ABSTRACT

Title of Document:	NUTRITIONAL AND PHYSIOLOGICAL CONTROL OF METABOLIC PATHWAYS THAT ALTER MILK PROTEIN AND LACTOSE SYNTHESIS BY THE MAMMARY GLAND
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The objectives of this research were to develop a [U-^{13}C]glucose tracer approach establishing the pathways and substrates for milk lactose and casein synthesis, and determine the influence of protein intake on murine mammary gland metabolism. Milk samples were collected after one, three and five days of feeding tracer (as 10% of dextrose). ^{13}C-Isotopic and isotopomer plateaus were attained by day three, establishing the time-course necessary for tracer feeding. 23% of lactose-derived glucose originated from sources other than blood glucose. Six paired (intake and pups equal) sets of lactating mice were fed either a normal (20%) or low (10%) protein diet. ^{13}C-mass isotopomer distribution (MID) in lactose-derived glucose and galactose did not differ, indicating common mammary metabolic pools. ^{13}C-MID in blood versus casein indicated significant mammary synthesis of glutamate (Normal:51%, Low:50%), alanine (Normal:32%, Low:29%), and serine (Normal:18%, Low:37%, $P < 0.05$), suggesting additional requirements for glucose and/or EAA for NEAA synthesis.

NUTRITIONAL AND PHYSIOLOGICAL CONTROL OF METABOLIC PATHWAYS THAT ALTER MILK PROTEIN AND LACTOSE SYNTHESIS BY THE MAMMARY GLAND

By

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Dedication

To my loving husband

Acknowledgements

First, to my advisor, Dr. Brian Bequette. Thank you for teaching me a fraction of your expertise. To learn all that you know would take a lifetime and I thank you for sharing your gift with me.

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List of Abbreviations

EAA	Essential Amino Acids
GC-MS	Gas Chromatography-Mass Spectrometry
h	Hour(s)
L	Low Dietary Protein Treatment
MIDA	Mass Isotopomer Distribution
min	Minute(s)
N	Normal Dietary Protein Treatment
NEAA	Non-essential Amino Acids
OAA	Oxaloacetate
PC	Pyruvate Carboxylase
PDH	Pyruvate Dehydrogenase
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate Carboxykinase
PEPCK-c	Cytosolic Phosphoenolpyruvate Carboxykinase
PEPCK-m	Mitochondrial Phosphoenolpyruvate Carboxykinase
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RT	Reverse Transcription
TCA	Tricarboxylic Acid
TTR	Tracer:Tracee Ratio

Chapter 1: Literature Review

Introduction

Over time, milk production continues to increase as the total number of cows decreases (Blayney, 2002). Dairy producers are increasingly dependent on improving the efficiency of milk production to reduce feed and other overhead costs. However, there comes a point when total production reaches a threshold due to management, genetic merit, or other constraints. In addition, as the demand for dairy products shifts focus to solid dairy products, byproducts, and specialty uses, emphasis is placed on milk components versus total production (Manchester and Blayney, 1997). For reasons that will be discussed, the current desire is for milk with higher protein, but lower fat and lactose contents. In addition, more attention is being placed on opportunities for the mammary gland to serve as a bioreactor in producing novel proteins (i.e. pharmaceuticals).

These new demands require a better understanding of how specific milk components are synthesized within the mammary gland. The dairy industry is focusing less on quantity and more on specialization; likewise, nutritionists and animal scientists are looking at the specifics of metabolism of milk production. Although the American dairy industry is far from using cows for bio-farming on a large scale, one can picture an industry where a farm will raise cows specifically to produce milk for cheese production, fluid milk consumption, or as a bioreactor for pharmaceutical production.

If the industry is to take steps towards more precise production, we must gain a better understanding of mammary gland metabolism; in particular, the pathways and substrates supporting milk component synthesis. We must fill in the missing pieces of the black box that is mammary gland metabolism. Why, for instance, does the mammary gland remove from the blood certain amino acids in amounts far in excess (i.e. essential amino acids (EAA)) of the requirement for synthesis of milk casein and through what metabolic pathways is this excess supply diverted? More importantly, how does the gland compensate for those amino acids whose uptake does not equal their output (i.e. the non-essential amino acids (NEAA)) glutamate, glutamine, serine, aspartate, and proline)? And finally, what are the precursors for milk lactose (glucose and galactose), and how might this be related to coordination involving the NEAA?

A unique approach that has been employed to investigate mammary gland metabolism involves the use of stable isotopes and gas-chromatography mass spectrometry (GC-MS). This approach allows tracking of the ^{13}C skeletons of [U-^{13}C]-substrates by analysis of the mass isotopomer distribution in substrates and products, measured by GC-MS. In the present research, [U-^{13}C]glucose (all 6 carbons in glucose are as ^{13}C) was used, and this acted as a tracer not only for glucose but also for dissecting pathways for NEAA and lactose synthesis, and tricarboxcylic acid (TCA) cycle metabolism. Furthermore, the use of stable isotopes has the advantage of being safer compared to the safeguards required when using radioisotopes.

Unique opportunities also exist to exploit molecular biology techniques. In the liver, phosphoenolpyruvate carboxykinase (PEPCK) exists as two isoforms located in the cytosol (PEPCK-c) and in the mitochondria (PEPCK-m). The

distribution of the PEPCK isoforms and their expression level in the liver serves as a key regulatory mechanism in the control of gluconeogenesis and TCA cycle metabolism. The discovery that both isoforms of PEPCK are found in the murine (García-Ruíz et al., 1983) and bovine (Scott et al., 1975) mammary glands raises the possibility that these enzymes may also be involved in lactose synthesis and in TCA cycle balancing of carbon flows. To date, the roles of the PEPCK isoforms in milk synthesis have yet to be elucidated. What, therefore, is the balance between these two isoforms in the mammary gland, and does this distribution serve a role in control of lactose synthesis versus TCA cycle metabolism and NEAA synthesis? Thus, another aim of this project was to determine the relationship between the gene expression (via quantitative real-time polymerase chain reaction (qRT-PCR)) of the two PEPCK isoforms in the murine liver and mammary gland, and how these changes relate to the use and flux of substrates within the mammary gland as determined by ^{13}C-mass isotopomer distribution analysis. By integrating metabolic flux data and enzyme expression in a key pace-setting step, one can obtain a more complete picture of mammary gland metabolomics. Thus, the combination of these two approaches offers a novel and groundbreaking opportunity to uncover the coordination of mammary gland metabolism, from gene expression to metabolic phenotype.

In order to study the concepts mentioned above *in-vivo*, an alternative to using the bovine species was utilized. Milk synthesis is an eloquent example of the "metabolic intersection" involving glycolysis, gluconeogenesis, fat and amino acid synthesis. This complexity is part of the reason that the intricacies of whole body metabolism still have yet to be fully uncovered (Reeds et al., 1997). Historical

approaches *in vitro* are limited in their potential application for two reasons. The first reason is due to the intersecting pathways of metabolism; changes in one pathway undoubtedly have ramifications in other pathways (Berthold et al., 1994; Reeds et al. 1997). Perturbations in one part of the system result in changes seen indirectly in other pathways. Secondly, compartmentalization of these interconnected pathways occurs on both a cellular and whole body level (Berthold et al., 1994; Reeds et al. 1997). To circumvent these challenges, the application of universally labeled stable isotopes and mass isotopomer distribution analysis (MIDA) via GC-MS (Berthold et al., 1994; Reeds et al. 1997) allows for monitoring of multiple pathways simultaneously. We employed a [U-^{13}C]glucose tracer feeding approach. Feeding was the desired mode of entry for the tracer because it would most closely reflect whole body metabolism. In addition, manipulation of milk composition on a large scale will likely involve nutritional modifications due to the practicality of on-farm approaches.

Feeding [U^{13}C]glucose to dairy cattle is impractical due to the amount of tracer needed to reach steady state. Therefore, the C57BL/6 mouse was chosen for our whole animal model. The mouse allowed for easy management, a manageable lactation cycle, and interesting comparisons can be made between the bovine and murine mammary glands. Additionally, at least in the case of amino acid and glucose metabolism, monogastrics and ruminants appear to have similar mechanisms for milk component synthesis (Katz and Wals, 1972; Mepham, 1982; Bequette et al., 1998; Rigout et al., 2002). Any differences will allow for species comparison. Thus we were confident in our choice of the C57BL/6 mouse as our model.

We made several hypotheses about the questions revolving around mammary gland metabolism. We hypothesized that, in addition to blood glucose, amino acids are a significant contributor to lactose synthesis. Furthermore, amino acids taken up in excess (the EAA) are the ones that not only contribute to lactose synthesis, but also to the synthesis of those NEAA not extracted by the mammary gland in sufficient amounts. PEPCK is hypothesized to play a key role in metabolic regulation of these processes in the mammary gland. The desire to alter milk composition and better understand mammary gland metabolism required further investigation of milk component synthesis.

Definition of the Problem

The Goal of Increasing Milk Protein Content and Yield

Between 1989 and 2002, the United States imported an average net of 23,463 metric tons of casein and casein derivatives (Cessna, 2004). Additionally, the consumption of dairy products such as cheese and cream continues to rise, while the consumption of fluid milk across the United States has declined (Putnam and Allshouse, 2003). Increasing the total solids content of milk favors an improved efficiency of production of these goods. According to Karatzas and Turner (1997), each increase in milk protein by 0.1% increases cheese yield by 0.164 kg. Eighty percent of milk protein casein is in the form of micelles, which affect the physical and chemical properties of the products made from casein derivatives (Smithers et al., 1991). By increasing milk protein concentration, the heat stability of milk is improved, and the amount and composition of curd formation during fermented milk product processing is enhanced (Dalgleish, 1993).

Value is added to milk when it is used to produce other dairy products for direct consumption, and also when milk proteins are produced which are used to improve taste, texture, and nutrition of popular processed foods. These milk ingredients are separated via centrifugation, membrane separation, ion exchange, chromatography or other means (Huffman and Harper, 1999). Once separated, casein and casein derivatives may be processed into commercial products such as nutrition bars, powered beverages, and frozen desserts, while whey products may be used in bakery or soup preparations (Huffman and Harper, 1999). Different milk proteins also have specific functions such as flavor-binding and texture enhancement.

