	p3	
Administered substrate: $[3-^{13}\text{C}]$ pyruvate												
$y = 0.1$												
0.90	NF											
0.50	NF											
0.09	1.0000	0.3927	-0.003235	0.4394	0.8867	0.8785	0.9563	0.07640	0.03958	0.07907	0.9898	
$y = 1$												
0.90	NF											
0.50	1.0000	0.1062	0.1476	0.2697	0.6624	0.6189	0.8031	0.2990	0.1407	0.3095	0.8312	
0.10	1.0000	0.1879	0.6025	0.1885	0.6798	0.6041	0.9352	0.05837	0.01901	0.06041	0.9680	
$y = 10$												
0.90	1.0004	0.002201	0.2542	0.06641	0.5539	0.4705	0.5782	0.4091	0.1046	0.4235	0.5985	
0.50	1.0000	0.01021	0.3003	0.04464	0.6699	0.3574	0.8067	0.1727	0.02930	0.1787	0.8350	
0.10	1.0000	0.01388	0.3029	0.03554	0.7364	0.2864	0.9407	0.02767	0.003731	0.02864	0.9736	

Conditions are the same as in Table 2, except that influx (efflux) of acetyl-CoA equals 0.28 (or zero, respectively) $\mu\text{mol} \cdot \text{g dry wt}^{-1} \cdot \text{min}^{-1}$. Isotopic enrichment of acetyl-CoA is higher than in Table 2 because of less dilution by unlabeled molecules of acetyl-CoA.

observing that the formula for y given by Eq. A26 is mathematically equivalent to Eq. A25 yet estimates y more accurately for all simulations (Table 3). To apply Eq. A26, the recycling parameter R is needed; we obtained R from the simulation by examining the rate of the influx into pyruvate from exogenous sources and the rate of anaplerosis. We infer that if a precise measurement of R could be made, then Eq. A25 could be replaced by an equation (i.e., Eq. A26) that is mathematically equivalent but more precise. On the other hand, computer simulations indicate that if relative anaplerosis (y) were to be estimated independently of R , then Eq. A26 would not be useful in estimating R because of the extreme sensitivity of the estimate to small errors in the measurement of g_4 and/or C3T (data not shown). Thus it is unlikely that Eq. A26 could be used to estimate R , given the limitations of precision of physical measurements.

Effects of Malate-Aspartate Shuttle on Estimation of Relative Anaplerosis

In a model of the metabolic fluxes across the mitochondrial membrane (at steady state),⁶ it is possible to derive the following equation for the ratio of the fractional enrichment of carbon C- i in cytosolic malate (x_3) to the fractional enrichment of C- i in mitochondrial malate (x_4) under conditions of continuous administration of isotopically labeled acetate and pyruvate unlabeled at C- i ($p_0 = 0$; see Fig. 5): $x_3/x_4 = [v_2 \cdot (v_0 + v_3)] / (v_0 \cdot v_2 + v_0 \cdot v_3 + v_2 \cdot v_3)$. If we allow administration of pyruvate isotopically labeled at C- i ($p_0 > 0$ in Fig. 5) as well as arbitrarily labeled acetate, we can derive the following formula: $x_3 - x_4 = (p_0 - x_1) \cdot (v_0/v_2)$, where x_1 is the fractional enrichment of C- i of cytosolic pyruvate (Fig. 5). From these formulas⁷ it is seen that, during administration of pyruvate unlabeled at C- i , cytosolic and mitochondrial malate will have approximately equal fractional enrichments at steady state (i.e., $x_3 \cong x_4$) if and only if either pyruvate influx into the cytosolic pool (v_0) or the rate of the flux catalyzed by cytosolic malic enzyme (v_3) is much slower than the rate of the malate-aspartate shuttle (v_2) (see Fig. 5): $v_0 \ll v_2$ or $v_3 \ll v_2$. Furthermore, even if pyruvate that is administered is isotopically labeled at C- i , sufficiently large flux through the malate-aspartate shuttle (v_2) compared with influx of pyruvate into the cytosolic pool (v_0)

⁶ Equations describing isotopic steady state follow. $x_1 \cdot (v_0 + v_4) = x_3 \cdot v_4 + p_0 \cdot v_0$; $x_3 \cdot (v_2 + v_4 + v_8) = x_5 \cdot (v_2 + v_8) + x_1 \cdot v_3$; $x_5 \cdot (v_2 + v_8) = x_3 \cdot v_8 + x_6 \cdot v_2$; $x_6 \cdot (v_2 + v_9) = x_4 \cdot (v_2 + v_9)$. From the last three equations, the following is derived: $x_3 \cdot (v_2 + v_4) = x_1 \cdot v_4 + x_4 \cdot v_2$. From the last equation and the first equation, the formula for x_3/x_4 is derived (assuming $p_0 = 0$), and (also) the formula for $x_3 - x_4$ (not assuming $p_0 = 0$).

⁷ Note that we assume equal exchanges of glutamate/aspartate compared with α -ketoglutarate/malate in heart (see Fig. 5), which has not been demonstrated in vitro or in vivo (11). The α -ketoglutarate/malate exchange is rapidly reversible, but the glutamate/aspartate exchange is not. Hence it is possible (in principle) that, at metabolic steady state, the stoichiometries of the two exchangers will be the same, but one must await experimental data. The equation relating the fractional enrichment of malate in the cytosol (x_3) with the enrichment in the mitochondrion (x_4) applies to our simplified model (Fig. 5) and may need to be modified as data are acquired.

will ensure that $x_3 \cong x_4$ (i.e., cytosolic and mitochondrial malate have the same fractional enrichment).

DISCUSSION

Our theoretical investigations show that current formulas using ratios of isotopic enrichments of carbons of glutamate or of isotopomers of glutamate (16, 17) will generally underestimate relative anaplerosis (y) in heart.⁸ These formulas derive from the pioneering work done by Weinman et al. (30) on the metabolism of [¹⁴C]acetate in hepatocytes. They assumed that anaplerotic flux was completely unlabeled and that differences in enrichment of mitochondrial and cytosolic metabolic pools were inconsequential. We reasoned that if the metabolic source of anaplerosis were isotopically labeled, these formulas would no longer give the correct value of relative anaplerosis (in heart). Indeed, our investigations support this conjecture. We further observed that it might be possible for the chemical reactants of anaplerosis (pyruvate in our examples) to become isotopically labeled by the actions of the CAC. If this were true, then an endogenous source of labeling of anaplerosis would amplify the problems caused by exogenous sources (e.g., [3-¹³C]pyruvate or [1-¹³C]glucose) and might even reduce the accuracy of formulas for anaplerosis during administration of compounds that do not directly label the source of anaplerosis (e.g., [2-¹³C]acetate).

