Application of stable isotopes and mass isotopomer distribution analysis to the study of intermediary metabolism of nutrients[1]

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ABSTRACT: Genomic investigations in animals have begun to reveal the metabolic and physiological functions of genes and protein products. However, a thorough understanding of the genomic roadmaps will require investigative approaches that yield qualitative and quantitative information on the activities, fluxes, and connectivity of pathways involved in nutrient use in farm animals; that is, the metabolic phenotype. Recently, the commercial availability of stable isotope (^{13}C, ^{15}N, ^{2}H)-labeled compounds and highly accurate mass spectrometers has made it possible to probe the details of metabolic pathways involved in macronutrient use. For years, the biological sciences have exploited uniformly ^{13}C-labeled substrates (e.g., glucose, amino acids, nucleic acids) and ^{13}C-mass isotopomer distribution (MID) in their metabolic investigations, whereas their use in the animal sciences is very limited. When [U-^{13}C] substrates are fed, infused, or added to cell incubations, the ^{13}C-skeletons distribute throughout metabolic networks. ^{13}C-Mass isotopomer distribution in intermediates and end products of the pathways provides a signature of the fluxes and activities of pathway enzymes traveled by the precursor molecule. This paper highlights aspects of animal nutrition and metabolism in which [U-^{13}C] substrates and MID can be applied to investigations of amino acid, carbohydrate, and fat metabolism. We will focus on [U-^{13}C] glucose as a tracer in chickens, fish, sheep, and cell cultures to investigate the interconnectivity of the pathways of macronutrient and nucleic acid metabolism, and provide demonstration of the central position of the Krebs cycle in preserving metabolic flexibility via anaplerotic and cataplerotic sequences. Exploitation of this approach in animal sciences offers endless opportunities to provide missing details of the biochemical networks of nutrient use that may prove to be strictly under genomic control.

Key words: amino acid, animal, glucose, mass spectrometry, nutrition, stable isotope

INTRODUCTION

There are several areas of animal nutrition in which our understanding of the underlying details of the routes of nutrient metabolism and the general contributions of macronutrient classes to energy generation is vague at best. Most of what is known has derived from studies on cells in culture, and it is clear that such systems are limited in their extrapolation to the whole tissue or whole animal. Thus, at present, there are only indirect clues as to how the metabolism of complex mixtures of dietary nutrients and blood substrates removed by target tissues (e.g., mammary gland, placental-fetal unit) is coordinated for tissue deposition and support of vital metabolic processes in the animal.

The emergence of technologies in mass spectrometry (**MS**), coupled with the application of novel stable isotope tracer approaches in whole animals and cell cultures, has opened the window into the dynamic processes underlying nutrient metabolism and the integration of associated metabolic networks (Owen et al., 2000). The present paper discusses some general aspects of MS and the interpretation of ^{13}C-mass isotopomer distribution (**MID**). We will highlight essential concepts in stable isotope application and provide examples of ^{13}C-labeling studies in animals to address questions related to dietary essentiality and the pathways of macronutrient (protein, carbohydrates, fat) use. Here, the goal is to define organic macronutrient needs as set by the metabolic signature of the animal. Furthermore, we will emphasize the approaches that can be taken with [U-^{13}C]-labeled tracers to expose the metabolic networks and the regulatory branch points in animal metabolism that will soon complement the vast and growing information at genomic level.

FEATURES OF MASS SPECTROMETRY AND MASS ISOTOPOMER DISTRIBUTION

This section will provide brief coverage of MS for measurement of stable isotope abundances in molecules. For further details, the reader is directed to several excellent reviews on MS techniques (Hellerstein and Neese, 1999), mathematical formalisms for resolving mass spectral data (Lee et al., 1991; Fernandez et al., 1996), and application of stable isotopes and MID to metabolic and nutrition research (Hellerstein and Neese, 1992; Berthold et al., 1994; Brunengraber et al., 1997; Reeds et al., 1997; Young and Ajami, 1999; Hellerstein, 2003).

Stable Isotopes vs. Radioisotopes

The metabolic information derived from the use of stable isotopes differs in many ways from that of radio-isotopes, and the best demonstration of this relates to their distinctly different analytical measurements. For example, quantification of radioisotopes is in terms of energy emitted from the loss of an electron from the nucleus (i.e., isotopic decay), with the energy emitted directly proportional to the number of radioactive atoms (^{14}C, ^{3}H, ^{35}S) present per sample or molecule weight (i.e., specific activity). Normally, specific activity of a whole molecule (e.g., pyruvate) is determined, and this has two particular disadvantages. First, the concentration of the molecule must be determined and the compound must be isolated for radioactive counting. Both of these analytical techniques can introduce error in the specific activity measurement. Second, with ^{14}C or ^{3}H tracers, total molecule specific activity is usually measured. For uniformly ^{14}C-labeled substrates such as glucose or glycerol, the disadvantage is that it is not possible to account for ^{14}C recycling back to the parent molecule. Although this information can be derived after chemical or enzymatic cleavage to yield specific molecular portions (e.g., glucose), the methods are labor intensive and for some molecules (e.g., [U-^{14}C] glycerol) it may not be possible. Consequently, use of certain radiolabeled compounds may limit information on the flux through and activity of metabolic pathways, and there are the issues of safety when handling radioactive compounds.