Two-thirds of total milk calcium is associated with casein micelles (Dalgleish, 1993). Therefore, it is plausible that altering milk protein levels would enhance milk calcium content. The increasing concern about diseases such as osteoporosis, caused by inadequate calcium in the diet, creates opportunity to encourage dairy product consumption, or potential calcium-enriched protein products (Huffman and Harper, 1999).

Another opportunity exists to use the bovine mammary gland as a bioreactor. Production of pharmaceuticals by transgenic animals, especially lactating animals, may serve to be more cost effective than conventional processing approaches due to the large production capacity and ease of harvest from the cow (Wall et al., 1997). Current pharmaceuticals of interest, which someday may be produced and harvested from milk, include blood clotting factors, antithrombin III, fibrinogen and human serum albumen (Wall et al., 1997). At present, although transgenic techniques have been used to increased production of specific proteins, the milk output of these

proteins is low and milk protein content remains relatively unchanged. Thus, for such transgenic approaches to become more efficient in terms of total milk protein produced, it will be necessary to define the underlying metabolic partition of amino acids and energy substrates towards casein versus lactose synthesis within the mammary gland. To date, our knowledge of substrate partition within the mammary gland is not yet complete.

Opportunities also exist for production of special value-added milk products based on separation and isolation techniques involving specific milk proteins (Huffman and Harper, 1999). The concept of value-added milk products is supported in part by the ability to increase milk protein, and a better understanding of milk protein production in the mammary gland. As fractionation techniques continue to get more sophisticated, individuals will be able to tailor particular milk proteins to meet the demands of specific nutritional needs. Etzel (2004) commented on the potential benefits of high protein diets, especially those high in EAA and branched-chain amino acids, of which dairy products contain high amounts. Due to the over-abundance of high carbohydrate, low nutritional value drinks in the United States, emphasis should be placed on nutritionally sound beverages such as milk. However, milk lactose intolerance is a major factor that contributes to lower fluid milk consumption.

The Goal of Decreasing Milk Lactose

Although the nutritional benefits to consuming dairy products are widely known, lactose intolerance prevents many individuals from consuming them. Manipulation of milk lactose content is another advantage to understanding milk

component production. More than 75% of the human population has some form of lactose intolerance due to lactase deficiency (Vilotte, 2002). The severity of intolerance varies greatly, and a study of 39 lactose intolerant individuals showed that many individuals illicit very little, if any, negative symptoms when they consume small amounts (7.0 g or less) of lactose (Vesa et al., 1996). Therefore, producing dairy products with lower levels of lactose would allow lactose intolerant individuals, especially those with mild intolerance, to enjoy more dairy products (Hertzler et al., 1996).

The advantage to producing milk with lower lactose levels is two-pronged. While lactose intolerant individuals might be able to enjoy low lactose milk, the other advantage would be milk with higher solids content. Lactose is the major osmotic regulator for milk and decreasing the water content of milk would decrease shipping costs, as well as add to the efficiency of dairy product processing (Karatzas and Turner, 1997; Vilotte, 2002).

Pursuit of these avenues for increasing the value of milk requires an understanding of the novel aspects involved, and a broader understanding of mammary gland metabolism and milk synthesis. The question now is not simply why we should pursue controlling mammary gland metabolism to shift component production, but how we should accomplish this goal. Mechanisms of altering milk production will be discussed in the next section. Overall, the ability to predictably alter the pathways of milk component synthesis will have an economic advantage for consumers, producers, and the dairy industry as a whole.

Historical Approaches to Altering Milk Composition

Although milk production has increased dramatically over the years as genetic selection and management have improved, the composition of milk has remained relatively constant. As has been discussed, there remain potential advantages to altering milk composition. Historically, approaches have involved nutritional supplementation, hormonal treatments, and other dietary means. More recently, genetic manipulation has become an experimental approach to altering milk composition on a small scale. Unfortunately, large scale use of genetics as a tool for altering milk composition is still a costly and inefficient answer (Wilmut et al., 1990; Yom and Bremel, 1993).

In the case of milk lactose, studies attempting to produce low lactose milk via genetic manipulation are difficult as some expression is needed for proper milk synthesis (Stacey et al., 1995; Karatzas and Turner, 1997). A way to get around this problem is to manipulate the gland to express a lactose-hydrolzying enzyme rather than blocking lactose production fully (Jost et al., 1999). Jost et al. (1999) successfully produced low-lactose milk using mice expressing an intestinal lactose-hydrolyzing enzyme in the mammary gland.

The number of progeny needed to test these applications in dairy animals is large, and large degrees of inbreeding may need to take place in order to perpetuate the genetic effects. Low rates of gene integration, poor embryo survival, and unpredictable gene behavior are some of the inefficiencies involved in genetic engineering of dairy cattle to alter milk composition (Wall et al., 1997). Once an egg is successfully injected (only 1 out of 1,600 injections in the case of dairy cattle),

there is only a 50% chance that the offspring will express the transgene (Wall et al., 1997). While production involving genetic manipulation may become relatively practical for high value products such as novel proteins, it is likely a long way off for basic manipulation of milk components.

Manipulation of milk production via dietary means seems to be an obvious suggestion, but to date, the results have been mixed. Responses in production through feeding of supplemental proteins or amino acids have not produced the desired milk protein production effects (Bequette et al., 1998). Duodenal infusion of casein, however, does shift milk components and increases milk protein content and yield (Guinard et al., 1994). Even higher increases in milk protein production, by 25%, are seen when cows are treated with an insulin clamp and supplied additional branched-chain amino acids (Mackle et al., 2000). Understanding how mammary gland metabolism is controlled in this extreme case could result in other means for the manipulation of milk components.

Overview of Milk Protein and Lactose Synthesis

Uptake of Amino Acids and Casein Synthesis by the Mammary Gland

The amino acids that are taken up by the ruminant mammary gland can be divided into three distinct groups: 1) Amino acids whose uptake is not sufficient to account for output in milk protein, 2) Amino acids whose uptake is in excess of the supply needed for milk protein synthesis, and 3) Amino acids whose supply is roughly equivalent to milk output (Mepham, 1982). All three groups of amino acids are taken up by the mammary gland via carrier systems in the basal/lateral membrane. The group of most interest to us is the first, which includes the NEAA glutamate,

glutamine, serine, aspartate, and proline. The deficient uptake of these amino acids requires, in consequence, that they must be synthesized *de novo* by the mammary gland. This most likely requires substrates that provide three to five-carbon skeletons such as those derived from the catabolism of several EAA and/or other sources such as glucose and triglyceride-glycerol. Indeed, several EAA (i.e. the branch-chain amino acids, threonine, and arginine) are extracted from the blood by the mammary gland in amounts much greater than needed for milk casein synthesis, and these are the likely contributors to NEAA synthesis (Annison and Bryden, 1999). However, for these EAA to contribute to the synthesis of the NEAA by the mammary gland requires exquisite coordination of their metabolism and proper balancing of carbon substrate flows. Blood glucose, another potential substrate for NEAA synthesis, is thought to be primarily used for lactose synthesis. The fate of glucose taken up by the mammary gland includes lactose, glycerol, and some amino acids (Katz and Wals, 1972). The fate of glucose for lactose synthesis will be discussed below.

Serine, alanine, glutamate, and aspartate can be synthesized from glucose; this was demonstrated by Katz and Wals (1972) in the rat when they found evidence of radioactive carbon (the source being ^{14}C labeled glucose) in these NEAA. Determining the proportion, if any, of glucose which contributes to NEAA synthesis, and thus milk protein synthesis, is one of the major objectives of this research. While some have suggested that the mammary gland is incapable of synthesizing amino acids from non-carbohydrate precursors (Scott et al., 1975), preliminary work from our group suggests that a portion of milk lactose may be derived from catabolism of amino acids (Bequette et al., 2005). Regardless of the absolute proportions of amino

acids being used for mammary gland hexoneogenesis (glucose, galactose) or glucose metabolism for amino acid synthesis, the mammary gland has the need for complex regulation of these processes. Exactly how the gland accomplishes this is still under investigation (Sunehag et al., 2002, 2003).

Glucose Metabolism and Milk Lactose Synthesis

The focus in this section will be on glucose once it reaches the mammary gland via the blood. Glucose is transported across the basal/lateral membrane by facilitated diffusion via the GLUT1 transporter. Glucose condenses with UDP-galactose via lactose synthetase, to form the milk sugar lactose. Glucose uptake to lactose output across the bovine mammary gland is approximately 1.45:1 and increases to 1.63:1 with duodenal glucose infusion (Rigout et al., 2002). The primary fate of mammary tissue glucose is milk lactose, while glucose may also be used for synthesis of some amino acids, as has already been mentioned. The question of whether glucose used for milk lactose is strictly from plasma, or is synthesized *de novo* in the mammary gland remains. The fates, in addition to lactose, of this glucose also remain undetermined. Early studies in dairy cows reported that only 70% of lactose is derived from blood glucose (Bickerstaffe et al., 1974). Sunehag et al. (2002, 2003) also reported that 80% of lactose comes from blood glucose during the fed state in humans.

Based on our preliminary work with bovine mammary explants incubated in various concentrations of glucose (0.67 to 27.7 mM), we observed that a variable proportion (33-97%) of the galactose in lactose is derived from non-glucose sources (Bequette et al., 2005). It was estimated that as much as 12% of the galactose in

lactose was derived from EAA (Figure 1, Bequette et al., 2005). In lactating women fed [U-^{13}C]glucose or [2-^{13}C]glycerol, Sunehag et al. (2002) reported that glucose accounted for 68% of galactose in lactose (in a fed state), while glycerol was also a contributor (20% of the remainder) to the synthesis of galactose. In a follow-up study in lactating women fed [1-^{13}C]galactose, 20% of lactose precursors derived from non-glucose sources in fed women, a proportion which increased to 40% upon fasting (Sunehag et al., 2003). In addition, they reported that different substrate pools account for differences in stable isotope labeling of glucose and galactose in the mammary gland (accounting for *de novo* synthesis). However, although these studies found that 20% of the non-glucose contribution was derived from glycerol, they could not account for the remaining 80% of the non-glucose source. Therefore, another objective of this project was to determine whether amino acid carbon-skeletons contribute to galactose and glucose in lactose, and whether dietary protein supply (i.e. amino acids) alters this contribution.