We demonstrated that the isotopic enrichment of pyruvate must be included in a general formula that accurately estimates relative anaplerosis from pyruvate, even with the assumption that cytosolic/mitochondrial compartmentation does not affect the estimate. By mathematical analysis and computer simulation, we showed that if [3-¹³C]pyruvate (or equivalently, [1-¹³C]glucose) or [2-¹³C]acetate is administered, then conventional formulas (16, 17) using the C1/C3, C2/C4 or isotopomer ratios of glutamate (Eqs. A18, A19, and A22) underestimate relative anaplerosis (Table 1). We propose a formula (Eq. A10) that is accurate during administration of [2-¹³C]acetate, [3-¹³C]pyruvate, and/or [1-¹³C]glucose, provided it is possible to measure the fractional enrichment of carbons C-2 and C-3 of pyruvate that is substrate for anaplerotic reactions. We recognize the difficulty in making this measurement, but the formula is extremely important in defining the relevant variables that must be considered in estimating the relative rate of anaplerosis. Our study demon-

⁸ For brevity, we have restricted our investigations to closed form (algebraic) formulas for relative anaplerosis from pyruvate in heart. Each "conventional" formula in Table 1, i.e., Eqs. A18, A19, and A22, is used in the interpretation of ¹³C NMR spectroscopic data in the peer-reviewed literature. We exclude the excellent work done on the CAC in liver, including analysis of labeling patterns of metabolic intermediates and of ¹³C mass isotopomers. Isotopomer analysis has recently been applied to oxidation (via PDH complex) of pyruvate that is exported from the CAC when anaplerosis occurs solely at succinyl-CoA (during metabolism of propionate) (9). This approach, in which a numerical solution for y is obtained (without an explicit algebraic formula), may prove most practical when applied to the problems described in this article. The existence of multiple pools of pyruvate remains a formidable problem that will need to be addressed by this advance in isotopomer analysis.

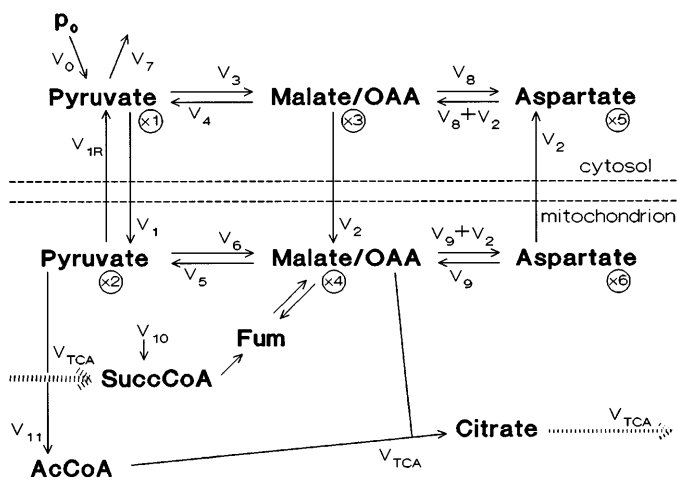


Fig. 5. Diagram of cytosolic vs. mitochondrial compartmentation of metabolic pools germane to our study. Metabolic fluxes shown are influx into cytosolic pyruvate pool that is substrate for anaplerosis (v_0); transport of pyruvate across the mitochondrial membranes (v_1 , v_{1R}); malate-aspartate shuttle (malate- α -ketoglutarate exchange, aspartate-glutamate electrogenic exchange, v_2); cytosolic malic enzyme (v_3 , v_4); mitochondrial malic enzyme (v_5); mitochondrial pyruvate carboxylase (v_6); efflux of pyruvate from the cytosolic pool that is substrate for anaplerosis (v_7); cytosolic aspartate aminotransferase (v_8); mitochondrial aspartate aminotransferase (v_9); propionyl-CoA carboxylase, methylmalonyl-CoA racemase, methylmalonyl-CoA isomerase (v_{10}); pyruvate dehydrogenase complex (v_{11}); and the citric acid cycle (v_{TCA}). Flux v_{10} represents metabolism of propionate (from fatty acids) and of methionine, isoleucine, and valine (18). For a given carbon position, fractional enrichment of molecules produced by flux v_0 equals p_0 . In deriving equations for the fractional enrichment of carbons of chemical intermediates at metabolic and isotopic steady state, it was assumed that $v_{1R} \cong 0$, $v_3 \cong v_4$, $v_5 = v_6 + v_{10}$, and $v_8 \cong v_9$. Not shown is one-half of the malate-aspartate shuttle, flux of α -ketoglutarate from mitochondrion into cytosol (coupled to malate transport), and flux of glutamate from cytosol into mitochondrion (coupled to aspartate transport). The cytosolic form of NADP^+ -dependent malic enzyme in rat or rabbit heart catalyzes both anaplerotic and cataplerotic reactions, whereas the mitochondrial form of enzyme catalyzes cataplerosis almost exclusively (27). In addition, there is substantial activity (in vitro) of pyruvate carboxylase in mitochondria isolated from rat heart (28), which may be important under specific conditions, such as increases in concentration of acetyl-CoA. Parametrization of metabolic diagram represents a simple case in which stoichiometry of exchange of α -ketoglutarate and malate matches exchange of glutamate and aspartate [as reported in perfused rat heart during perfusion with glucose and insulin at metabolic steady state (25)].

strates that enrichment of the pool of pyruvate that is substrate for anaplerosis will severely decrease the accuracy in estimates of relative anaplerosis by use of conventional methods (16, 17), no matter how small the mass of the pool of pyruvate.

Isotopomer distributions are more difficult to analyze than ratios of fractional enrichment of individual carbons. The assumptions underlying contemporary isotopomer analysis in heart (16) are 1) instant equilibration of OAA with fumarate and 2) no reincorporation of ^{13}C CO₂ by anaplerotic reactions. An additional assumption is not inherent in the equations (16) but is a simplification adopted in all analyses (16, 17), namely, 3) if molecules of exogenous pyruvate are not doubly labeled at C-2 and C-3, then anaplerosis from pyruvate does not contain molecules that are doubly labeled at

both carbons C-2 and C-3. However, our simulations (see Table 3) and mathematical analysis (see Eq. A28) demonstrate that molecules of OAA doubly labeled at C-2 and C-3 are produced by the actions of the CAC during administration of $[2\text{-}^{13}\text{C}]$ acetate or $[3\text{-}^{13}\text{C}]$ pyruvate. From published steady-state data (16) and our Eq. A29, we calculate that 65.5% (46.3%) of molecules of OAA in the perfused rat heart are doubly labeled at C-2 and C-3 during administration of $[2\text{-}^{13}\text{C}]$ acetate (or $[3\text{-}^{13}\text{C}]$ pyruvate, respectively). Therefore, one cannot ignore anaplerosis of doubly labeled molecules possessing ^{13}C at both positions C-2 and C-3, unless one assumes that recycling of carbons of pyruvate does not occur (i.e., one assumes that $R = 0$). In fact, we derive a formula (Eq. A31) for the fraction of molecules of pyruvate doubly labeled at C-2 and C-3 at steady state (specifically referring to the pool of pyruvate that is substrate for anaplerosis). We show that reincorporation of labeled carbon fragments into the CAC (cataplerosis followed by anaplerosis) can have a significant impact on the accuracy of the formulas used to estimate relative anaplerosis.