By contrast, the stable isotopes (^{13}C, ^{2}H, ^{15}N, ^{18}O, and ^{34}S) commonly used in nutrient metabolism studies are heavier by 1 or 2 atomic mass units, and this allows differentiation of molecules containing 1 or more labeled atoms as determined by mass differences on an MS. It should be noted that stable isotope-enriched compounds are available only for those elements having naturally occurring heavy isotopes. Mass spectrometers are the analytical instruments that allow for measurement of molecular masses in a variety of matrices (gas, liquid, solid) after their conversion into ions. In brief, introduction of a sample containing a molecule(s) of interest into the MS generates ionized (positive or nega-

Table 1. Mass isotopomer abundances (moles of tracer per 100 mol of tracee) and the possible combinations of positions of ^{13}C in hepatic pyruvate from hybrid striped bass (n = 10) fed a diet containing 20% dextrin, with 18% of diet dextrin as [U-^{13}C] dextrin (Bequette et al., 2005b)[1]

Mass isotopomer	^{12}C and ^{13}C positions	Moles
M+0:	^{12}C-^{12}C-^{12}C	100
M+1:	^{13}C-^{12}C-^{12}C	3.59
	^{12}C-^{13}C-^{12}C	
	^{12}C-^{12}C-^{13}C	
M+2:	^{12}C-^{13}C-^{13}C	3.21
	^{13}C-^{13}C-^{12}C	
	^{13}C-^{12}C-^{13}C	
M+3:	^{13}C-^{13}C-^{13}C	5.40

[1]For pyruvate labeled with ^{13}C, data are presented as moles of the isotopomer (M+n) per 100 mol of tracee (M+0), where M+0 represents the unlabeled pyruvate and M+n (n = 1, 2, and 3 for pyruvate) represents the ^{13}C-containing isotopomers.

tive charges) fragment ions that can be selectively sorted by their mass-to-charge (m/z) ratio, recorded, and a graphic representation produced of the ions separated according to their m/z ratio. For a given native or derivatized molecule under the same MS ionization conditions, the molecule will fragment predictably to yield an identical ion spectrum signature. When measurements require information on the stable isotope content and distribution in a molecule, generally the largest molecular weight fragment ion and its associated heavier isotopomers (tracer containing) are selectively monitored, whereas smaller fragment ions not containing all potential molecular positions of the isotopes are disregarded. Subsequently, the ion abundances are normalized as the percentage of the most abundant (unlabeled) species.

The normalized crude ion abundances of an enriched sample molecule must be corrected for the measured natural abundance (^{13}C, 1.11%; ^{2}H, 0.015%; ^{15}N, 0.365%; ^{18}O, 0.204%; and ^{34}S, 4.22%) of stable isotopes present in the original molecule and that contributed by the derivative. This correction is easily made by subtraction when the molecule contains only one labeled atom. However, when the analyte is a mixture of labeled and unlabeled molecules where, for example with pyruvate (Table 1), 1, 2, and 3 of the ^{12}C (unlabeled) atoms in pyruvate molecules have been replaced (enriched) with ^{13}C, more sophisticate mathematical approaches are required to correct the measured elemental distribution of the stable isotope (Lee et al., 1991; Fernandez et al., 1996). For pyruvate labeled with ^{13}C, data are presented as moles of the isotopomer (M+n) per 100 mol of tracee (M+0), where M+0 represents the unlabeled pyruvate and M+n (n = 1, 2, and 3 for pyruvate) represents the ^{13}C-containing isotopomers. Note that the mole ratio representation is analogous to the specific activity formalism used for radioisotopes.

Interpreting Mass Isotopomer Distribution

As an example of MID and the type of metabolic information that can be derived, we will focus our discussion mainly on the use of [U-^{13}C] glucose. Since the mid-1980s, stable isotopes and MID have been used extensively in laboratory animal and human metabolic research (Kalderon et al., 1986; Kelleher, 1986; Pascual et al., 1998; Wykes et al., 1998). These authors identified the distinct advantages of stable isotopes over radioisotopes in probing the details of glucose metabolism in vivo and, latterly, demonstrated that operation of the Krebs cycle could also be probed with [U-^{13}C$_6$] glucose. In contrast to radioisotopes, use of [U-^{13}C$_n$] substrates with ^{13}C-MID analysis of downstream metabolites allows for the use of a single isotope, rather than multiple radioisotopes, to acquire simultaneous information on individual carbon fluxes.

Table 1 shows the ^{13}C-MID of hepatic pyruvate sampled from fish fed [U-^{13}C$_6$] dextrin (glucose) for 1, 3, or 5 d (Bequette et al., 2005b). Both the relative and absolute labeling of isotopomers of metabolites (i.e., plasma [M+1...M+6]glucose, hepatic [M+1...M+3]pyruvate) had been achieved by 1 d of feeding the dextrin tracer, and so the means of all fish are given. The ^{13}C-mass isotopomer labeling pattern of hepatic pyruvate indicated that pyruvate was derived from 3 sources or pathways: 1) directly from glycolysis ([M+3]pyruvate, all 3 carbons labeled), 2) synthesis of pyruvate ([M+1]-[M+2]pyruvate) from glucose carbon recycled via phosphoenolpyruvate carboxykinase (**PEPCK**) and (or) via operation of citrate lyase-malic enzyme, and 3) synthesis of pyruvate ([M+0]pyruvate) from unlabeled carbon skeletons (e.g., amino acids, glycerol). Because the MS measures differences in molecular mass, it is not possible to determine the position of the ^{13}C label in the molecule. For example, for both the [M+1] and [M+2]pyruvate, there are 3 possible positional ^{13}C-isotopomers, whereas for the [M+0] and [M+3] species of pyruvate, only one isotopomer exists where all carbons contain either ^{12}C or ^{13}C, respectively. However, because [M+3]pyruvate can derive only from glycolysis, the relationship of [M+3]pyruvate to [M+6]glucose (plasma) gives the proportion of hepatic pyruvate flux derived directly from glucose catabolism. By contrast, if radioactive [U-^{14}C] glucose had been given, measurement of the whole-molecule ^{14}C-specific activity of pyruvate would have led to an overestimation of pyruvate flux from glucose. Further, valuable metabolic information on the rate of and sources for gluconeogenesis and operation of the Krebs cycle could have been overlooked if [U-^{14}C] glucose had been used.