Shifts in Milk Components

The mammary gland itself shows us proof that a complex relationship between milk protein and lactose exists. While the concentration of lactose remains relatively constant throughout lactation, milk protein gradually increases. Milk protein concentration reaches its peak at approximately day 100 of lactation, while

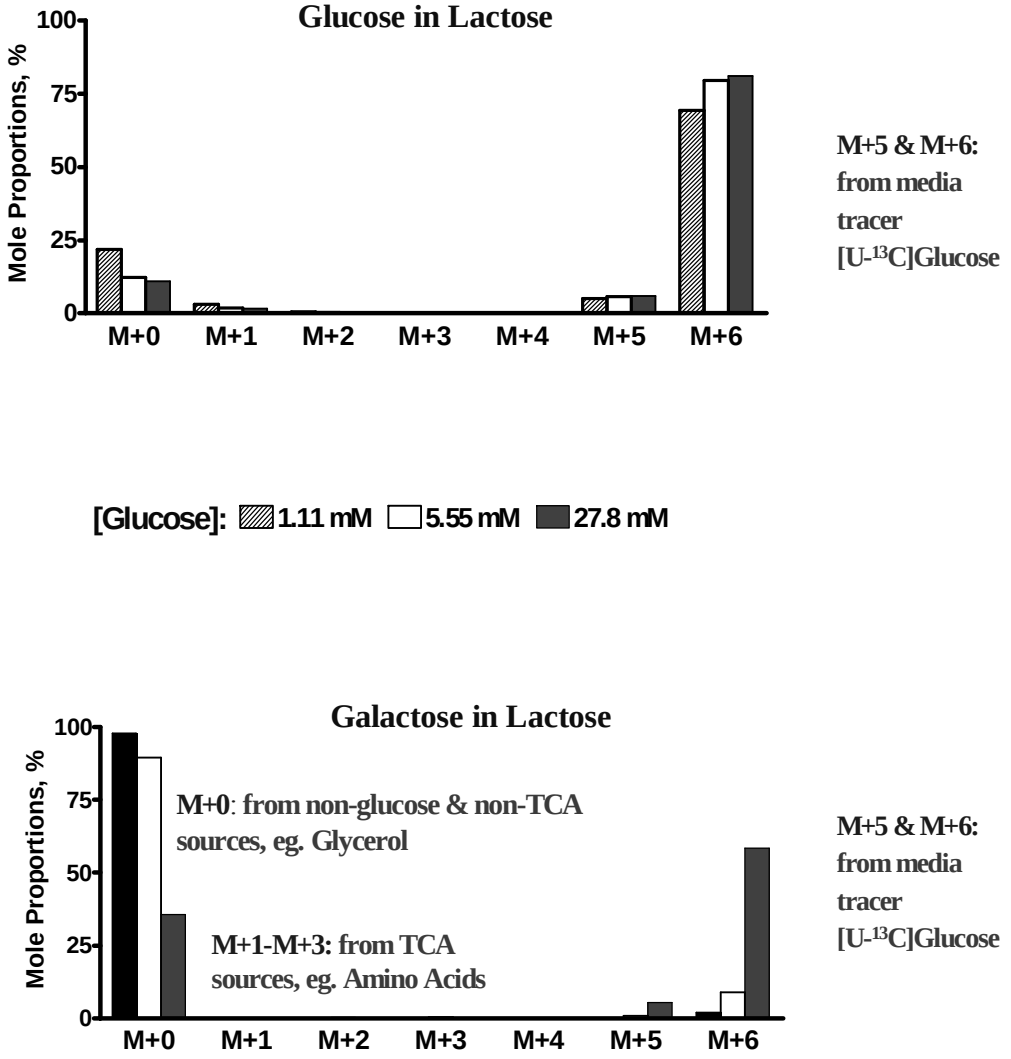


Figure 1. Source of Glucose and Galactose in Bovine Mammary Explants**.**

Explants were incubated with varying levels of [U-^{13}C]glucose and mass isotopomer distribution is shown (Mole proportions, %) for glucose and galactose from milk lactose. [M+X] refers to the enrichment of molecules with X number of ^{13}C molecules (versus ^{12}C). Mole proportions of 33-97% for [M+0] in galactose represent the proportion derived from non-glucose and non-TCA intermediates. From Bequette et al., 2005.

milk lactose content remains fairly invariable (Gáspárdy et al. 2004). Additionally, across species as well as individuals, milk protein and lactose concentrations are inversely related (Mepham, 1984). Even amongst breeds there is variation in milk protein (i.e. 3.8% for Jersey versus 3.19% for Holstein) (Karatzas and Turner, 1997). Significant seasonal effects can also be seen, independent of stage of lactation, as hot, humid months correlate with lower milk protein percentages (Allore et al., 1997; Heinrichs et al., 1997). Therefore, as illustrated in these discrepancies, there are differences in the use of substrates within the mammary gland and shifts in component production accordingly. Having the ability to alter these shifts, by understanding their mechanisms, would be beneficial.

Changes in milk components are also observed in response to hormones and dietary ingredients as mentioned above. For example, dairy cows subjected to a hyperinsulinemic-euglycemic clamp have been used to increase plasma insulin levels while maintaining glucose homeostasis. Insulin infusion increases the percentage of protein in milk (Molento et al., 2002). An increase in plasma insulin parallels the above-mentioned increase in milk protein as lactation progresses (Molento et al., 2002). In addition, an even greater response to insulin was seen when treatment included bovine somatotropin (bST) administration (Molento et al., 2002). In dairy cows subjected to the insulin clamp, Mackle et al.(2000) observed a dramatic increase in milk protein content and yield (+28%) which appeared to be supported by an increase in EAA extraction by the mammary gland. By contrast, the clamp had only minimal effects on milk lactose. The latter is surprising given that the clamp decreased (70% versus 50%) the calculated proportion of glucose used for milk

lactose synthesis and furthermore that milk fat synthesis was reduced by almost 50%. Thus, glucose extracted by the udder was used for purposes other than for lactose synthesis and for the generation of reducing equivalents for fatty acid synthesis. Again, the question of how the gland regulates these metabolic shifts to affect the production of greater amounts of milk protein, but not lactose, is of great interest and one which has yet to be fully explained.

These latter studies and others (Griinari et al., 1997) make a strong case that the mammary gland of the dairy cow is not functioning at its full capacity, especially in regard to milk protein production. Common amongst the latter studies involving the insulin clamp is the increase in blood flow, and therefore amino acid supply, to the mammary gland. Yet, even in those studies where amino acid supply to the mammary gland is increased by protein intake or by post-ruminal infusion, there are often small or no effects on milk protein production (Bequette et al., 1998). Milk protein response is dependent upon site of delivery, stage of lactation, and basal dietary protein level (Bequette et al., 1998). However, when dairy cows were given duodenal casein infusions, milk protein content and yield were increased with each level of casein infusion (Guinard et al., 1994). In that study, casein infusion also increased milk lactose output (Guinard et al., 1994). Interestingly, these authors also observed that the uptake of NEAA increased to a greater extent (177%) with casein infusion than did the uptake of EAA (97%). What role the excessive uptake of NEAA served, other than for casein synthesis, would be purely speculative without a more thorough characterization of the partition of amino acid carbon skeletons within the mammary gland.

The Role of PEPCK

PEPCK and TCA Cycle Metabolism

The enzyme PEPCK may be at the center of milk protein and lactose synthesis. This catalyzes the formation of phosphoenolpyruvate (PEP) from oxaloacetate (OAA) during hepatic gluconeogenesis. PEPCK is a key enzyme involved in glucose synthesis in the liver and kidney, and triglyceride-glycerol synthesis (glyceroneogenesis) in white adipose tissue and the small intestine (Hanson and Reshef, 1997). Two isoforms of the enzyme, cytosolic and mitochondrial, are expressed (Hanson and Patel, 1994). The two isoforms are encoded by separate genes, and have high sequence similarity, which is conserved among species. For example, there is 90% similarity among the rat, human, and mouse PEPCK-c coding regions (Hanson and Reshef, 1997).

While the cytosolic PEPCK enzyme favors the use of amino acids for gluconeogenesis via the TCA cycle, the mitochondrial PEPCK promotes dependence on glycerol and lactate for gluconeogenesis, or hexogenesis in the case of the mammary gland. For instance, if TCA cycle intermediates, specifically malate, are sequestered in the cytosol for carbon use in gluconeogenesis by high PEPCK-c activity, a greater proportion of amino acid carbon will be used for carbohydrate synthesis (Figure 2). Furthermore, with higher PEPCK-m activity relative to PEPCK-c, we expect greater channeling of amino acids and other substrates that enter the TCA cycle at or beyond succinyl-CoA, towards α-ketoglutarate, and thus glutamate for casein synthesis.