The accuracy of Eq. A10 (Table 1) for estimating relative anaplerosis is not affected by recycling of carbons between pyruvate and pools of the CAC. The assumptions underlying Eq. A10 follow. Reactions of the CAC and the transfer of carbons among metabolic pools are given in Figs. 1–3. Mitochondrial/cytosolic compartmentation of metabolic pools may be ignored, i.e., corresponding pools in the mitochondria and in the cytosol are assumed to have the same fractional enrichments. We restrict our consideration to anaplerotic reactions that solely use pyruvate as substrate. This excludes oxidation of fatty acids having an odd number of carbons, because the terminal 3-carbon fragment of β -oxidation is metabolized to succinyl-CoA (18). However, metabolism of fatty acids such as palmitate that contain an even number of carbons is allowed. In the current analysis, we assume that there is no influx into pools of the CAC other than that shown in Fig. 1. For example, we do not consider the exchange of molecules of the pool of glutamate with plasma glutamate. Further work will extend our analysis to the more general case.

Two factors complicate measurement of the rate of anaplerosis in heart. First, it has been observed (13) that there may exist a pool of glutamate in heart that is metabolically inactive (i.e., whose molecules do not exchange carbons with α -ketoglutarate that is a constituent of the CAC). We suggest two methods for estimating the true fractional enrichment of carbons of glutamate (i.e., excluding the metabolically inactive pool), either use of isotopomer analysis of glutamate or use of the fractional enrichment of C-2 of mitochondrial acetyl-CoA and C-4 of glutamate (see APPENDIX B). This is admittedly a difficult problem for which we do not have a complete solution at the present time. Nonetheless, it is still meaningful to discuss theoretical results in terms of the fractional enrichments of the carbons of

glutamate or of the positional isotopomers of glutamate. Such discussion can easily be translated into the case in which the true enrichment of glutamate can be estimated only imprecisely. The second difficulty in modeling the CAC is the presence of multiple pools of pyruvate in the cytosol (13, 22). However, there is experimental evidence that cytosolic alanine has the same fractional enrichment as the cytosolic pool of pyruvate that feeds the mitochondrial pyruvate (12, 13, 22, 26), which may be useful in formulas for y that require knowledge of the fractional enrichment of this pool of cytosolic pyruvate.

It is of interest to apply *Eq. A10* to published data on the isolated mammalian heart perfused with $[3-^{13}\text{C}]$ pyruvate and to recalculate the value of y . In the isolated rabbit heart perfused with 2.5 mM $[3-^{13}\text{C}]$ pyruvate and no glucose (13), our formula yields 18% as the estimate of y (after correction for a pool of metabolically inactive glutamate) compared with the value of 10% obtained with *Eq. A18*. In the isolated rat heart perfused with 1 mM $[3-^{13}\text{C}]$ pyruvate and 5 mM glucose (3), the value of y obtained with our formula is 14% compared with the value obtained with the classical formula (*Eq. A18*) of 4% (correction for metabolically inactive glutamate pool could not be made). As predicted from simulations (Table 2), the classical formula (*Eq. A18*) that uses the ratio of enrichments of C-2 to C-4 of glutamate underestimates the true value of relative anaplerosis by $\sim 50\%$. In our calculations using *Eq. A10*, we assumed that alanine and pyruvate that is substrate for anaplerosis are in equilibrium and that C-2 of these compounds is unenriched (12, 13, 22). Therefore, the values obtained with *Eq. A10* in these examples are lower bounds, which would be increased if the fractional enrichment of C-2 of pyruvate were greater than zero. As we have mentioned, we do not make any assumptions concerning the fractional enrichment of carbons of lactate.

The malate-aspartate shuttle may affect estimates of relative anaplerosis, even at isotopic steady state. Several investigators have provided evidence that, in the perfused rat heart, the rate of the malate-aspartate shuttle is fast enough that analysis of the dynamics of enrichment of glutamate could be used to estimate the absolute rate of the CAC as well as the rate of anaplerosis, without regard for compartmentation (3, 4, 32). Other investigators have shown that the malate-aspartate shuttle is rate limiting for transport of α -ketoglutarate and glutamate between the mitochondrion and cytosol, thereby affecting the dynamics of labeling of glutamate (4, 34). However, for the purposes of this paper, only the steady-state behavior is relevant; we did not consider the dynamics of isotopic labeling. With the assumption that the source of cytosolic glutamate is either export from mitochondria or transamination with cytosolic α -ketoglutarate, the fractional enrichment of glutamate would be identical in mitochondria and cytosol at steady state, and equal to the fractional enrichment of (mitochondrial) α -ketoglutarate. Indeed, Lewandowski et al. (14), using NMR spectroscopy, found that in extracts from perfused rabbit hearts the fractional

enrichment of C-4 of α -ketoglutarate and that of glutamate were equal.

We investigated the effect of the rate of the malate-aspartate shuttle on formulas for estimation of relative anaplerosis which assume that differences in fractional enrichment between cytoplasmic and mitochondrial metabolic pools of the CAC are negligible. Analysis of a model of the metabolic fluxes shows that if the mass flux of the malate-aspartate shuttle is much greater than the rate of influx of cytosolic pyruvate ($v_2 \gg v_0$ in Fig. 5), then the difference in enrichment of cytosolic and mitochondrial pools of malate is negligible and the formulas may be applied. Alternatively, if the influx of pyruvate is unlabeled (and the influx of acetate is isotopically labeled), then it suffices that the mass flux of the malate-aspartate shuttle greatly exceed the mass flux catalyzed by cytosolic malic enzyme ($v_2 \gg v_3$ in Fig. 5). Thus we have derived sufficient conditions for the application of models of the CAC that are used to derive algebraic formulas for estimation of relative anaplerosis.

Recommendations

In acid extracts obtained from glucose-perfused or acetate-perfused isolated mammalian hearts, the lack of NMR resonances for pyruvate can be explained by the low concentration of pyruvate under these conditions (23). We suggest that the enrichment of pyruvate is important, although perhaps difficult to measure. Instead, either conditions that enlarge the pool of pyruvate ought to be selected (so that an assessment of the fractional enrichment of pyruvate can be made),⁹ or an indirect estimation of the fractional enrichments of carbons of pyruvate ought to be made, i.e., fractional enrichment of carbons of alanine (12, 13, 22, 26). Use of acetate should be discouraged under conditions that cause the concentrations of pyruvate and alanine to become too small to be observed with NMR spectroscopy. Administration of $[3-^{13}\text{C}]$ lactate, $[3-^{13}\text{C}]$ pyruvate, or $[1-^{13}\text{C}]$ glucose may allow for estimation of the fractional enrichment of carbons of pyruvate from the NMR spectrum of alanine (13, 31). Together with the fractional enrichment of carbons of glutamate, this may enable relative anaplerosis (y) to be estimated with greater accuracy (*Eq. A10*).