A particular advantage of using [U-^{13}C$_6$]glucose, [U-^{13}C$_3$]lactate, or [U-^{13}C$_3$]pyruvate as metabolic probes is that, when these tracer substrates are fed, infused into the blood, or added to cell incubations, ^{13}C-labeled carbon skeletons from their catabolism are introduced into intermediary metabolic pathways, namely gluconeogenesis-glycolysis, glycogenesis, the Krebs cycle, fatty acid synthesis, glyceroneogenesis, and nonessential amino acid synthesis. When interpreting results from [U-^{13}C$_n$]-labeled tracers, it is important to note that appearance of [U-^{13}C$_n$] isotopomers can only derive from the administered tracer (e.g., dietary [U-^{13}C$_6$]dextrin, n = 6). Therefore, when [U-^{13}C$_n$] compounds ([M+n]) are administered, appearance of the [M+n] isotopomer in, for example, plasma glucose when [U-^{13}C$_6$]glucose is administered (Figure 1), represents the proportion of the compounds derived from the intact tracer. As a cautionary note, a preliminary tracer infusion or a modeling exercise should be undertaken to establish the length of tracer infusion to achieve plateau labeling of the primary (e.g., arterial plasma glucose) and any secondary (e.g., hepatic pyruvate) metabolite pools of interest. Failure to achieve isotopic and isotopomer steady states at time of plasma and tissue sampling will lead to gross errors in estimation of flux and metabolic partitioning.

The plasma glucose data in Figure 1 (Sunny et al., 2004; Bequette et al., 2005a,b) are from studies in which we administered [U-^{13}C$_6$] glucose to hybrid striped bass (dietary [U-^{13}C] dextrin), young chicks (dietary [U-^{13}C] glucose), and sheep (intravenous [U-^{13}C] glucose) for several days (see figure notes). There are several points we would like to make. First, when the [U-^{13}C] glucose was fed to fish and chicks, appearance of [M+6] plasma glucose represented the contribution of absorbed dietary [U-^{13}C$_6$] glucose to plasma glucose flux. Similar precursor to product relationships can be used to determine dietary essentiality of nutrients (see below). Second, as noted above, an important advantage in the use of [U-^{13}C$_n$] tracers is the metabolic detail that can be gleaned from the extent and patterns of the ^{13}C recycled. In Figure 1, the appearance of [M+1], [M+2], and [M+3] isotopomers in plasma glucose resulted from the flux through phosphoenolpyruvate (i.e., recycled glucose trioses) to glucose synthesis. However, the [M+2] isotopomer in glucose derives exclusively from recycling of glucose ^{13}C via oxaloacetate (**OAA**); this is a reflection of the activity of PEPCK in the cytosolic compartment. Therefore, a crude measure of the relative recycling of glucose carbon via phoshoenolpyruvate vs. recycling via OAA to phoshoenolpyruvate can be assessed from the ratio of [M+3] to [M+2]glucose. Note that the levels of tracer infused should be kept to a minimum to reduce the probability of two ^{13}C$_3$-pyruvates recombining to form an [M+6]glucose.

The ratio [M+1]-[M+2]glucose to hepatic [M+1]-[M+2]OAA (via hepatic aspartate) gives the proportion of glucose derived via Krebs metabolism (recycling). This latter value underestimates recycling to the extent that the 3-carbon (pyruvate, lactate, and alanine) and OAA pools are diluted with other unlabeled sources (e.g., amino acids, glycerol), but this contribution (i.e., dilution) can be accounted for mathematically (Wykes et al., 1998). Furthermore, the pathways of glucose metabolism and the relative activities of pyruvate dehydrogenase vs. pyruvate carboxylase and gluconeogene-

A. Fish (65 g of BW)

B. Chicks (45 g of BW, 2 d old)

C. Sheep (35 kg of BW)
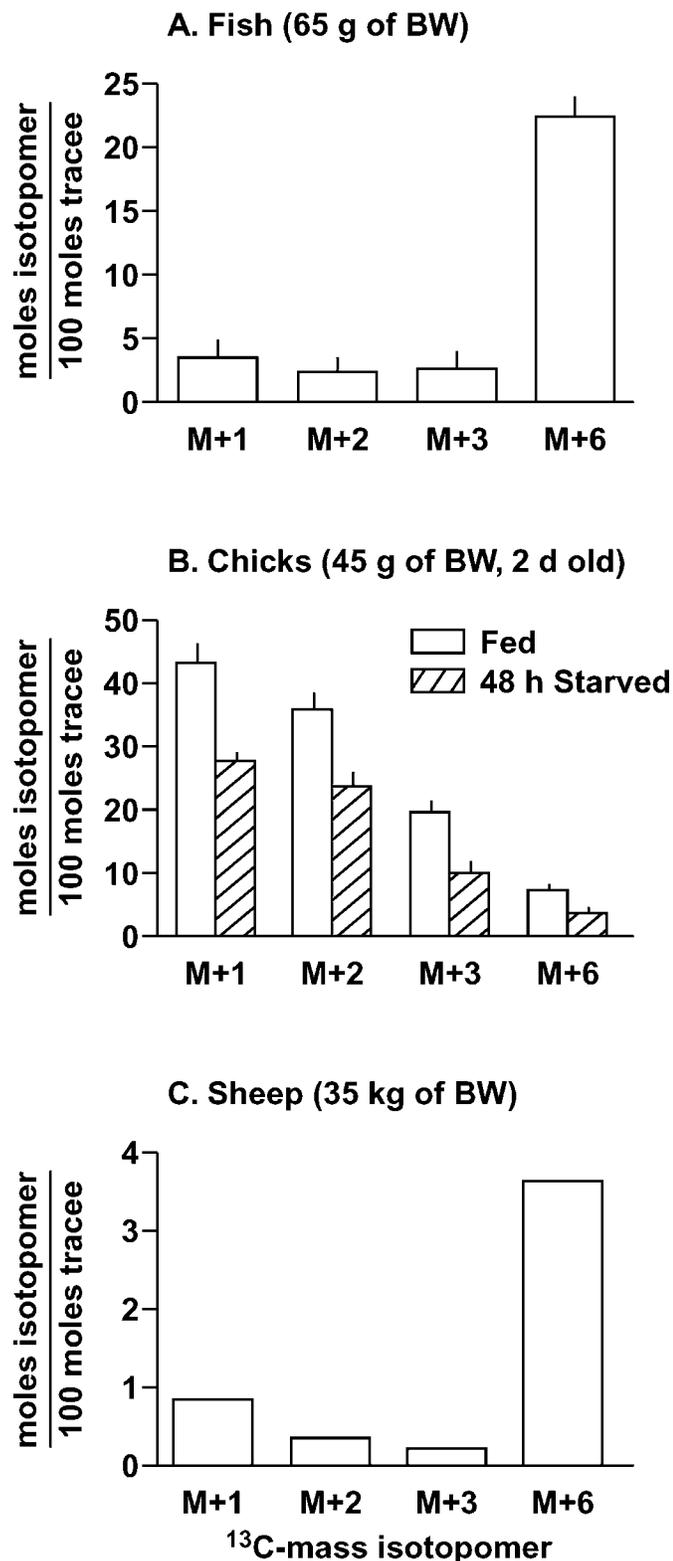
Figure 1. ^{13}C-Mass isotopomer distribution in plasma glucose from A) hybrid striped bass (n = 10; Bequette et al., 2005b), B) 2-d-old chicks (n = 5 per treatment; Sunny et al., 2004), and C) a sheep (B. J. Bequette and S. W. El-Kadi, unpublished data) fed [U-^{13}C] dextrin (fish) or [U-^{13}C] glucose (chicks), or intravenously infused with [U-^{13}C] glucose (sheep).