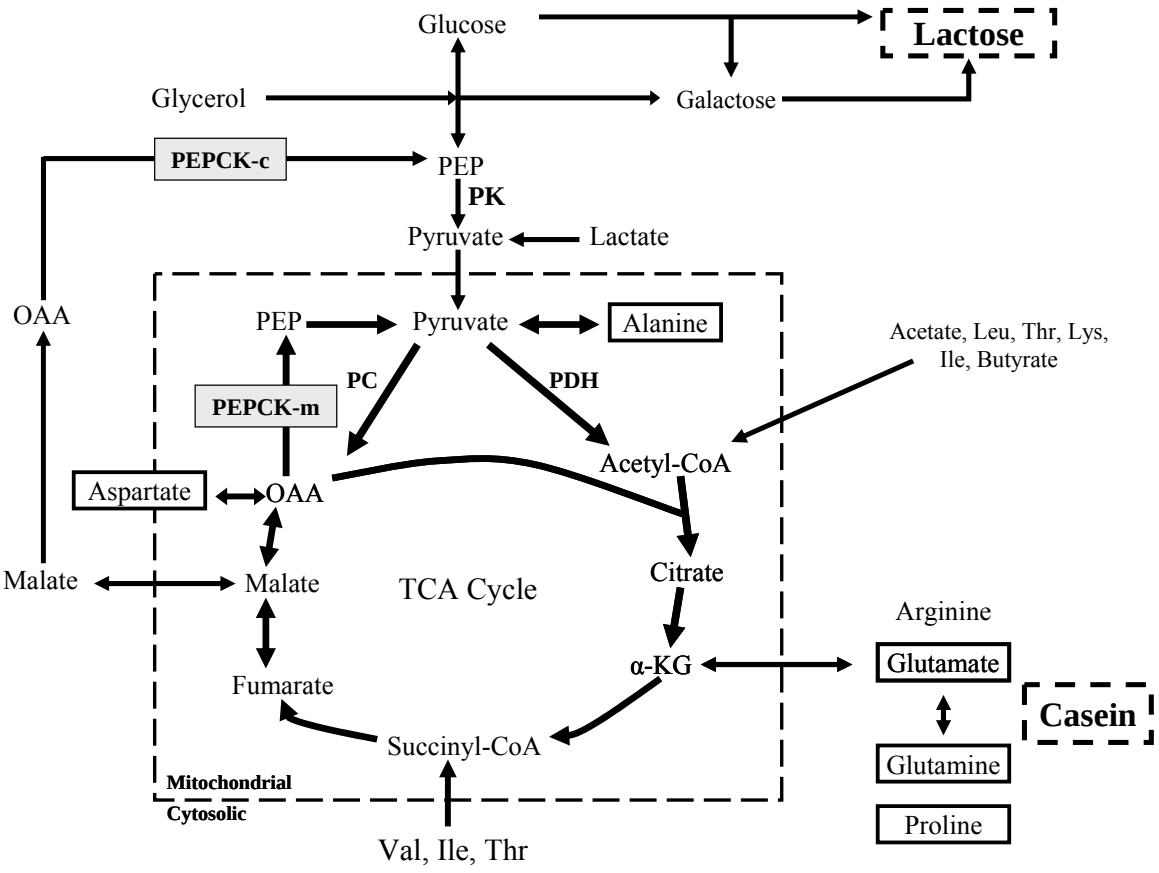


Figure 2. Major Metabolic Pathways and Contributors to the TCA Cycle.

Amino acids within solid-line boxes are taken up in proportions less than milk output.

PEP = phosphoenolpyruvate
PC = pyruvate carboxylase enzyme
PDH = pyruvate dehydrogenase enzyme complex
OAA = oxaloacetate
α-KG = α-keto glutarate

Not all intermediates are shown.
PEPCK-c favors the use of amino acids for gluconeogenesis via the TCA cycle, and PEPCK-m promotes dependence on glycerol and lactate for gluconeogenesis, or hexogenesis. Higher PEPCK-m activity relative to PEPCK-c, channels amino acids and other substrates entering the TCA cycle at or beyond succinyl-CoA, towards α-ketoglutarate, and thus glutamate for casein synthesis.

PEPCK-c serves as a cataplerotic enzyme in relation to the TCA cycle. Cataplerosis refers to the removal of TCA cycle intermediates from the system (Owen et al., 2002) for synthesis of glucose, glycerol and NEAA. In addition, besides serving as a key regulator in the balance of TCA cycle metabolism, PEPCK serves to regulate the metabolic flux through the glycolytic and gluconeogenic pathways, at least in the liver (Magnuson et al., 2003). We therefore asked how these observations are related to the potential roles of PEPCK in the mammary gland.

PEPCK in the Mammary Gland

Previous work has examined the relationship of PEPCK-c and PEPCK-m isoforms in the liver, however, work in mammary tissue has been limited. PEPCK activity has been reported in the mammary gland of several species including bovine, guinea pig, and rat (Baird, 1969; García-Ruíz et al., 1983; Jones et al., 1989). Baird (1969) proposed that the presence of PEPCK activity in the mammary gland suggests that this tissue participates in carbohydrate metabolism at the level of the TCA cycle, and that could serve a role in the mammary gland for the synthesis of lactose from non-glucose precursors.

In a study conducted in guinea pigs, PEPCK-m was found to increase 43-fold (10 times the rate of tissue expansion) in the mammary gland at the onset of lactation (Jones et al., 1989), whereas PEPCK-c increased at a rate only slightly more than the increase in tissue size, or at a 2-fold increase per gram of mammary tissue (Jones et al., 1989). Overall, as lactation progressed, the mitochondrial form of the enzyme became the dominant regulator of metabolism. To date, the role(s) of the PEPCK isoforms in milk synthesis has not been determined. One possibility is that higher

mammary gland PEPCK-m expression and activity relative to PEPCK-c serves to support greater shuttling of EAA carbon-skeletons towards NEAA synthesis via intermediates of the TCA cycle. Interestingly, different effects are seen in rats, in which PEPCK-c changes most dramatically (García-Ruíz et al., 1983).

Work on rats by García-Ruíz et al. (1983) showed that the cytosolic form of PEPCK in the mammary gland varies the most as demonstrated by the dramatic increase (40-fold) at the onset of lactation, and thus seems to have more of an influence on mammary gland metabolism than PEPCK-m. In the same study, the activity of PEPCK-c in the rat dams decreased rapidly and dramatically (95%) after just 48 hours (h) of separation from the pups. Lobato et al. (1969) suggested that this may be due in part to a decrease in plasma prolactin observed at weaning. These results show that, unlike the guinea pig, it is the cytosolic rather than mitochondrial form of PEPCK that is highly regulated and whose activity changes rapidly based on the nutritional and/or physiological state of the rat.

How these patterns may change in bovine mammary tissue is still unknown. Milk from guinea pigs has 6.3% protein on average (Oftedal, 1984). The bovine mammary gland produces milk with only 3% protein, half that of the guinea pig (Oftedal, 1984). Therefore, based on the enzyme activity needed to support TCA cycling, there should be a smaller increase in PEPCK-m in bovine tissue. Portions of the nucleotide sequences for the two isoforms (cytosolic and mitochondrial) of this enzyme have been sequenced from a bovine mammary gland cDNA library, according to a recent BLAST search of the *Bos Taurus* gene index (http:// compbio.dfci.harvard.etu/tgi) by E. Connor (USDA, pers. comm.). Preliminary work

for this project, presented in Chapter 2, also demonstrated appreciable gene expression of the two isoforms of PEPCK in the bovine mammary gland. This is the first report of mRNA expression of the two forms of the PEPCK enzyme in the bovine mammary gland. This work suggests, given greater mRNA expression, that it is the mitochondrial form in the mammary gland that is responding to metabolic changes in dairy cows. In fact, in support of these observations, Scott et al. (1975) reported that PEPCK-m represented 88% of the total PEPCK activity in bovine mammary tissue.

PEPCK in Other Tissues, Including the Liver

Interestingly, PEPCK-c is expressed in white adipose tissue and this finding led to the demonstration that PEPCK-c serves a key role in glyceroneogenesis and thus fatty acid turnover and storage in adipose tissue (Hanson and Reshef, 1997). The kidney cortex and small intestine also illustrate PEPCK-c activity (Hanson and Reshef, 1997). PEPCK obviously also plays a critical role in glucose synthesis in the liver, serving as the first committed step in gluconeogenesis. Across all tissues, it appears that, for the most part, PEPCK-c expression and ultimate activity varies based on metabolic state while PEPCK-m remains constitutively regulated (Hanson and Reshef, 1997). An exception, in the guinea pig, has already been mentioned (Jones et al., 1989).

Liver PEPCK-c serves a cataplerotic role in relation to the TCA cycle for the synthesis of glucose, glycerol and other products (Owen et al., 2002; Hanson and Reshef, 2003). Unlike the guinea pig mammary gland, it seems as though rat liver PEPCK-c, rather than PEPCK-m, undergoes greater responses during metabolic

changes (Jones et al, 1989). Again, this seems to suggest very different control of the two forms in each of the two tissues. Indeed the patterns seen in the rat liver are more common amongst species and the guinea pig seems to be something of an anomaly (Jones et al., 1989).

The balance of the role of the two forms of the enzyme in the liver is well characterized in birds. PEPCK-c is not expressed in the avian liver (Hanson and Patel, 1997). However, both forms are present in the kidney (Hanson and Patel, 1997). This supports the known metabolism of birds which includes little gluconeogenesis from amino acids in the liver, but rather lactate as the source (Hanson and Patel, 1997). The kidney, therefore, is the main site of glucose synthesis from amino acids in birds.

While post-hatch birds have no hepatic PEPCK-c activity, the rat, mouse, and hamster livers have 90% PEPCK-c activity in relation to total PEPCK activity (Hanson and Patel, 1994). Most other mammalian species have similar (close to 50:50) PEPCK-c to PEPCK-m distribution in liver (Hanson and Patel, 1994). Hanson and Patel (1994) noted that, with the exception of adult birds, the relative activities of the enzymes are the same in each tissue within each species. In dairy cows, Agca et al. (2002) found that liver PEPCK-c gene expression increased during transition to lactation. We hypothesize that a similar result would be observed for mammary gland PEPCK, if the bovine follows the pattern of similar tissue responses within species. Greenfield et al. (2000) also noted an increase in hepatic PEPCK-c at day 28 of lactation in dairy cows. She et al. (2000) classified PEPCK-c as being a key regulator in hepatic regulation when they observed mice that were able to maintain

normal plasma glucose levels with decreased PEPCK-c function, but altered fat synthesis and raised metabolic enzyme expression (acetyl-CoA oxidase and fatty acetyl-CoA dehydrogenase) with complete absence of PEPCK-c. While PEPCK-c is involved in gluconeogenesis and glyceroneogenesis in several tissues, it is interesting to propose that this enzyme functions as a regulatory point for lactose and glycerol synthesis in the mammary gland.

PEPCK Regulation

Control of the activity of the two forms of the enzyme is dependent on the relative synthesis and degradation of the enzyme because there have been no allosteric regulators of the enzymes identified (Hanson and Reshef, 1997). Diet and hormones have been shown to regulate only PEPCK-c, and not PEPCK-m (Chakravarty et al., 2005). Both forms appear to use a divalent metal ion bound to the active site as an activator (Hanson and Patel, 1994). While pertinent points will be mentioned here, full reviews of the topics of regulation of the PEPCK enzyme can be found in Hanson and Reshef (1997), and Chakravarty et al. (2005).