APPENDIX A. MATHEMATICAL EQUATIONS AND DERIVATIONS

Formulas for Estimating Rates of Flux at Metabolic and Isotopic Steady State

Our mathematical analysis presumes the metabolic pathways shown in Figs. 1 and 2 and the details concerning transfer of carbons within the relevant pathways shown in Fig. 3. One can show (see Fig. 3) that at isotopic steady state

⁹ In rat hearts perfused with glucose and insulin, the addition of acetate caused the ratio of moles of alanine to moles of glutamate plus glutamine to be 3.5%, whereas the addition of pyruvate caused the ratio to be 34% (8).

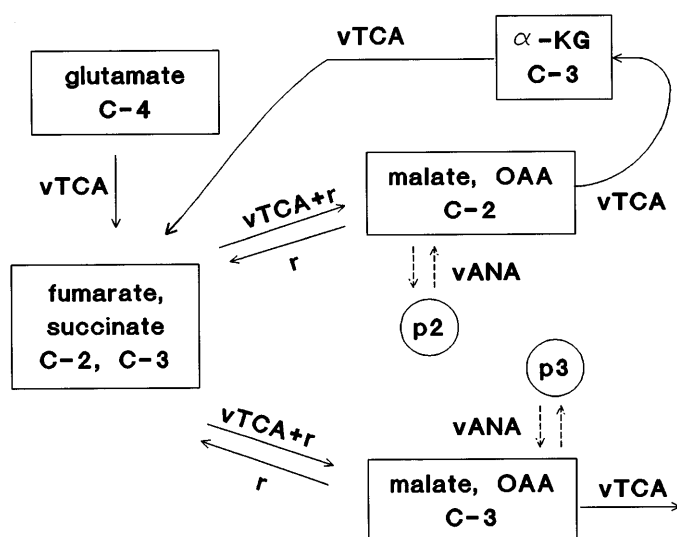


Fig. 6. Model of atomic (carbon) flow between selected pools of citric acid cycle that is useful for illustrating derivation of Eqs. A7-A9. As in Fig. 1, succinate and fumarate are combined into a single compartment, as are malate and OAA. Each box represents the set of carbons at a given position (e.g., C-4 glutamate) or positions (e.g., C-2 and C-3, succinate and fumarate). v_{TCA} , Rate of flux catalyzed by α -ketoglutarate dehydrogenase complex; r , rate of flux from OAA to fumarate, catalyzed by malate dehydrogenase and by fumarase; v_{ANA} , rate of anaplerosis (equal to rate of cataplerosis).

the following equations hold

$$\begin{aligned} o1 = c6, o2 = g3, o3 = g2, o4 = g1, a1 = g5, \\ \text{and } a2 = g4 \end{aligned} \quad (\text{A1-A6})$$

where o_i is the fractional enrichment of C- i of OAA; g_i is the fractional enrichment of C- i of glutamate; a_i is the fractional enrichment of C- i of acetyl-CoA (the carbonyl carbon is C-1); and $c6$ is the fractional enrichment of the carboxyl carbon of citrate that is covalently bonded to the carbon to which is attached to the -OH group (cit6 in Fig. 3; same as the carbon position of citrate that receives C-1 of OAA in Fig. 1). All fractional enrichments pertain to pools of metabolites that are metabolically active (see APPENDIX B).

The equations describing the fractional enrichments of carbons of the pools of the CAC are derived from examination of Fig. 6

$$s2 \cdot 2 \cdot (v_{\text{TCA}} + r) = (g4 + o2) \cdot v_{\text{TCA}} + (o2 + o3) \cdot r \quad (\text{A7})$$

$$o2 \cdot (v_{\text{ANA}} + v_{\text{TCA}} + r) = p2 \cdot v_{\text{ANA}} + s2 \cdot (v_{\text{TCA}} + r) \quad (\text{A8})$$

$$o3 \cdot (v_{\text{ANA}} + v_{\text{TCA}} + r) = p3 \cdot v_{\text{ANA}} + s2 \cdot (v_{\text{TCA}} + r) \quad (\text{A9})$$

where $s2$ equals the fractional enrichment of carbons C-2 and C-3 of succinate, and p_i equals the fractional enrichment of C- i of the pool of pyruvate that is substrate for anaplerotic enzymes. Letting y equal the ratio of v_{ANA} to v_{TCA} in Fig. 2, one can derive the following from Eqs. A1-A9

$$y = (g4 - g2)/(g2 + g3 - p2 - p3) \quad (\text{A10})$$

provided $g2 + g3 - p2 - p3 \neq 0$, and

$$g2 - g3 = (p3 - p2) \cdot v_{\text{ANA}} / (v_{\text{TCA}} + r + v_{\text{ANA}}) \quad (\text{A11})$$

where r is the rate of the reverse flux catalyzed by fumarase (from malate to fumarate).

Examination of carbons C-1 and C-4 of succinate and OAA in Fig. 3 and of the description of flux rates in Fig. 6 allows for the derivation of the following equations

$$s1 \cdot 2 \cdot (v_{\text{TCA}} + r) = (g5 + o3) \cdot v_{\text{TCA}} + (o1 + o4) \cdot r \quad (\text{A12})$$

$$o1 \cdot (v_{\text{ANA}} + v_{\text{TCA}} + r) = p1 \cdot v_{\text{ANA}} + s1 \cdot (v_{\text{TCA}} + r) \quad (\text{A13})$$

$$o4 \cdot (v_{\text{ANA}} + v_{\text{TCA}} + r) = p4 \cdot v_{\text{ANA}} + s1 \cdot (v_{\text{TCA}} + r) \quad (\text{A14})$$

where $s1$ equals the fractional enrichment of the carbons C-1 and C-4 of succinate, and $p4$ equals the fractional enrichment of CO_2 that is substrate for anaplerotic reactions. From Eqs. A12-A14 and A1-A6, one can derive the following formula involving $p4$ (provided that $y \neq 0$)

$$p4 = [(y + 1) \cdot (g1 + c6) - p1 \cdot y - g2 - g5] / y \quad (\text{A15})$$

Formula for Recycling Parameter R

We assume metabolic steady state, so that pool sizes are unchanging and hence anaplerosis and cataplerosis are equal ($v_{\text{TCA}} = v_{\text{CATA}}$). If Q_i equals the fractional enrichment of carbon C- i of the pyruvate influx (Fig. 2), then at isotopic steady state, for $i = 1, 2, \text{ and } 3$,

$$o_i \cdot v_{\text{CATA}} + Q_i \cdot \text{pyrInflux} = p_i \cdot (\text{pyrInflux} + v_{\text{CATA}}) \quad (\text{A16})$$

where o_i and p_i are the fractional enrichments of carbon C- i of OAA and pyruvate, respectively. From Eq. A16 and the definition of R (see Fig. 2), one can derive the following formula for R (provided that $o_i \neq Q_i$)

$$R = (p_i - Q_i) / (o_i - Q_i) \quad (\text{A17})$$

Equation A17 also holds if o_i , p_i , and Q_i denote fractional enrichments of certain positional isotopomers of OAA, intracellular pyruvate, and exogenous pyruvate, e.g., $[2,3-^{13}\text{C}]$ oxaloacetate, $[2,3-^{13}\text{C}]$ pyruvate, and exogenous $[2,3-^{13}\text{C}]$ pyruvate, respectively (see Eq. A30 below).