sis (pyruvate carboxylase-PEPCK) vs. glycolysis (pyruvate kinase) can be assessed by measurement of the relative labeling of α-ketoglutarate (or intracellular glutamate; Fernandez and Des Rosiers, 1995), OAA (via intracellular aspartate), and pyruvate (or via intracellular alanine; Wykes et al., 1998).

Note that in all three species (Figure 1), in particular young chicks, a good portion of glucose entry into plasma was due to glucose carbon recycling. This recycling component does not represent gluconeogenesis inasmuch as the source of carbon skeletons derived from plasma glucose originally. Furthermore, it was of interest that the metabolic handling of glucose in fish and sheep were similar, and this is probably a reflection of the protein- and fatty acid- (volatile fatty acids in ruminants) dominated intakes or absorption (ruminants) of these species.

The last point we would like to make is the importance of administering the [U-^{13}C] compound (e.g., glucose, nucleic acids, fatty acids, amino acids) for a sufficient period to allow ^{13}C to enter into and return from the various intracellular metabolic pathways and to achieve plateau labeling of compartments (e.g., plasma glucose, DNA, RNA, protein). Achievement of isotope equilibration will be reflected in isotopomer steady state when the relationship of the various [M+n] isotopomers remains constant over time. In the case of glucose pools in vivo, <6 h is adequate to achieve steady state for measurements of gluconeogenesis, but >1 d may be necessary when [U-^{13}C] glucose is to be used as a global tracer for nonessential amino acid synthesis and Krebs metabolism (e.g., the fish, chick, and sheep studies in Figure 1). For labeling of nucleic acid pools, the length of administration to achieve plateau labeling in RNA pools may require >6 d, and for DNA pool labeling, even longer (Berthold et al., 1995).

METABOLIC CONNECTIVITY, NUTRIENT METABOLISM, AND END-PRODUCT SYNTHESIS

A quick glance at a metabolic chart and it becomes abundantly clear that central, and even secondary, pathways of macronutrient metabolism intersect with great regularity. In particular, the pivotal position of the Krebs citric acid cycle, through which the pathways arising from glucose, amino acid, and fatty acid metabolisms collide (Figure 2). Consequently, changes in the activity of enzymes or substrate flows through a sequence of enzyme reactions can readily induce changes in pathway fluxes that seem only remotely connected. This is, of course, not a novel concept, and much has been published on metabolic control theories and their eventual applications (Wildermuth, 2000). Nonetheless, what we would like to highlight is that the intertwining of carbohydrate, fatty acid, and amino acid metabolisms is as much a reflection of the dietary components as the proportions and amounts of dietary mac-
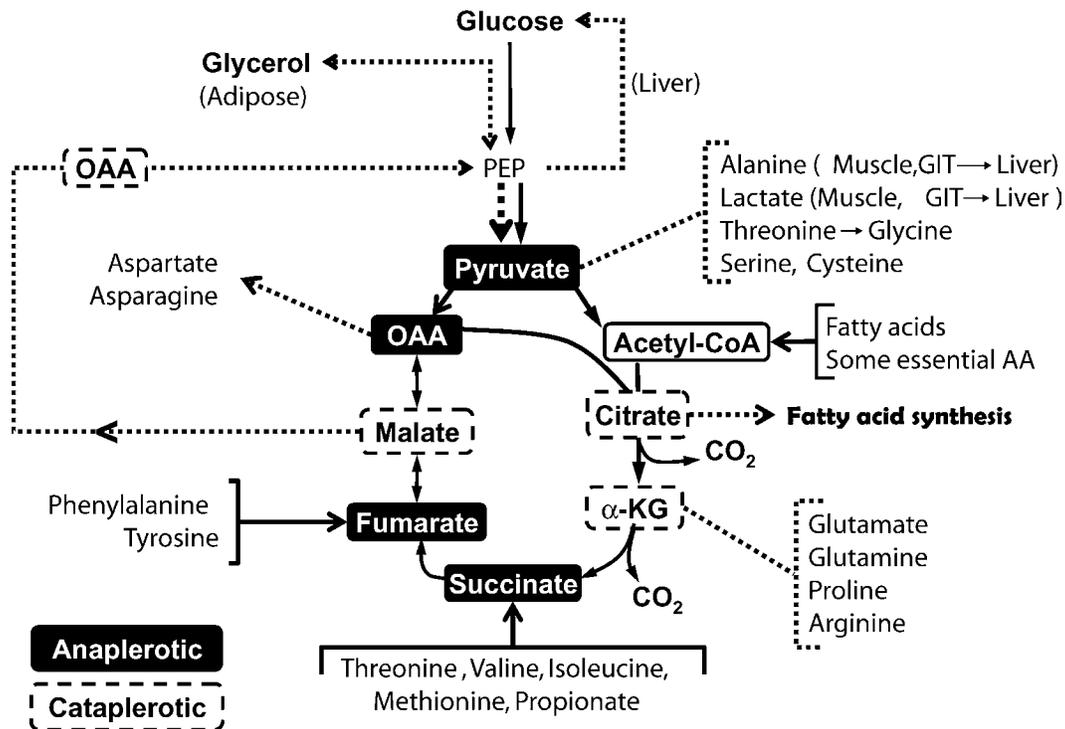
Figure 2. Diagram representing the anaplerotic and cataplerotic sequences that connect Krebs cycle metabolism to gluconeogenesis, fatty acid metabolism, and nonessential AA synthesis. α-KG = α-ketoglutarate; OAA = oxaloacetate; PEP = phosphoenolpyruvate.

ronutrients in the diet are needed to balance the networks of metabolism.