The ratio of the two forms of the enzyme varies among species, and regulation is tissue-dependent. Evidence of hormonal regulation via control of gene transcription rate and mRNA stability of PEPCK-c has been found in some tissues (Hanson and Reshef, 1997). Of note is the inverse relationship found between plasma insulin concentrations and total PEPCK activity in the liver and mammary gland of rats (García-Ruíz, 1983). Additional evidence shows that insulin is an inhibitor of PEPCK-c gene expression in the liver (Hanson and Reshef, 1997; Chakravarty et al.,

2005). On the other hand, acidosis increases PEPCK-c activity in the liver (Hanson and Reshef, 1997).

PEPCK-c is not active in the mammalian fetal liver but initiation of PEPCK-c activity is necessary post-natally for gluconeogenesis and survival, even if PEPCK-m activity is adequate (Hanson and Reshef, 1997). This shows that the newborn requires PEPCK-c to support the metabolism of amino acids to glucose rather than glucose synthesis through the PEPCK-m pathway (Hanson and Reshef, 1997). These observations are consistent with a decrease in insulin at birth, in consequence reducing the negative effects of insulin on PEPCK-c gene expression (Hanson and Reshef, 1997) and resultant increased capacity for gluconeogenesis.

Lobato et al. (1985) suggested that prolactin is also a regulator of PEPCK-c in rats. They demonstrated that when prolactin secretion was inhibited, PEPCK-c activity decreased by 50%, mimicking the effects of insulin (Lobato et al., 1985). In their study, similar results were seen in liver and mammary gland PEPCK, suggesting similar regulatory mechanisms, and another possible route for manipulation of milk components (Lobato et al., 1985).

The hyperinslinemic-euglycemic clamp causes shifts in milk production and milk components. Since insulin inhibits PEPCK-c in the liver, and treatment with insulin and bST increase milk protein levels, then these hormones may be inhibiting mammary gland PEPCK-c the same as in the liver. Evidence for this was shown in lactating dairy cows (Velez and Donkin, 2004) and calves (Hammon et al., 2005) when there was an increase in liver PEPCK upon treatment with bST. This causes a shift in the TCA cycle towards use of glucose for amino acid synthesis versus lactose

synthesis. By inhibiting PEPCK-c, one expects to see enhanced milk protein synthesis due to greater channeling of carbon from EAA (Val, Thr, Ile) towards glutamate and glutamine synthesis (i.e. casein synthesis) (Figure 2). Understanding changes in the relative activities of PEPCK-c and PEPCK-m, will help explain the relationship of milk protein and lactose concentration throughout lactation. These relationships form the basis for future manipulation of milk components by dietary or other means.

[U-^{13}C]Glucose Tracer Approach and MIDA

The use of stable isotopes has allowed for precise quantification of carbon flow through the numerous pathways of metabolism. Stable isotopes (versus radioactive) allow one to distinguish between molecules containing 1, 2, etc. atoms of the isotope of interest. These molecules are referred to as [M+*n*]-containing species, with the *n* referring to the number of heavy isotope atoms. For example, isotopomers derived from the use of [U-^{13}C]glucose where all 6 carbons are found as ^{13}C, result in TCA cycle intermediates with one, two, or three ^{13}C atoms ([M+1, [M+2], etc.). The abundance of these molecules can be accurately measured via high-resolution nuclear magnetic resonance spectrometry, or GC-MS (Reeds et al., 1997).

Naturally occurring "unlabelled" compounds do have a predictable amount of "heavy" atoms. Fortunately, mathematical models allow for adjustment of abundances which take into account the naturally occurring isotope (Hachey, 1994). Corrections can also be made accounting for the fact that universally labeled tracers will not be 100% pure. Therefore, also by mathematical means, the final enrichment accurately portrays the amount of labeling coming only from the tracer molecule

itself. This MIDA then allows for estimation of glucose recycling, gluconeogenesis, and the entry points for intermediates of the TCA cycle, among other things.

As an example, [M+3] pyruvate can only result from the direct breakdown of [U-^{13}C]glucose tracer provided. If this [M+3] pyruvate is resynthesized to an [M+3]-containing molecule of glucose, then the [M+3]:[M+6] glucose ratio is a minimum estimation of glucose that has been derived from pyruvate (Berthold et al., 1994). This is an underestimation because entry of unlabelled sources into the TCA cycle contributes to [M+1] and [M+2]-containing molecules of pyruvate, which can then also be synthesized to glucose. Calculations taking into account these dilution factors have been developed and allow for a more accurate portrayal of TCA cycle metabolism (Berthold et al., 1994; Reeds et al., 1997, Pascual et al., 1997, 1998).

Pascual et al. (1997, 1998), Wykes et al. (1998), Haymond and Sunehag (2000), and Sunehag et al. (2002) are just a few who have used the above MIDA approach to estimate various contributors to metabolism. It is by this approach that Sunehag et al. (2002) determined that not all lactose is coming from blood glucose in humans. They used combinations of [U-^{13}C]glucose molecules, [2-^{13}C] glycerol molecules, and [1-^{13}C]galactose molecules to determine that 20% of glucose in the human breast and 40% of galactose is synthesized *de novo* (Sunehag et al., 2002, 2003). Pascual et al. (1997) measured extensive glucose recycling in the rat liver and determined that lipids are the major hepatic energy source in rats. These studies illustrate the power of MIDA techniques, and we have employed some of these same techniques and calculations for determining various relationships in murine mammary gland metabolism.

The desire to manipulate milk composition by increasing protein and decreasing milk lactose, coupled with the observations that not all milk glucose and galactose derives from blood glucose, and that EAA may be used for milk lactose synthesis in the mammary gland called for application of the MIDA approach to explore mammary gland metabolism. These conclusions, along with the potential regulatory role of PEPCK formed the basis for these experiments.

This project aimed to address three overall objectives:

1) To determine the length of time (days) for feeding of the [U-^{13}C]glucose to achieve isotopic plateau (steady state) in milk and tissue samples taken from lactating mice;
2) To determine whether the differences in mammary gland metabolism caused by altering dietary protein intake are due to shifts in the partitioning of nutrients (glucose and EAA) within the mammary gland to support lactose versus casein synthesis; and
3) To establish the role of the PEPCK isoform enzymes (cytosolic and mitochondrial) in the partitioning of nutrients within the mammary gland, specifically their involvement in the dietary protein response.

These objectives would be met via two experiments, the first being a pilot study in order to validate the MIDA and PEPCK gene expression analysis techniques.

Chapter 2: Development of a [U-^{13}C]glucose feeding approach to examine the metabolic pathways and substrates used to support milk lactose and casein synthesis by lactating C57BL/6 mice

Introduction

There are a few key observations that illustrate that the coordination of milk synthesis is not fully understood. First, though it seems logical that most, if not all, glucose in milk lactose comes from blood glucose, evidence suggests that other precursors may be contributing to milk lactose. These "other" precursors have been shown in part to be glycerol in the case of humans (Tigas et al., 2002; Sunehag et al., 2003) and dairy cows (Scott et al., 1975). This requires that the mammary gland synthesizes lactose *de novo* from sources other than absorbed glucose, and we suggest that amino acids may serve as an additional source of glucose carbon.

Our second observation of mammary gland metabolism is that several NEAA (especially glutamate/glutamine, aspartate/asparagine, serine and proline) are taken up by the mammary gland in quantities less than what is required for milk synthesis (Fleet and Mepham, 1985). The commonsensical source for these amino acids is *de novo* synthesis in the mammary gland with EAA serving as the main precursors. However, the interconnectivity of glucose and amino acid metabolism requires intricate regulation of several pathways involved in milk synthesis. There is the potential for involvement of the use of glucose for NEAA synthesis.

The complexity of the central pathways of carbon metabolism encompasses intersections of glycolysis, gluconeogenesis, and fatty acid metabolism; all

converging at the TCA cycle. Furthermore, the arms of these pathways involve glucose carbon, as well as amino acids, triglycerides (glycerol, fatty acids), and the intermediates associated with the recycling (turnover) of these metabolites. This "metabolic intersection" is part of the reason that the intricacies of whole body metabolism still have to be fully uncovered (Reeds et al., 1997).

Many approaches *in vitro* are limited in their potential application for two reasons. First, because metabolic pathways intersect with great regularity within and between cells (tissues), changes in one pathway undoubtedly will have ramifications on the fluxes through other pathways (Berthold et al., 1994; Reeds et al., 1997). Perturbations in the system are likely to result in changes seen indirectly in other pathways. Secondly, compartmentalization of these interconnected pathways occurs on both cellular and whole body levels (Berthold et al., 1994; Reeds et al., 1997). A way to circumvent some of these challenges involves the application of universally labeled stable isotopes in vivo, and MIDA performed by GC-MS or another separation technique integrated with a mass spectrometer (Berthold et al., 1994; Reeds et al., 1997).

Unlike radio-labeled isotopes, the use of stable isotopes allows for measurement of isotopically labeled molecules versus single isotopic atoms (Reeds et al., 1997). One of the most convenient and most commonly employed tracers is [U-^{13}C]glucose (Reeds et al., 1997), the tracer chosen for this study. Through measurements of isotopomer enrichments, one can determine rather easily how much of a given molecule was derived intact from the tracer. Any full-labeled (i.e. [M+6] glucose) molecule must have come directly from the fully-labeled tracer (i.e. [U-

^{13}C]glucose). The advantages of MIDA are fully utilized, however, by isotopomer distribution analysis since molecules that are not fully labeled can only result from products which have been recycled from the initial tracer. Measurement of [M+1], [M+2], and [M+3]-containing molecules, including adjustments made for isotopomer dilution via recycling, provides rich information of how the ^{13}C atoms provided by the tracer have been recycled through these complex pathways of metabolism. This allows for calculation of the extent of gluconeogenesis, sources of amino acid synthesis, and other metabolic contributions to TCA cycle metabolism.

The mammary gland serves as an ideal model for employment of the MIDA technique. Additionally, the desire to understand mammary gland metabolism in order to alter milk component production within the gland makes a stronger case for exploitation of these techniques in this area. Feeding of the tracer is the desired mode of isotope delivery because it gives the most complete picture of whole body metabolism in relation to mammary gland metabolism. In addition, future manipulation of milk production is likely to involve nutritional aspects on the basis of economy and management. Therefore, from the practical standpoint, how metabolism is regulated from digestion, through absorption and onward, is our greatest interest.