Classical Formulas That Assume Instant Randomization of OAA

Suppose that molecules of OAA are instantly equilibrated with molecules of fumarate. Then, at isotopic steady state, regardless of the isotopic labeling of exogenous substrate, the following equalities hold: $o1 = o4$ and $o2 = o3$; and hence, by Eqs. A1-A4, $g1 = c6$ and $g2 = g3$. If one assumes that anaplerosis is unlabeled at C-2 and C-3, so that $p2 = 0$ and $p3 = 0$, one can derive the following formula by substituting into Eqs. A10 and A15 and rearranging

$$g2/g4 = 1/(2 \cdot y + 1) \quad (\text{A18})$$

If we assume that anaplerosis is unlabeled at C-1 ($p1 = 0$), that reincorporation of $[^{13}\text{C}]\text{CO}_2$ does not occur ($p4 = 0$), and that acetyl-CoA is not isotopically labeled at C-1 ($g5 = 0$, see Eq. A5), then we can derive the following formula

$$g1/g3 = 1/(2 \cdot y + 2) \quad (\text{A19})$$

Equations A18 and A19 were originally derived for hepatic metabolism (30).

In our model of the CAC (which does not include instant equilibration), we may derive a formula similar to Eq. A18. If we assume that molecules of exogenous pyruvate are not labeled at either C-2 or C-3, then by examination of Fig. 3 we may infer that $g2 = g3$, and from Eqs. A10 and A17 we may

derive the following equation

$$g2/g4 = 1/[2 \cdot y \cdot (1 - R) + 1] \quad (A20)$$

Formula for y With Positional Isotopomer Analysis

The formula for the C-3 triplet is the following (17)

$$C3T = g4 \cdot g4 / (1 + y) \quad (A21)$$

and therefore

$$y = (g4 \cdot g4 / C3T) - 1 \quad (A22)$$

where C3T is the fraction of molecules of glutamate that are (triply) labeled at C-2, C-3, and C-4 divided by the fractional enrichment of glutamate C-3

$$C3T = \frac{([2,3,4-^{13}\text{C}]glutamate) + [1,2,3,4-^{13}\text{C}]glutamate + [1,2,3,4,5-^{13}\text{C}]glutamate + [2,3,4,5-^{13}\text{C}]glutamate)}{[3-^{13}\text{C}]glutamate} \quad (A23)$$

This fraction can be obtained by dividing the area of the triplet at C-3 of glutamate by the area of the entire C-3 resonance of glutamate in the ¹³C NMR spectrum.

Using the same model of the CAC but including the effects of labeled anaplerosis from pyruvate, we have derived the following formula (see Eqs. A28 and A29)

$$C3T = g4 \cdot g4 / (1 + y) + (p23 \cdot g4 \cdot y) / [g3 \cdot (1 + y)] \quad (A24)$$

where p23 is the mole fraction of the pool of pyruvate that is doubly labeled at C-2 and C-3 and is substrate for anaplerotic reactions. Solving for y , one obtains

$$y = g3 \cdot (g4 \cdot g4 - C3T) / (g3 \cdot C3T - g4 \cdot p23) \quad (A25)$$

provided that $(g3 \cdot C3T - g4 \cdot p23) \neq 0$. As we show (see below) for the 1-pool model of pyruvate metabolism (Fig. 2), in the special case that exogenously administered pyruvate is not doubly labeled at both C-2 and C-3 (although endogenously generated [2,3-¹³C]pyruvate and [1,2,3-¹³C]pyruvate are allowed), our formula simplifies to the following

$$y = [(g4 \cdot g4 / C3T) - 1] / (1 - R) \quad (A26)$$

where R is the recycling parameter for pyruvate described above and $(0 \leq R < 1)$. Comparing Eq. A24 with Eq. A21 and Eq. A26 with Eq. A22, we conclude that, in the formulas derived from isotopomer analysis, there are implicit assumptions that p23 = 0 and that $R = 0$.

Generation of Doubly Labeled OAA

We next derive a formula for o23, the fraction of molecules of OAA that are labeled at both C-2 and C-3 at isotopic steady state. These molecules derive from molecules of α -ketoglutarate that are isotopically labeled at C-3 and C-4 or from molecules of pyruvate that are doubly labeled at C-2 and C-3 (see Fig. 3). Assume instant equilibration of OAA with malate, fumarate, and succinate. From the balance of inflow and outflow of molecules of OAA (see Fig. 2), we derive the following equation

$$o23 \cdot (v_{TCA} + v_{ANA}) = p23 \cdot v_{ANA} + a2 \cdot o2 \cdot v_{TCA} \quad (A27)$$

Substituting for a2 and o2 (by Eqs. A2 and A6), the following

formula is obtained

$$o23 = (g3 \cdot g4 + p23 \cdot y) / (1 + y) \quad (A28)$$

where $y = v_{ANA} / v_{TCA}$. Equation A28 demonstrates that $o23 \geq g3 \cdot g4 / (1 + y)$, establishing a lower bound for o23.

Derivation of Eq. A24

By definition of C3T, $g3 \cdot C3T$ equals the fraction of molecules of glutamate that are isotopically labeled at C-2, C-3, and C-4. The atoms in positions C-2, C-3, and C-4 of a molecule of glutamate derive from carbons C-3 and C-2 of OAA and from C-2 of acetyl-CoA, respectively (see Fig. 3). Therefore, recalling that $a2 = g4$ (see Eq. A6), one may write the following equation to describe isotopic steady state

$$g4 \cdot o23 = g3 \cdot C3T \quad (A29)$$

Solving for y in Eqs. A28 and A29, one obtains Eq. A24.

Derivation of Eq. A26

If exogenously labeled pyruvate is not doubly labeled at C-2 and C-3, then by Eq. A17

$$R = p23 / o23 \quad (A30)$$

Eliminating o23 from Eqs. A29 and A30, and solving for p23, one obtains

$$p23 = R \cdot g3 \cdot C3T / g4 \quad (A31)$$

With use of Eq. A31 to substitute for p23 in Eq. A25, one derives Eq. A26.