Furthermore, in farm animal metabolism, even as new information is collated on DNA, messenger RNA, and protein expressions, it is becoming increasingly clear that changes occurring at these levels of organization must be interpreted in the context of a true functional analysis. In this connection, to permit the establishment of cause and effect relationships between genomic changes and tissue accretion, details of the quantitative and qualitative metabolite flux through pathways will also be required.

Anaplerosis and Cataplerosis: Maintaining Metabolic Balance

Anaplerotic sequence was a phrase used by Hans Kornberg in 1966 (Kornberg, 2003) to describe "... a series of enzymatic reactions or pathways that replenish the pools of metabolic intermediates in the tricarboxylic acid cycle." With respect to the Krebs cycle, anaplerosis is necessary to replenish intermediates lost to gluconeogenesis, fatty acid synthesis, glyceroneogenesis, and nonessential amino acid synthesis (Figure 2). These latter processes deplete carbon skeletons from the Krebs cycle, and are termed cataplerotic sequences. The twin processes of anaplerosis and cataplerosis carry out three important functions that we wish to highlight in the nutrition and metabolism of animals.

Determining Amino Acid Essentiality

First, in discussing the nutritional adequacy of nonessential amino acids, it is important to note that nearly 100% of the dietary supplies of aspartate, glutamate, and glutamine are metabolized by the gastrointestinal tract (Windmueller and Spaeth, 1975, 1976; Stoll et al., 1998, 1999). Consequently, postabsorptive supplies of these amino acids for net protein deposition must derive from synthesis de novo. What are the sources of carbon skeletons that must be metabolized to provide a net supply of carbon skeletons for synthesis of these nonessential amino acids? Recall that aspartate synthesis occurs solely from the Krebs cycle intermediate OAA; therefore, glucose metabolized via pyruvate carboxylase to OAA or metabolism of some essential amino acids (e.g., isoleucine, valine, threonine) prior to OAA, but not beyond this point (e.g., acetyl-CoA), are the only sources of new carbon skeletons. By contrast, glutamate and glutamine can be synthesized from the Krebs cycle intermediate α-ketoglutarate or from catabolism of proline or arginine. It is clear that, if essential amino acids are involved, this has implications for dietary amino acid intakes and the pattern of amino acid supply.

There are two tracer approaches that can be taken to address this question. Berthold et al. (1991) fed a diet containing [U-^{13}C]-labeled algal biomass (protein, carbohydrates, lipids) to a laying hen, collected eggs over the course of 27 d, and collected tissues from the hen at termination. Their results were in general

agreement with the categories of nutritional essentiality and nonessentiality, except that all proline and 76% of cysteine deposited in the hen tissues and in the egg had derived solely from the diet (i.e., fully conserved carbon skeleton, [U-^{13}C] M+n), therefore behaving like an essential amino acid. By contrast, there was virtually no fully ^{13}C-labeled (i.e., [U-^{13}C$_4$] or [U-^{13}C$_5$]) aspartate, glutamate, and glutamine in the egg and hen, whereas lower molecular weight ^{13}C isotopomers (i.e., [M+1], [M+2], and [M+3]) were present. Therefore, 89 to 94% of these amino acids were synthesized de novo via operation of the Krebs cycle and entry of ^{13}C-labeled precursors derived from the algae. In this respect, because the [U-^{13}C] biomass contained [U-^{13}C] amino acids and [U-^{13}C] carbohydrate (primarily glucose), the source of carbon for nonessential AA was equivocal.

An alternative approach to determine dietary essentiality is based on the use of [U-^{13}C] glucose whose subsequent metabolism may lead to appearance of [^{13}C] isotopomers in nonessential amino acids via cataplerotic sequences of the Krebs cycle; that is, an inside-out approach. This technique has been employed to good effect in human infant studies (Jaksic et al., 1994; Miller et al., 1995), and we have also applied the approach to studies in vivo with young chicks (Figure 3; Sunny et al., 2004) and carnivorous fish (Figure 4; Bequette et al., 2005b), and in vitro with bovine mammary explants (Figure 5; Bequette et al., 2005a). The comparison between the omnivorous chicks and the carnivorous fish provides a striking comparison of the different metabolic strategies these species utilize for amino acid synthesis, and the sources of substrates and pathways of metabolism they employ to derive energy (see below). In the chicks, which were fed a 65% dextrose diet (18% as [U-^{13}C$_6$] glucose), no aspartate had apparently derived from dietary aspartate absorption by the intestines. Instead, glucose carbon accounted for 41 to 43% of aspartate synthesis with the remainder derived from sources metabolized through pyruvate (e.g., serine, alanine, threonine). By contrast, 34% of glutamate and glutamine derived from dietary protein, with another 28% synthesized from glucose carbon and the remainder again derived from substrates metabolized through the pyruvate pool. The fish results were in contrast to those of the chick. In the hybrid striped bass (20% dietary dextrin, 18% as [U-^{13}C] dextrin) glucose carbon contributed only 6 to 8% to aspartate, glutamate, and glutamine fluxes. Even though as much as 30% of these amino acids was derived from dietary absorption, most (62%) of the supply of carbon skeletons for synthesis of these amino acids derived from other amino acids, primarily those entering the Krebs cycle prior to OAA.