Though the bovine is the commercial target species of interest, several aspects hinder the use of this species at least in a wide-scale tracer feeding approach. Cost prohibits the use of feeding tracers to dairy cattle in amounts necessary for measurable isotopic enrichment in various components (milk, blood, tissue). However, mammary gland metabolism in the broadest sense is assumed to vary little

among species, at least in the case of lactose and amino acid metabolism. Uptake versus output of amino acids for milk casein is similar for monogastrics and ruminants. Across species, uptake of amino acids such as glutamate, glutamine, serine, proline and alanine is far less than what is required for milk casein synthesis (Mepham, 1982; Bequette et al., 1998). The rat mammary gland appears to convert 65% of the glucose extracted from the blood into lipid and 25% into lactose, with oxidation accounting for the rest (Katz and Wals, 1972). The bovine mammary gland has a net glucose balance across the gland of 1.45 g/g (uptake:output) (Rigout et al., 2002). The difference in milk synthesis between monogastrics and ruminants involves milk-fat synthesis due to ruminant incorporation of acetate from rumen fermentation (Katz and Wals, 1972). Based on this information, we can safely assume that the aspects of mammary gland metabolism that we are specifically interested in will vary little between species, and any differences would allow for species comparisons. Therefore, one objective of this study was to confirm the validity of the use of C57BL/6 mice as an appropriate model for mammary gland metabolism.

In order to utilize isotopic enrichment data to determine the contribution of various carbon intermediates to metabolic pools, four assumptions must be met. First and foremost, the tracer must be indistinguishable from the unlabelled substrate (tracee) by the body during metabolism. Second, the isotope must be delivered in amounts that do not alter substrate metabolism. Thirdly, in order to measure metabolic flux and nutrient turnover, a steady state in isotope plateau must be ensured. Finally, in order to measure rates of metabolic recycling (via glucose and

the TCA cycle), steady state must also be reached in lower mass isotopomers relative to the fully labeled molecule. Assumptions one and two are satisfied through selection of a universally labeled glucose molecule as the tracer. In order to satisfy assumptions three and four, the time-course required to reach isotopic and isotopomer plateaus in mouse tissues and milk need to be determined. Due to the high cost of [U-^{13}C]glucose (the tracer chosen based on assumptions one and two), we wanted to limit the amount of tracer required in subsequent studies. Our specific hypothesis, based on previous work in our lab, was that within three days, isotopic plateau would be reached in milk and tissues. The feeding of stable [U-^{13}C]glucose in future studies would be based on the findings of the pilot study.

Finally, we also probed for PEPCK expression in the mammary gland and liver of lactating mice to confirm that we could combine enzymatic gene expression data with the metabolism data gleaned from the MIDA approach. PEPCK serves as an important regulator of metabolism, in part, because it exists in two isoforms (cytosolic and mitochondrial), which serve important roles in maintaining compartmentalization of the metabolic pathways mentioned earlier (Agca et al., 2002). In particular, PEPCK-c is thought to determine the flux of carbon through glycolytic versus gluconeogenic pathways (Magnuson et al., 2003). Activity specific to both forms has been found in the guinea pig, rat, and bovine mammary glands, with regulatory patterns suggesting an important function in the mammary gland for milk synthesis (García-Ruíz et al., 1983; Lobato et al., 1985; Scott et al., 1985; Jones et al., 1989). Since PEPCK-c promotes channeling of carbon out of the mitochondria and towards gluconeogenesis or glyceroneogeneis, while PEPCK-m promotes the use

of amino acid carbon for NEAA synthesis or oxidation, the balance between these two isoforms could hold a key to how the complex regulation of interconnecting metabolic pathways occurs within the mammary gland. Simply put, our ultimate objective is to determine how PEPCK might be related to the use of carbon for amino acid or lactose synthesis. Since our other species of interest is the dairy cow, we sought to confirm gene expression of both forms of PEPCK in the bovine liver and mammary gland. Additionally, demonstrated presence of the isoforms in the murine liver and mammary gland will allow for additional comparisons between these two species.

Materials and Methods

Animal Care

The details of this experiment were reviewed and accepted by the University of Maryland Institutional Animal Care and Use Committee (Protocol #R-05-57). A total of six C57BL/6 mice, obtained from the University of Maryland Department of Animal and Avian Sciences breeding colony, were used for this experiment. For breeding, three females were placed in a cage with one male. Several days prior to the estimated date of parturition, female mice were placed into individual plastic shoebox cages. Mice were monitored daily for food intake and health status, and fresh water was provided every other day. Cages were cleaned and sterilized once every week. At the time of sampling for this pilot study, the four lactating mice were all aged three months and weighed 23.92 grams (±.99 g). Two additional mice were not lactating at the time of sampling.

Diets and Feeding

Prior to parturition, mice were fed a standard lab animal diet in pelleted form (Lab Diet 5001 Rodent Diet, PMI Nutrition International, LLC, Brentwood, MO). Mice were switched to a purified, formulated diet (Table 1) within two days of parturition which contained adequate levels of protein (20%), energy (kJ/kg) and vitamins. The lactation diet was formulated to meet the nutrient recommendations of the National Research Council's Dietary Recommendations for Laboratory Animals (NRC, 1995). Diet ingredients were mixed using a hand mixer, and enough water was added to form a soft dough. Pellets (approximate wet weight, 8.57g) were formed by pressing into a tablespoon measuring spoon and left to dry at room temperature (at least 48 hr). Pellets (approximate dry weight, 6.23g) were weighed and fed to mice *ad libitum*.

Within two days of parturition, all mice were exclusively fed *ad libitum* the purified, formulated diet. Prior to that, a mixture of purified diet and standard lab animal diet was fed in order to acclimate the mice to the new diet. Mice adapted to the formulated diet within one day, and daily intakes were recorded. Mice were monitored twice daily for pup arrival. The day that pups were first noticed in the nest was considered day 1 of lactation. On day 9 (n=3) or day 12 (n=1) of lactation, 10% of the diet dextrose was replaced with [U-^{13}C]glucose tracer, and fed to the mice for 5 days. All other ingredients in the diet remained the same. One non-lactating mouse was also placed on the tracer diet and the other was fed the non-labeled diet for determination of background enrichments for GC-MS analysis.

Table 1. Composition of Experimental Diets

Ingredient	Unlabelled Diet g/100g	Labelled Diet g/100g
Dextrose[1]	60.00	54.00
Vit. Free Casein[1]	20.00	20.00
Solka-Floc[2]	5.00	5.00
Soybean Oil[1]	9.00	9.00
AIN 93 Mineral Mix[3]	4.00	4.00
AIN 76 Vitamin Mix[4]	1.50	1.50
Methonine[1]	0.30	0.30
Choline Bitartrate[1]	0.20	0.20
[U-^{13}C]Glucose[5]	-	6.00

[1]Source: Dyets, Inc.

[2]Source: International Fiber Corporation

[3]Source: MPBio

[4]Source: ICN Biomedicals, Inc.

[5]Source: Cambridge Isotope Laboratories, Inc.

Experimental Design and Sample Collection

The experimental design was longitudinal with time as the explanatory variable (one, three, or five days of tracer feeding). The milk from lactating mice was sampled after one, three, and five days of feeding the diet containing [U-^{13}C]glucose. After one, three, and five days of feeding the labeled diet, dams were separated from their pups for two h prior to hand-milking. At the time of milking, mice were gently restrained by hand and 0.2 ml of oxytocin (1 IU/ml concentration in sterile saline) was given intra-abdominally. Nipples were manually stimulated for several minutes (min) and then milked using a vacuum pump constructed essentially as described by Teter et al. (1990). Briefly, the teat cup was constructed from a silanized glass Pasteur pipette end (8-mm external diameter, 6-mm internal diameter, 4-cm in length), which was passed through a rubber bunge into a plastic vial (50 ml volume, 3.25cm diameter, 8.5cm in length) connected to Teflon tubing at the opposite end. The Teflon tubing was connected through a rubber bunge to a 1-L flask, which was held under vacuum by a water aspirator (Figure 3). The mild suction created enough vacuum to gently draw milk from the teat canal. Suction could be adjusted by placing a thumb over a hole in the side of the plastic vial.

Once several drops of milk formed in the glass collection tube, milk was removed from the glass tube by capillary action into a hematocrit tube, sealed with Critaseal and labeled appropriately. When milking was completed, the dams were returned to their pups. After the day five milking, dams were euthanized by isoflurane asphyxiation (approximately two h after milking). Blood was then sampled via