Analysis of Errors in Formulas for y

We define the relative error of an estimate to be the difference between the estimate and the actual value divided by the actual value, i.e., relative error = (estimated value - actual value) / actual value. The advantage of using this measure of error is that it is independent of the actual value of the estimate, thereby allowing comparison of estimates of y for widely differing values of y . We obtain the following formulas for the relative error (Rel Err) in Eqs. A18 and A19

$$\text{Rel Err (Eq. A18)} = - (p2 + p3) / (g2 + g3) \quad (A32)$$

$$\text{Rel Err (Eq. A19)} = [-p1 - p4 - (g5/y)] / (2 \cdot g1) \quad (A33)$$

Equation A33 assumes that OAA is instantly equilibrated with fumarate, whereas Eq. A32 compares the accuracy of Eq. A18 with a formula that applies to the general case of incomplete equilibration, i.e., $g2$ ($p2$) may be unequal to $g3$ ($p3$). If exogenously administered pyruvate is not labeled at either C-2 or C-3, then pyruvate derives its label solely from cataplerosis, and hence $p2 = p3$ (at isotopic steady state) and $g2 = g3$, by Eq. A11. Therefore, for this special case, and with the assumption of the 1-pool model of pyruvate metabolism (Fig. 2), the relative error in Eq. A18 equals $-p2/g3$, which equals $-R$ (by Eq. A17).

The formula for the relative error in Eq. A22, obtained by comparison of that equation with Eq. A24 (which we take to be the "correct" formula, assuming instant equilibration of OAA and succinate) is $\text{Rel Err (Eq. A22)} = -p23/o23$. If exogenously administered molecules of pyruvate are not doubly labeled at C-2 and C-3 (see discussion of Eq. A17), then the relative error in Eq. A22 equals $-R$.

Differential Equations for Fractional Enrichments of Carbons

We next present the parameters of the ordinary differential equations that were solved using MLAB (2) for Tables 2 and 4 (see Figs. 1–3). The equations were run under conditions of metabolic steady state until isotopic steady state was reached. The rates of influx (efflux) of acetyl-CoA and pyruvate from their respective pools are acInf and pyrInf (acEff, pyrEff), respectively. The rates of CAC, anaplerosis (equal to cataplerosis), and flux catalyzed by aspartate aminotransferase and by the pyruvate dehydrogenase (PDH) complex are denoted by v_{TCA} , v_{ANA} , v_{TA} , and PDH, respectively. The rate of flux from malate to fumarate catalyzed by fumarase is denoted by r . Values for the flux rates (in $\mu\text{mol} \cdot \text{g dry wt}^{-1} \cdot \text{min}^{-1}$) are v_{TCA} (8.28), v_{TA} (22.9), v_{ANA} ($y \cdot v_{\text{TCA}}$, where y is the desired relative anaplerosis), PDH (0.8 or 8.0 for administration of [^{13}C]acetate or [^{13}C]pyruvate, respectively), and acInf (8.28 or 0.28 for Tables 2 and 4, respectively). The remaining parameters are computed by the following equations (see Fig. 2)

$$\text{pyrEff} = (1 - R) \cdot (v_{\text{ANA}}/R) - \text{PDH} \quad (\text{A34})$$

$$r = 10 \cdot v_{\text{TCA}} \quad (\text{A35})$$

$$\text{acEff} = \text{PDH} - v_{\text{TCA}} + \text{acInf} \quad (\text{A36})$$

$$\text{pyrInf} = \text{pyrEff} + \text{PDH} \quad (\text{A37})$$

where $v_{\text{ANA}} = v_{\text{CATA}}$ (with assumption of metabolic steady state), and $R > 0$ (with assumption that $v_{\text{CATA}} > 0$). If $v_{\text{CATA}} = 0$, then the formula for pyrEff (Eq. A34) would not be required (and in this case pyrEff is a free variable). To ensure a feasible simulation, we must have a nonnegative value of the rate (pyrEff) of efflux from the pyruvate pool, implying that the following condition must hold: $(1 - R)/R \geq \text{PDH}/v_{\text{ANA}}$, which is equivalent to $R \leq v_{\text{ANA}}/(\text{PDH} + v_{\text{ANA}})$. This upper bound for the recycling parameter R causes some values of R to determine simulations that are not feasible for given rates of anaplerosis and of flux catalyzed by PDH (see Tables 2–4).

The (constant) parameters representing the concentrations of metabolic pools are M_{cit} (citrate), M_{akg} (α -ketoglutarate), M_{fum} (fumarate and succinate, combined pools), M_{mal} (malate and OAA, combined pools), M_{pyr} (pyruvate), M_{accoa} (acetyl-CoA), M_{asp} (aspartate), and M_{glu} (glutamate). The parameter representing the enrichment of carbon $C-i$ in the influx into the pyruvate pool is pyrC_iIN , e.g., pyrC1IN , and into the pool of acetyl-CoA is acC_iIN (e.g., acC1IN). The parameter representing the fractional enrichment of CO_2 that is substrate for anaplerotic reactions is p_4 (We let $p_4 = 0$ in our simulations). The values for the pool sizes (in $\mu\text{mol/g dry weight}$) are M_{cit} (2.03), M_{akg} (0.16), M_{fum} (0.32), M_{mal} (0.3223), M_{pyr} (0.2), M_{accoa} (0.02), M_{asp} (2.02), and M_{glu} (20.87).

We let the following variables denote the fractional enrichment of carbon $C-i$ of citrate, α -ketoglutarate, fumarate, malate, pyruvate, acetyl-CoA, aspartate, and glutamate, respectively: citC_i , akgC_i , fumC_i , malC_i , pyrC_i , accoaC_i , aspC_i , and gluC_i . In the following equations, the independent variable (*time* t) is shown for the derivatives [e.g., $\text{citC1}'(t)$] but not for the dependent variables (e.g., citC1). The initial value of each of these variables is zero in our simulations. These ordinary differential equations were solved using MLAB (2)

$$\text{citC1}'(t) = (v_{\text{TCA}} \cdot \text{malC4})/M_{\text{cit}} - (v_{\text{TCA}} \cdot \text{citC1})/M_{\text{cit}}$$

$$\text{citC2}'(t) = (v_{\text{TCA}} \cdot \text{malC3})/M_{\text{cit}} - (v_{\text{TCA}} \cdot \text{citC2})/M_{\text{cit}}$$

$$\text{citC3}'(t) = (v_{\text{TCA}} \cdot \text{malC2})/M_{\text{cit}} - (v_{\text{TCA}} \cdot \text{citC3})/M_{\text{cit}}$$

$$\text{citC4}'(t) = (v_{\text{TCA}} \cdot \text{accoaC2})/M_{\text{cit}} - (v_{\text{TCA}} \cdot \text{citC4})/M_{\text{cit}}$$

$$\text{citC5}'(t) = (v_{\text{TCA}} \cdot \text{accoaC1})/M_{\text{cit}} - (v_{\text{TCA}} \cdot \text{citC5})/M_{\text{cit}}$$

$$\text{citC6}'(t) = (v_{\text{TCA}} \cdot \text{malC1})/M_{\text{cit}} - (v_{\text{TCA}} \cdot \text{citC6})/M_{\text{cit}}$$