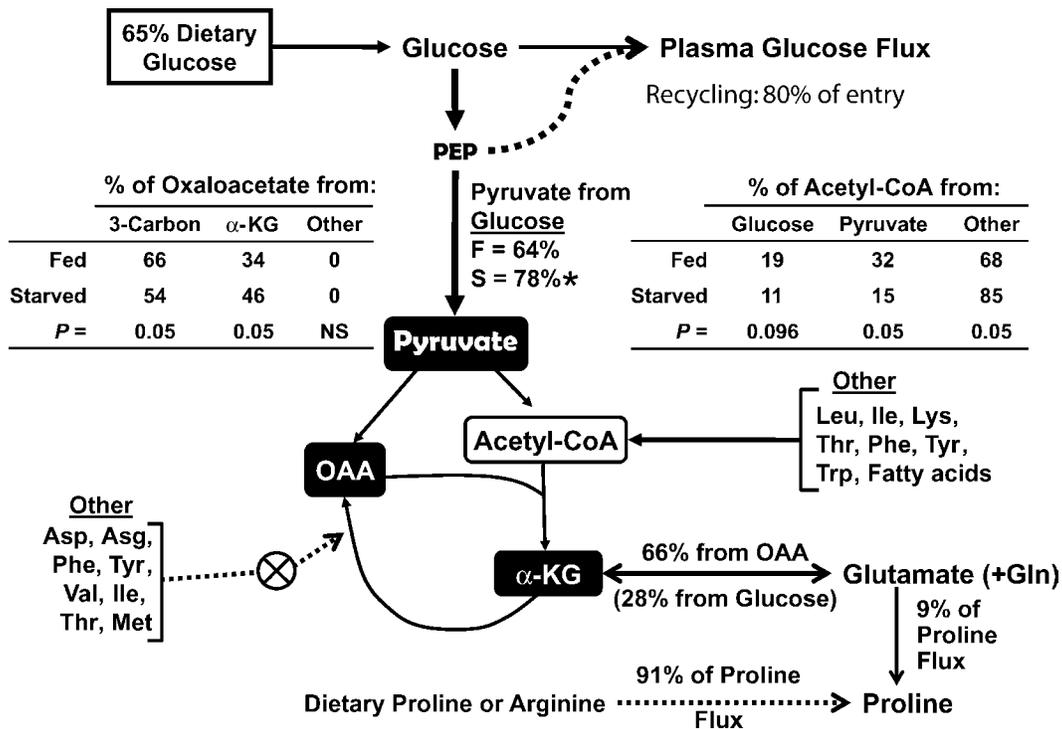## Considering Nutrients as Energy Sources

The second function we will consider is the role of anaplerotic and cataplerotic sequences to ensure complete and efficient oxidation of carbon substrates (Figure 2). In discussing the overall contribution of carbon substrates to energy generation in the Krebs cycle, it is important to recognize that complete oxidation of a carbon skeleton necessitates its terminal entry into the Krebs cycle via acetyl-CoA. Let us take the example of an acetyl-CoA unit from β-oxidation of an even-chained fatty acid. The acetyl-CoA unit will be completely metabolized to CO_2 on the first turn of the Krebs cycle, and all that is required is that a unit of OAA condenses with the acetyl-CoA input. Because this OAA unit is subsequently regenerated through function of the cycle, no further inflows of OAA units (e.g., from pyruvate or aspartate) are needed to condense with another unit of acetyl-CoA. However, as mentioned previously, the Krebs cycle is also a source of carbon skeletons for nonessential amino acid synthesis as well as for fatty acid and glucose synthesis. These reactions deplete the cycle of carbon skeletons, and so anaplerotic inputs are necessary to regenerate the pool of OAA units. The primary anaplerotic reaction is conversion of pyruvate to OAA via pyruvate carboxylase, but in some tissues (e.g., intestines; Reeds et al., 1996, 1997; Stoll et al., 1999) anaplerotic substrates may also enter via α-ketoglutarate (e.g., glutamine, glutamate) to fulfill this requirement, and serve as terminal oxidative substrates. This brings us back to the beginning, and the requirement that complete catabolism and yield of energy from carbon substrates requires, ultimately, that they be terminally oxidized via the acetyl-CoA pool.
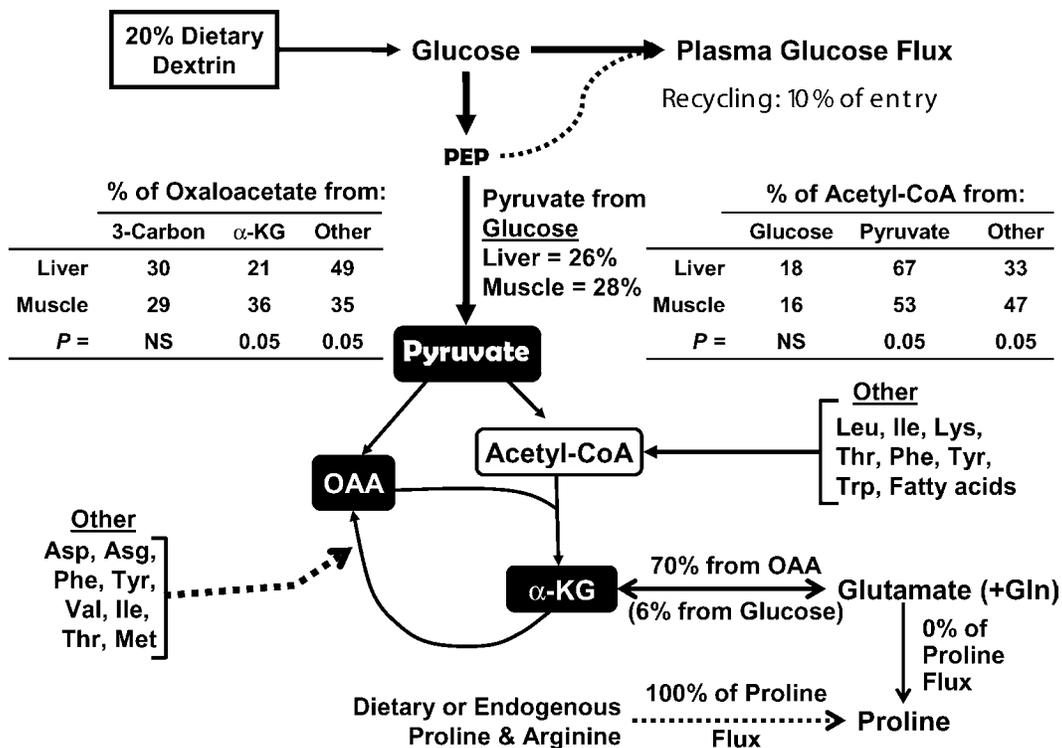
In our work investigating the limits to dietary starch use by carnivorous fish (hybrid striped bass), we further examined the carbon substrate flows into and out of the Krebs cycle by monitoring the ^{13}C-MID patterns of intermediates of the Krebs cycle (Figure 4). Figure 3 gives similar measurements made in the 2-d-old chick (liver). It was particularly noteworthy that in both the chick and fish, and for the fish tissue comparisons, the contribution of glucose to acetyl-CoA flux was minor compared with other substrates, indicating that dietary and endogenous glucose supplies are not major oxidative substrates. And, although glucose was a major contributor to the anaplerotic reaction at OAA in the chicks, this role was assumed by amino acid carbon inflows in the fish. With respect to the carnivorous fish, we are currently examining whether, at higher intakes of carbohydrate (>20% dextrin), the partition of glucose is shifted more toward the oxidative and (or) anaplerotic routes to spare amino acids from complete or partial catabolism. What is clear, however, is that catabolism of glucose to pyruvate in the fish is not as limiting as the enzymatic pathways (e.g., pyruvate dehydrogenase, pyruvate carboxylase) that control entry of pyruvate into the Krebs cycle.