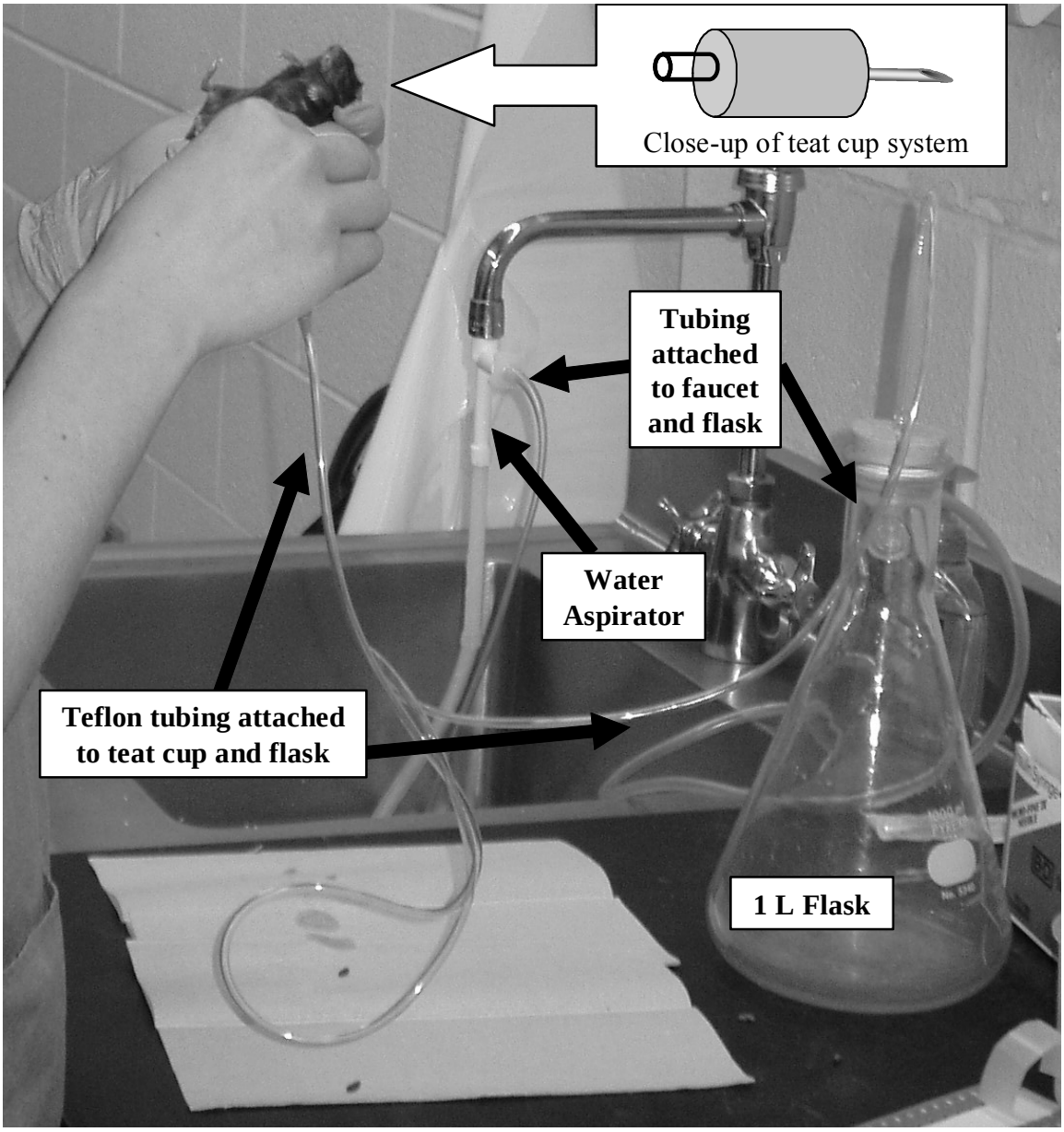


Figure 3. Milking System.

System for milking mice shown is as essentially described by Teter et al. (1990).

the portal vein, and stored at -20°C for later analysis. Tissues collected were stored at -20 °C or below (-80°C for tissues used for RNA extraction).

For tissue collection, a mid-line incision was made through to the abdominal cavity to expose the visceral tissues and the mice were placed on an ice-cold marble tile. Blood samples were taken fresh from the portal vein with a 21-gauge needle and immediately transferred to heparinized tubes prior to storage. The whole liver, both kidneys and the small intestines (from the pylorus to the illeal-cecal junction) were immediately removed, wrapped in tin foil, and submerged into liquid nitrogen. Muscle tissue from the rear hind legs and only the lactating mammary glands were dissected and stored as above. Tissues were subsequently pulverized to a powder using a freezer-mill (SPEX CertiPrep 6850 Freezer/Mill), and lyophilized to dryness prior to processing for GC-MS analysis.

Sample Preparation and Analysis via GC-MS

GC-MS Analysis of Glucose and Galactose from Milk Lactose

Immediately following collection into capillary tubes, whole milk samples were placed in a microcapillary centrifuge at maximum velocity for 15 min to separate fat from other milk components. Milk fat percentage was estimated using a hematocrit ("creamatocrit") by measuring between the bottom and top of the fat layer. Tubes were then stored frozen (-20 °C). Subsequently, the top fat layer in each capillary tube was separated from the nonfat layer (lactose and proteins) by scoring the tube at the fat-nonfat layer interface and gently breaking the tube. The fat layer tube was placed into a 1.5-mL centrifuge tube and stored frozen. The non-fat layer was removed from its tube section using a blunt needle attached to a 1.0-mL syringe,

and forcing air through to dispense the nonfat layer into a 1.5-mL centrifuge tube. To separate lactose from milk proteins, 1.0 ml of distilled H_2O was added to the nonfat layer and vortexed. Approximately two drops of 0.1 N HCl was added to the sample to bring the pH to 4.0-4.5; the sample was vortexed and then centrifuged (12,000 rpm, 10 min) to precipitate milk caseins. The supernatant (containing milk lactose) was transferred into another tube and 0.1 N NaOH (approximately one drop) added to bring the solution to pH 7.0. The lactose-containing supernatant was then centrifuged again for 10 min to remove additional precipitates (debris) and the supernatant was transferred to another tube and stored frozen until enzymatic cleavage of lactose to free glucose and galactose. The remaining casein pellet was washed with 1.0 mL distilled H_2O to remove residual free amino acids, and subsequently frozen at -20°C until further processing.

Lactose was enzymatically cleaved to glucose and galactose according to methods as described by Sunehag et al. (2002, 2003), but with some modification. The modification was that the lactose portion from milk was transferred to an 8-mL glass vial (Wheaton, Millville, NJ) and to this was added 50 µL (15 U) of glucose oxidase (Sigma, St. Louis, MO) plus 20 µL (15 U) of bovine catalase (Sigma). The vial was capped and the sample incubated overnight at room temperature. The addition of the glucose oxidase step served to convert free glucose and galactose, which may be derived from blood or another mammary gland compartment, to their corresponding lactones. On GC-MS, these compounds elute elsewhere, and do not interfere with measurement of lactose–derived glucose and galactose by GC-MS. The next morning, the solution was filtered through a minispike acrodisc syringe

filter (Pall Corporation, Ann Arbor, MI) to remove the glucose oxidase and catalase enzymes. Next, 50 µL (7 U) of ß-galactosidase (Sigma) was added, the vial capped, and the sample incubated for 1 h at room temperature. The solution was then filtered as above, decanted into a 3.0-mL Reacti-vial, and blown down to dryness at room temperature under a gentle stream of N_2. To the dried sample, two drops of distilled H_2O were added to dissolve the dry material, followed by the addition of 1.0 mL of freshly made 0.38 M sulfuric acid in acetone. After incubation at room temperature for 1 h, the solution was transferred to a 20 × 150-mm screw cap culture tube, 2.0 mL of 0.44 M sodium carbonate was added and vortexed. Next, 2.0 mL of a saturated sodium chloride solution was added and the solution vortex mixed. After vortex mixing, 3.0 mL of ethyl acetate was added, the sample vortexed vigorously for 10 min, followed by centrifugation (2,000 × g, 15 min). The top ethyl acetate layer, containing glucose and galactose, was drawn off and the extraction procedure repeated to ensure complete extraction of glucose and galactose. The combined ethyl acetate extractions were transferred to a Reacti-vial and blown down to dryness at room temperature under a stream of N_2. To this dried residue was added 50 µL each of ethyl acetate and acetic anhydride, the vial tightly capped and heated at 60 ºC for 30 min. This derivatization procedure converts glucose and galactose to their respective isopropylidene-pentaacetate forms. The derivatized samples were then transferred to gas chromatography vial inserts (Agilent Technologies, Palo Alto, CA) prior to analysis by GC-MS under electrical ionization mode (HP 5973N Mass Selective Detector, Agilent Technologies). The gas chromatography column (HC-5; 30 m x 0.25 m i.d.; Alltech Associates, Inc., Deerfield, IL) conditions were as

follows: 100º C initial temperature, followed by a ramping rate of 10 ºC/min until 200 ºC, followed by a rate of 30 ºC/min to a final temperature of 280 ºC and held for 1 min. Total run time was 13.67 min with α-galactose eluting at 9.85 min and β-glucose at 10.35 min. Selective ion monitoring was conducted for ions 287 to 293 for both glucose and galactose.

GC-MS Analysis of Blood Glucose

Approximately 0.2-mL aliquots of blood were used. To each aliquot was added 0.6 mL ice-cold 12% (wt/vol) sulphosalicyclic acid, and samples were vortexed vigorously prior to centrifugation at 13,000 rpm for 10 min. The acid-supernatant was applied to a neutral pH cation exchange resin (AG 50-X8, 100-200 mesh, H^+ form, Bio-Rad Laboratories, Richmond, CA), which binds positively charged molecules, such as amino acids, while allowing neutral glucose to pass through the resin. Thus, the initial eluate plus an additional 1 mL distilled H_20 wash were collected into an 8.0-mL Wheaton sample vial for derivitization of glucose. Amino acids were eluted from the resin by addition of 2 ml of 2 M NH_4OH plus 1 mL distilled H_20 and collected into a 4 mL Wheaton vial (see below). A 300-µL aliquot of the glucose portion reduced to dryness at room temperature under a gentle stream of N_2 gas. Glucose was converted to the isopropylidene-pentaacetate derivative prior to selective ion monitoring of m/z 287-293 by GC-MS as described above for lactose-derived glucose and galactose.

GC-MS Analysis of Plasma NEAA

The amino acid-containing fraction from above was subsequently frozen and lyophilized to dryness. The procedure for conversion of amino acids to their hepta-fluorobutylamide derivative is based on the methods described by MacKenzie and Tenaschuk (1979). The amino acids were transferred from the Wheaton vials to 0.6-mL Reacti-vials by rinsing twice with 150 µL water. We found that water, rather than 0.1 N HCl resulted in more efficient hepta-fluorobutylamide formation due to the less acidic environment. For esterification, 200 µL of a 10:1 ratio of ice-cold iso-butanol (Aldrich. St. Louis, MO) and acetyl chloride (Sigma) were added to the V-vials, vortex mixed, capped and heated at 90 ºC for 30 min. Note: Care was taken to keep the iso-butanol and acetyl chloride solutions ice-cold upon mixing to prevent explosive reactivity. If large amounts of debris or precipitate formed during this step, the sample was centrifuged at 13,000 rpm for 10 min prior to the next steps. After heating, the sample was blown down to dryness under a gentle stream of N_2 gas at 40 ºC. To the dry reside was added 50 µL each of ethyl acetate and heptafluorobutyl anhydride (Sigma), vortex mixed and heated at 90 ºC on the Reacti-Therm for 15-20 min. After cooling to room temperature, samples were blown down to dryness at room temperature under a gentle stream of N_2 gas. Next, the sample was taken up in 60 µL of ethyl acetate prior to GC-MS analysis under negative chemical ionization conditions (HP 5973N Mass Selective Detector, Agilent Technologies). For gas chromatography separation, the column (30m; HP-1; 0.25-µm film thickness; Agilent Technologies) conditions were: an initial temperature of 100 ºC, ramping to 190 ºC at 10.0 ºC/min, followed by a 30.0 ºC/min to 300 ºC, held for 2.0 min. Table 2 shows the peak retention times and ions monitored for the NEAA.