$$\begin{aligned} \text{akgC1}'(t) &= (v_{\text{TCA}} \cdot \text{citC1} + v_{\text{TA}} \cdot \text{gluC1})/M_{\text{akg}} \\ &\quad - (v_{\text{TCA}} + v_{\text{TA}}) \cdot \text{akgC1}/M_{\text{akg}} \end{aligned}$$

$$\begin{aligned} \text{akgC2}'(t) &= (v_{\text{TCA}} \cdot \text{citC2} + v_{\text{TA}} \cdot \text{gluC2})/M_{\text{akg}} \\ &\quad - (v_{\text{TCA}} + v_{\text{TA}}) \cdot \text{akgC2}/M_{\text{akg}} \end{aligned}$$

$$\begin{aligned} \text{akgC3}'(t) &= (v_{\text{TCA}} \cdot \text{citC3} + v_{\text{TA}} \cdot \text{gluC3})/M_{\text{akg}} \\ &\quad - (v_{\text{TCA}} + v_{\text{TA}}) \cdot \text{akgC3}/M_{\text{akg}} \end{aligned}$$

$$\begin{aligned} \text{akgC4}'(t) &= (v_{\text{TCA}} \cdot \text{citC4} + v_{\text{TA}} \cdot \text{gluC4})/M_{\text{akg}} \\ &\quad - (v_{\text{TCA}} + v_{\text{TA}}) \cdot \text{akgC4}/M_{\text{akg}} \end{aligned}$$

$$\begin{aligned} \text{akgC5}'(t) &= (v_{\text{TCA}} \cdot \text{citC5} + v_{\text{TA}} \cdot \text{gluC5})/M_{\text{akg}} \\ &\quad - (v_{\text{TCA}} + v_{\text{TA}}) \cdot \text{akgC5}/M_{\text{akg}} \end{aligned}$$

$$\text{gluC1}'(t) = (v_{\text{TA}} \cdot \text{akgC1})/M_{\text{glu}} - (v_{\text{TA}} \cdot \text{gluC1})/M_{\text{glu}}$$

$$\text{gluC2}'(t) = (v_{\text{TA}} \cdot \text{akgC2})/M_{\text{glu}} - (v_{\text{TA}} \cdot \text{gluC2})/M_{\text{glu}}$$

$$\text{gluC3}'(t) = (v_{\text{TA}} \cdot \text{akgC3})/M_{\text{glu}} - (v_{\text{TA}} \cdot \text{gluC3})/M_{\text{glu}}$$

$$\text{gluC4}'(t) = (v_{\text{TA}} \cdot \text{akgC4})/M_{\text{glu}} - (v_{\text{TA}} \cdot \text{gluC4})/M_{\text{glu}}$$

$$\text{gluC5}'(t) = (v_{\text{TA}} \cdot \text{akgC5})/M_{\text{glu}} - (v_{\text{TA}} \cdot \text{gluC5})/M_{\text{glu}}$$

$$\begin{aligned} \text{fumC1}'(t) &= (\text{akgC2} + \text{akgC5}) \cdot v_{\text{TCA}}/(2 \cdot M_{\text{fum}}) \\ &\quad + (\text{malC1} + \text{malC4}) \cdot r/(2 \cdot M_{\text{fum}}) - [\text{fumC1} \cdot (v_{\text{TCA}} + r)]/M_{\text{fum}} \end{aligned}$$

$$\begin{aligned} \text{fumC2}'(t) &= (\text{akgC3} + \text{akgC4}) \cdot v_{\text{TCA}}/(2 \cdot M_{\text{fum}}) \\ &\quad + (\text{malC2} + \text{malC3}) \cdot r/(2 \cdot M_{\text{fum}}) - [\text{fumC2} \cdot (v_{\text{TCA}} + r)]/M_{\text{fum}} \end{aligned}$$

$$\begin{aligned} \text{fumC3}'(t) &= (\text{akgC3} + \text{akgC4}) \cdot v_{\text{TCA}}/(2 \cdot M_{\text{fum}}) \\ &\quad + (\text{malC2} + \text{malC3}) \cdot r/(2 \cdot M_{\text{fum}}) - [\text{fumC3} \cdot (v_{\text{TCA}} + r)]/M_{\text{fum}} \end{aligned}$$

$$\begin{aligned} \text{fumC4}'(t) &= (\text{akgC2} + \text{akgC5}) \cdot v_{\text{TCA}}/(2 \cdot M_{\text{fum}}) \\ &\quad + (\text{malC1} + \text{malC4}) \cdot r/(2 \cdot M_{\text{fum}}) - [\text{fumC4} \cdot (v_{\text{TCA}} + r)]/M_{\text{fum}} \end{aligned}$$

$$\begin{aligned} \text{pyrC1}'(t) &= (\text{pyrC1IN} \cdot \text{pyrInf} + v_{\text{ANA}} \cdot \text{malC1})/M_{\text{pyr}} \\ &\quad - (v_{\text{ANA}} + \text{PDH} + \text{pyrEff}) \cdot \text{pyrC1}/M_{\text{pyr}} \end{aligned}$$

$$\begin{aligned} \text{pyrC2}'(t) &= (\text{pyrC2IN} \cdot \text{pyrInf} + v_{\text{ANA}} \cdot \text{malC2})/M_{\text{pyr}} \\ &\quad - (v_{\text{ANA}} + \text{PDH} + \text{pyrEff}) \cdot \text{pyrC2}/M_{\text{pyr}} \end{aligned}$$

$$\begin{aligned} \text{pyrC3}'(t) &= (\text{pyrC3IN} \cdot \text{pyrInf} + v_{\text{ANA}} \cdot \text{malC3})/M_{\text{pyr}} \\ &\quad - (v_{\text{ANA}} + \text{PDH} + \text{pyrEff}) \cdot \text{pyrC3}/M_{\text{pyr}} \end{aligned}$$

$$\begin{aligned} \text{accoaC1}'(t) &= (\text{acC1IN} \cdot \text{acInf} + \text{PDH} \cdot \text{pyrC2} \\ &\quad - (v_{\text{TCA}} + \text{acEff}) \cdot \text{accoaC1})/M_{\text{accoa}} \end{aligned}$$