## MAMMARY GLAND METABOLIC BALANCE FOR MILK COMPONENT SYNTHESIS

In this last section, we would like to provide what we believe is a unique example of how application of a

65% Dietary Glucose → **Glucose** → **Plasma Glucose Flux**

Recycling: 80% of entry

PEP

Pyruvate from Glucose
F = 64%
S = 78%*

Pyruvate

% of Oxaloacetate from:

	3-Carbon	α-KG	Other
Fed	66	34	0
Starved	54	46	0
P =	0.05	0.05	NS

% of Acetyl-CoA from:

	Glucose	Pyruvate	Other
Fed	19	32	68
Starved	11	15	85
P =	0.096	0.05	0.05

OAA

Acetyl-CoA

Other
Leu, Ile, Lys, Thr, Phe, Tyr, Trp, Fatty acids

Other
Asp, Asg, Phe, Tyr, Val, Ile, Thr, Met

α-KG

66% from OAA
(28% from Glucose)

Glutamate (+Gln)

9% of Proline Flux

91% of Proline Flux
Dietary Proline or Arginine ·········> **Proline**

Figure 3. The contribution of glucose and other substrates to the fluxes of Krebs cycle intermediates in the liver of 2-d-old chicks either fed a 65% dextrose diet (n = 5, 15% of dextrose as [U-^{13}C] glucose) or starved (n = 5, 50 mg of [U-^{13}C] glucose given 3 times per day by gavage; Sunny et al., 2004). α-KG = α-ketoglutarate; 3-carbon = pyruvate-alanine-lactate; F = fed; S = starved; OAA = oxaloacetate; PEP = phosphoenolpyruvate.

20% Dietary Dextrin → **Glucose** → **Plasma Glucose Flux**

Recycling: 10% of entry

PEP

Pyruvate from Glucose
Liver = 26%
Muscle = 28%

Pyruvate

% of Oxaloacetate from:

	3-Carbon	α-KG	Other
Liver	30	21	49
Muscle	29	36	35
P =	NS	0.05	0.05

% of Acetyl-CoA from:

	Glucose	Pyruvate	Other
Liver	18	67	33
Muscle	16	53	47
P =	NS	0.05	0.05

OAA

Acetyl-CoA

Other
Leu, Ile, Lys, Thr, Phe, Tyr, Trp, Fatty acids

Other
Asp, Asg, Phe, Tyr, Val, Ile, Thr, Met

α-KG

70% from OAA
(6% from Glucose)

Glutamate (+Gln)

0% of Proline Flux

100% of Proline Flux
Dietary or Endogenous Proline & Arginine ·········> **Proline**

Figure 4. The contribution of glucose and other substrates to the fluxes of Krebs cycle intermediates in the liver and muscle of hybrid striped bass (n = 10) fed a diet containing 20% dextrin, with 18% of dietary dextrin as [U-^{13}C] dextrin (Bequette et al., 2005b). α-KG = α-ketoglutarate; 3-carbon = pyruvate-alanine-lactate; OAA = oxaloacetate; PEP = phosphoenolpyruvate.
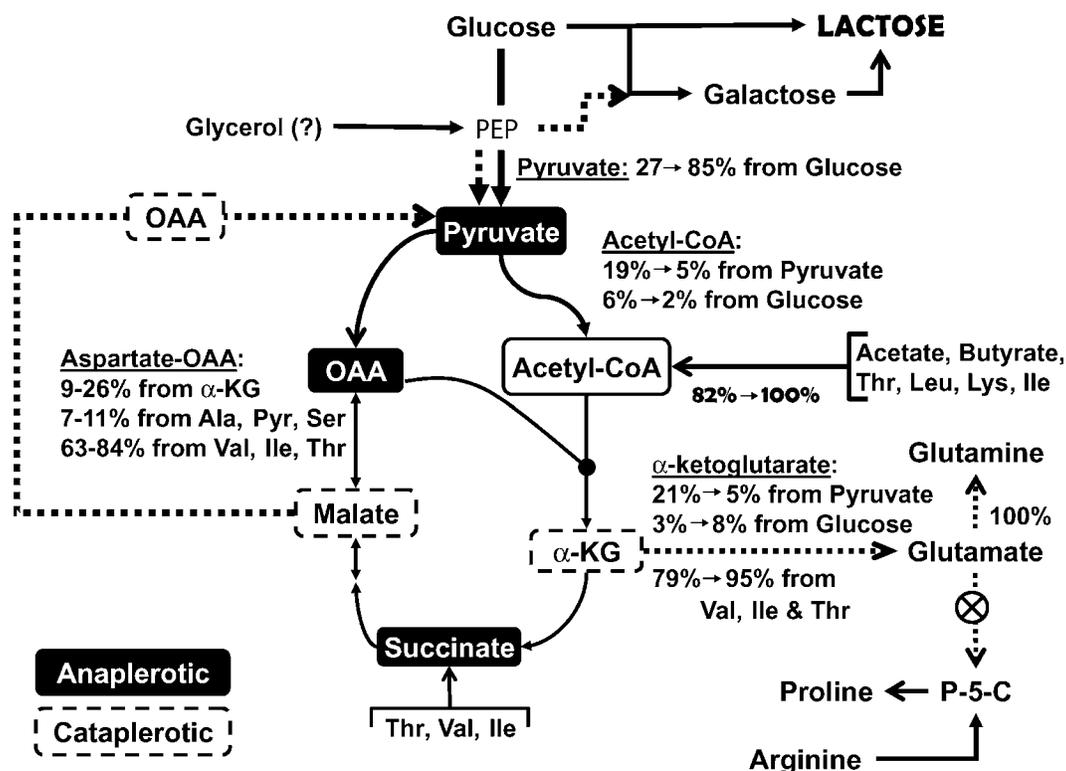
Figure 5. The contribution of glucose and other substrates to galactose and nonessential amino acid synthesis and to the fluxes of Krebs cycle intermediates in bovine mammary explants (150 mg) incubated in medium containing all essential amino acids and increasing (0.67 to 27.7 mM) concentrations of [U-^{13}C] glucose (Bequette et al., 2005a). α-KG = α-ketoglutarate; 3-carbon = pyruvate-alanine-lactate; OAA = oxaloacetate; 5-C, = pyroline-5-carboxylate; PEP = phosphoenolpyruvate.

[U-^{13}C] tracer can be used to dissect multiples aspects of metabolic connectivity and product synthesis. An elegant demonstration of elaborate metabolic integration to achieve a physiological function is milk protein, fat, and lactose synthesis by the lactating mammary gland. The lactating mammary gland extracts from the blood supply a range of precursor substrates that are greater than, equal to, or less than their requirement for synthesis of milk components. How does the mammary gland achieve metabolic balance? Inevitably, the mammary gland must catabolize the excess substrates and coordinate the flux of their carbon skeletons toward pathways that lead to synthesis of those precursors not extracted in adequate amounts.

Two observations from the literature provide some clues to the sources of substrates. First, the net removal of lysine, leucine, threonine, valine, isoleucine, and arginine by the sow (Trottier, 1997) and ruminant (Bequette et al., 1998) mammary glands are in variable excess of milk protein synthesis requirements, whereas the removals of glutamate, glutamine, serine, and proline (and often alanine) are considerably less (by 40 to 100%) than requirements. Therefore, this latter group of nonessential amino acids must be synthesized de novo by the mammary gland, which necessitates catabolism of substrates 3- to 5-carbons in length. Second, studies in dairy cows (Bickerstaffe et al., 1974) and in lactating women (Sunehag et al., 2002) indicate that only ~60 to 80% of galactose in lactose, but 100% of glucose in lactose, derives from plasma glucose. In turn, the remainder of the galactose must be synthesized de novo from 3- to 5-carbon substrates. In lactating women, glycerol has been shown to be a precursor for de novo galactogenesis (Sunehag et al., 2002), but this did not account for all of the galactose carbon.

We investigated, using bovine mammary explants incubated (0.5 to 6 h) with [U-^{13}C$_6$] glucose (0.67 to 27.7 mM), whether glucose supplies a portion or all of the galactose for lactose synthesis. This also gave us the opportunity to determine the sources of precursor carbon for nonessential amino acid synthesis, and those amino acids metabolized by the Krebs cycle for generation of energy (Bequette et al., 2005a; Figure 5). We wish to focus on two general observations. First, [U-^{13}C] isotopomers were detected in lactose-bound glucose and galactose and mostly [M+1]-[M+2] isotopomers were detected in all the nonessential amino acids. The notable exceptions were proline and arginine, which suggests that these were not synthesized via the Krebs cycle. Rather, the deficit in proline removal by the mammary gland (no proline was added to incubations) must have derived from catabolism of arginine via pyrroline-5-carboxylate. Within the physiological range of plasma glucose concentrations (1.11 to 5.55 mM), most (46 to

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86%) of the galactose in lactose was synthesized de novo from nonglucose carbon sources. It was also of interest that only the [M+1] and [M+2] isotopomers were detected in the nonessential amino acids, which indicated that a very minor portion (3 to 8%) of their synthesis was from glucose and that the bulk of nonessential amino acid carbon skeletons derived from catabolism of essential amino acids that were provided in the incubation media (i.e., threonine, valine, and isoleucine).

The second important observation was that, in addition to the appearance of [M+6]galactose, we also observed significant unlabeled [M+0]galactose and, importantly, there was some appearance of [M+1]-[M+3] isotopomers in galactose, but not in glucose. The appearance of these isotopomers in galactose can only occur if there was sufficient PEPCK activity present in the cytosolic compartment to promote the shuttling of Krebs cycle intermediates toward hexoneogenesis or for glyceroneogenesis (guinea pig, Jones et al., 1989; rat, Garcia-Ruiz et al., 1983). When account is taken of the dilution of the ^{13}C in the 3-carbon and OAA pools, we estimate that as much as 12% of galactose synthesis derived from essential amino acid catabolism. Overall, the results of this investigation provided evidence of the role of essential amino acids in the synthesis of large amounts of nonessential amino acids and, perhaps to some extent, their metabolism to provide carbon skeletons for lactose synthesis. Furthermore, the results provided support for the presence of PEPCK activity in the cytosolic and mitochondrial compartments, which now requires verification of these pace-setting enzymes by quantitative gene analysis.

IMPLICATIONS

In this brief coverage of stable isotope tracers and mass isotopomer distribution analysis, we provided examples of the types of metabolic information that can be derived when this approach is applied to questions related to the nutritional and metabolic essentiality of macronutrients and their pathways of use in farm animals. In so doing, we also hoped to encourage more widespread application of these techniques in the animal sciences, in particular because it is becoming more evident that the goal of providing the complete details of gene function will only be achieved by verification of the metabolic phenotype underlying the genomic signatures.

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