$$\begin{aligned} \text{accoaC2}'(t) &= (\text{acC2IN} \cdot \text{acInf} + \text{PDH} \cdot \text{pyrC3} \\ &\quad - (v_{\text{TCA}} + \text{acEff}) \cdot \text{accoaC2}) / M_{\text{accoa}} \\ \text{malC1}'(t) &= (v_{\text{ANA}} \cdot \text{pyrC1} + (v_{\text{TCA}} + r) \cdot \text{fumC1} \\ &\quad + v_{\text{TA}} \cdot \text{aspC1}) / M_{\text{mal}} - (v_{\text{ANA}} + v_{\text{TCA}} + v_{\text{TA}} + r) \cdot \text{malC1} / M_{\text{mal}} \\ \text{malC2}'(t) &= (v_{\text{ANA}} \cdot \text{pyrC2} + (v_{\text{TCA}} + r) \cdot \text{fumC2} \\ &\quad + v_{\text{TA}} \cdot \text{aspC2}) / M_{\text{mal}} - (v_{\text{ANA}} + v_{\text{TCA}} + v_{\text{TA}} + r) \cdot \text{malC2} / M_{\text{mal}} \\ \text{malC3}'(t) &= (v_{\text{ANA}} \cdot \text{pyrC3} + (v_{\text{TCA}} + r) \cdot \text{fumC3} \\ &\quad + v_{\text{TA}} \cdot \text{aspC3}) / M_{\text{mal}} - (v_{\text{ANA}} + v_{\text{TCA}} + v_{\text{TA}} + r) \cdot \text{malC3} / M_{\text{mal}} \\ \text{malC4}'(t) &= (v_{\text{ANA}} \cdot \text{p4} + (v_{\text{TCA}} + r) \cdot \text{fumC4} \\ &\quad + v_{\text{TA}} \cdot \text{aspC4}) / M_{\text{mal}} - (v_{\text{ANA}} + v_{\text{TCA}} + v_{\text{TA}} + r) \cdot \text{malC4} / M_{\text{mal}} \\ \text{aspC1}'(t) &= (v_{\text{TA}} \cdot \text{malC1}) / M_{\text{asp}} - (v_{\text{TA}} \cdot \text{aspC1}) / M_{\text{asp}} \\ \text{aspC2}'(t) &= (v_{\text{TA}} \cdot \text{malC2}) / M_{\text{asp}} - (v_{\text{TA}} \cdot \text{aspC2}) / M_{\text{asp}} \\ \text{aspC3}'(t) &= (v_{\text{TA}} \cdot \text{malC3}) / M_{\text{asp}} - (v_{\text{TA}} \cdot \text{aspC3}) / M_{\text{asp}} \\ \text{aspC4}'(t) &= (v_{\text{TA}} \cdot \text{malC4}) / M_{\text{asp}} - (v_{\text{TA}} \cdot \text{aspC4}) / M_{\text{asp}} \end{aligned}$$

APPENDIX B. CORRECTION FOR A POOL OF GLUTAMATE THAT DOES NOT CONTRIBUTE TO MITOCHONDRIAL METABOLISM

In the isolated, perfused rat heart metabolizing pyruvate, lower fractional enrichment (FE) of C-4 of glutamate compared with the methyl carbon of acetyl-CoA has been observed, indicating the existence of a pool of glutamate that does not mix with the mitochondrial glutamate produced from α -ketoglutarate by aminotransferase reactions (13). For purposes of exposition, these glutamate molecules shall be called "metabolically inactive" (with respect to exchange of carbons with α -ketoglutarate, which is a constituent pool of the CAC). From the mechanism of transfer of carbons in the CAC (5), it can be shown that the FE of C-2 of acetyl-CoA (the methyl carbon, denoted a_2 in APPENDIX A) equals the FE of C-4 of glutamate (denoted g_4 in APPENDIX A), if all acetyl-CoA molecules are available for the reaction catalyzed by citrate synthase and all glutamate molecules are in exchange with mitochondrial α -ketoglutarate. In other words, the ratio f_{active} of the mass of metabolically active glutamate to the mass of total glutamate (metabolically active as well as inactive glutamate) equals $g_{4\text{obs}}/a_2$, where a_2 equals the true FE of C-2 of acetyl-CoA and $g_{4\text{obs}}$ equals the observed FE of C-4 of glutamate (ratio of moles of glutamate possessing ^{13}C at carbon C-4 divided by moles of total glutamate, including metabolically inactive molecules). It is assumed the FE of carbons of acetyl-CoA can be correctly determined. To obtain the actual fractional enrichment of carbon positions or of positional isotopomers of glutamate, multiply the observed FE by $1/f_{\text{active}}$.

Alternatively, provided there is sufficient enrichment of acetyl-CoA, conventional isotopomer analysis (16) can be used to obtain $1/f_{\text{active}}$, the correction factor for converting "observed" FEs of glutamate carbons or of glutamate isotopomers to "true" FEs (whose values are not affected by the amount of metabolically inactive glutamate). This application of isotopomer analysis to the problem of correcting for metaboli-

cally inactive glutamate is new and original.¹⁰ In APPENDIX A, we derive the following equation for the C-3 triplet of glutamate (see Eq. A29), using the definitions of isotopomer fractions as presented in Ref. 16

$$\text{C3T} = o_{23} \cdot g_4/g_3$$

where o_{23} is the fraction of molecules of OAA that possess ^{13}C at both carbon positions C-2 and C-3. By similar analysis, one can derive the following equations for the C-4 quartet (C4Q), a C-4 doublet (C4D34), the C-2 quartet (C2Q), and a C-2 doublet of glutamate (C2D23)

$$\text{C4Q} = \text{Fc3} \cdot g_3/g_4$$

$$\text{C4D34} = \text{Fc2} \cdot g_3/g_4$$

$$\text{C2Q} + \text{C2D23} = o_{23}/g_2$$

where Fc2 equals the fraction of molecules of acetyl-CoA labeled at C-2 but not at C-3, and Fc3 equals the fraction of molecules of acetyl-CoA labeled at both C-2 and C-3. In the last four equations, the values of g_2 , g_3 , and g_4 represent true values that would be obtained if all glutamate molecules were metabolically active. However, we observe that the ratio of two true FEs (e.g., g_3/g_4) equals the ratio of the corresponding observed FEs (i.e., $g_{3\text{obs}}/g_{4\text{obs}}$), regardless of the amount of metabolically inactive glutamate. By solving these four equations simultaneously, we obtain two expressions for $1/f_{\text{active}}$

$$1/f_{\text{active}} = \text{C3T} \cdot g_{3\text{obs}}/[g_{2\text{obs}} \cdot g_{4\text{obs}} \cdot (\text{C2Q} + \text{C2D23})]$$

$$1/f_{\text{active}} = (\text{C4D34} + \text{C4Q})/g_{3\text{obs}}$$

where $g_{2\text{obs}}$, $g_{3\text{obs}}$, and $g_{4\text{obs}}$ are observed FEs obtained by dividing the moles of glutamate molecules having ^{13}C at position C-2 (or C-3, C-4, respectively) by the moles of total glutamate (including glutamate whose carbons do not exchange with α -ketoglutarate that is a constituent pool of the CAC).

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¹⁰ Our application of isotopomer analysis relaxes several of the assumptions of conventional analysis (16). We allow for recycling of pyruvate ($R > 0$), finite reverse flux catalyzed by fumarase (finite r/v_{TCA}), and reincorporation of $[^{13}\text{C}]\text{CO}_2$ by anaplerotic reaction ($p_4 > 0$, see APPENDIX A).

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