Table 2. Ions monitored for individual amino acids in plasma, milk casein, and liver tissue

Amino Acid	Ions Monitored[1] (m/z)	Retention Time (min)
Alanine	310 - 324	5.95
Glycine	307 - 310	6.14
Serine	533 - 536	8.22
Proline	347 - 350	10.08
Glutamine	361 - 364	11.10
Methionine	381 - 384	11.78
Aspartate	421 - 424	12.45
Glutamate	435 - 438	13.81

[1]Ions monitored were [M+0] - [M+3] for all amino acids, and represented the N(O,S)-Heptofluorobutyrl Isobutyryl amino acid derivatives according to MacKenzie and Tenaschuk (1979).

GC-MS Analysis of NEAA from Milk Casein

Analysis of NEAA from milk casein was essentially as described above for blood, with a few modifications. The isolated milk casein pellets were dissolved in 200 μL of 0.1 N NaOH and vortex mixed. One-hundred microliters of this solution was added to a hydrolysis tube containing 4 mL of 2 M HCl, and the sample was hydrolyzed for 24 h at 100 ºC. Following hydrolysis, the sample was applied to cation exchange resin (AG 50-X8, 100-200 mesh, H^+ form, Bio-Rad Laboratories). The resin was rinsed with excess (6.0 mL) water to elute the acid, followed by the addition of 2 × 2.0 mL of 2 M ammonium hydroxide and 1.0 mL of H_20 to elute amino acids. The latter fraction was collected into a glass vial, frozen, and lyophilized to dryness. The lyophilized amino acids were transferred to a V-vial in 400 μL of H_20. From this point, the samples were derivatized and analyzed for isotope enrichment by GC-MS as for blood amino acids.

GC-MS Analysis of Keto Acids and Lactate in Tissue

For GC-MS determination of the isotopomer labeling of TCA cycle keto-acids and lactate from tissues (mammary, liver, muscle, and whole intestines) the method of Des Rosiers et al. (1994) was employed. Approximately 40 mg of freeze-dried, pulverized tissue was placed into a 12 × 77-mm borosilicate culture tube, 3 mL of ice-cold 8% sulfosalicylic acid was added, and the sample was homogenized for 1 min with a VWR Powermax AHS 200 homogenizer. Samples were then centrifuged for 15 min at 4000 rpm (4 °C), and the supernatant was transferred to a 9.0-mL screw cap test tube. The pH of the samples was adjusted to pH of 6-7 with 2 M KOH (Sigma),

sonicated for approximately 15 min and then incubated for 60 min at 65 ºC. After incubation, the samples were acidified to pH <2.0 with 2 N HCl, followed by the addition 4 g of NaCl and vortex mixed. The keto-acids and lactate were back-extracted (2 times) into 3 mL of ethyl acetate and the ethyl acetate extractions combined into an 8-ml V-vial. The extract was dried under 2 psi N_2 at 40 ºC. To the dried residue was added 60 µL of a 1:1 mixture of ethyl acetate and N-methyl-N(t-butyldimethylsilyl)triflouroraoacetamide + 1 % dichlorodimethylsilane and the sample was incubated at 90 ºC for 20 min. This lead to the formation of tert-butyldimethylsilyl derivatives of the keto-acids and lactate, which were then monitored under electrical ionization mode by GC-MS analysis. TCA cycle intermediates were separated by gas chromatography (HP 6890; Agilent Technologies) using a fused silica capillary column (HC-5; 30 m x 0.25 m i.d.; Alltech Associates, Inc.) with helium as carrier gas (1.0 mL/min). The gas chromatography column was programmed as follows: an initial temperature of 100 ºC, with ramping by 30 ºC/min to 190 ºC, followed by 10 ºC/min ramping to 270 ºC, and finally 30 ºC/min to a final temp of 300 ºC with a 3 min hold time. Table 3 shows ions monitored and retention times. Various split modes, ranging from 1:1 to 25:1, were used for compounds with varying concentrations. An unknown source of ion contamination led to unusual labeling patterns in fumarate, preventing its analysis.

Table 3. Ions monitored for individual TCA cycle intermediates in mammary gland, liver, muscle, and intestinal tissues

	Ions Monitored[1] (m/z)	Retention Time (min)
Lactate	[M+0] **261** - [M+4] **265**	5.85
Pyruvate	[M+0] **274** - [M+3] **277**	6.40
Succinate	[M+0] **289** - [M+4] **293**	7.44
Fumarate	[M+0] **287** - [M+4] **291**	7.61
Malate	[M+0] **419** - [M+4] **423**	10.03
Oxaloacetate	[M+0] **432** - [M+4] **436**	10.54
α-ketoglutarate	[M+0] **446** - [M+5] **451**	11.37

[1]Ions monitored were *tert* -butyldimethylsilyl derivatives of the intermediates according to Des Rosiers et al. (1994)

GC-MS Analysis of Plasma Lactate

In order to determine the labeling of blood lactate, 0.5 mL of the water eluate from the cation exchange resin (see above) was used. This portion was acidified (50 µL of concentrated HCl) and lactate back extracted twice with 1.0 mL ethyl acetate. The combine ethyl acetate extractions were transferred to a V-vial and dried under a stream of N_2 gas at 40 ºC. For derivatization, 70 µL of N-methyl-N(t-butyldimethylsilyl)triflouroraoacetamide + 1 % dichlorodimethylsilane plus 30 µL of ethyl acetate were added and the sample incubated for 20 min at 90 ºC. Ions monitored (HP 6890; Agilent Technologies) for lactate were 261-264 with a retention time of 9.96 min. The column (HC-5; 30 m x 0.25 m i.d.; Alltech, Inc.) conditions were as follows: an initial temperature of 100 ºC, with ramping by 20 ºC/min to 120 ºC, followed by 5 ºC/min ramping to 160 ºC, and finally 50 ºC/min to a final temp of 280 ºC with a 1 min hold time.

Calculations and MIDA

Enrichments of single ions determined from GC-MS analysis were subjected to correction for the difference between theoretical and measured values based on natural isotopic abundance and the purity of our [U-^{13}C]glucose (Hachey, 1994; Fernandez et al., 1995). [M+X] refers to the enrichment of molecules with X number of ^{13}C molecules (versus ^{12}C). For example, [M+6]glucose refers to a glucose molecule with all six carbons labeled as ^{13}C. This glucose molecule is six mass units heavier than a fully unlabelled glucose molecule. Enrichments are reported as mol/100 mol of tracee, or tracer:tracee ratio (TTR), and means appear ± 1 standard deviation.

The following calculation was determined:

Proportion of glucose in milk lactose derived from blood glucose (Sunehag et al., 2002):

[M+6] lactose-glucose / [M+6] blood-glucose (eq. a)

Sample Preparation and Analysis via qRT-PCR

RNA Extraction

Due to the sensitive nature of RNA and the potential for contamination by RNAses, special care was taken with tissues collected for mRNA analysis. From the dissected tissues (liver and mammary) a sample of approximately 0.2 g was sub-sampled for total RNA isolation. Care was taken not to collect sub-sample sizes larger than 0.5 cm in any dimension as per the RNA*later* protocol provided by Ambion (Ambion, Inc., Austin, TX). Samples were submersed in 1.0 mL RNA*later* and placed at 4 °C overnight. Excess RNA*later* was decanted off and samples were frozen at -20 °C until total RNA extraction.

Approximately 100 mg of tissue was placed in 1.0 ml of TRIzol in a 5-mL collection tube. Samples were homogenized for 1 min each and then left to sit at room temperature for 5 min. The probe was washed between each sample with three molecular grade H_20 washes, three 70% ethanol washes, followed by a final TRIzol wash. Following homogenization, 200 µl of chloroform was added and the samples were left to sit at room temperature for 10 min, vortexing occasionally. Samples were then centrifuged for 15 min at 4 ºC. The top aqueous layer (RNA- containing) was then placed in a 1.5-ml RNAse-free microcentrifuge tube and 0.50 ml isopropyl

alcohol was added for RNA precipitation at room temperature for 10 min. Samples were then centrifuged for 10 min at 4 ºC to pellet the RNA. After the supernatant was poured off and discarded, 1.0 ml cold 75% ethanol was used to wash the RNA pellet by pipetting vigorously and vortexing the sample. The resuspended pellet was then centrifuged for 5 min at 4 ºC, after which the ethanol was removed carefully with a pipette. Tubes were then left open under a fume hood for further evaporation of the ethanol. After drying, 200 µl of molecular grade H_20 was added and the samples were placed at 55 ºC with continuous shaking to resuspend the RNA pellet. Some samples required higher dilutions and vigorous pipetting to breakup the RNA pellets. Samples were then heated for 5 min at 65 ºC to denature the RNA.

The samples were DNAse treated prior to qRT-PCR using the Turbo DNA-*free* kit (Ambion, Inc.). Concentrations of RNA were then determined on a NanoDrop spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, DE). RNA quality was assessed by running on a Bioanalyzer to look for degradation products (2100 BioAnalyzer, Agilent Technologies).

Primer Design

Primer3 software (Whitehead Institute/MIT Center for Genome Research) was used to design primers for the cytosolic and mitochondrial forms of the murine PEPCK enzyme. High sequence homology between the two forms required that sequences fall near the 3'end of the gene. Primers chosen can be found in subjected to a BLAST search in order to confirm proper gene selection. Sequencing of polymerase chain reaction-amplification products confirmed amplification of the desired gene targets. Sequencing was performed using a CEQ 8000 automated

Table 4 and were supplied by IDT, Inc. (Coralville, IA). Primer sequences were then

Table 4. Forward and reverse primer sequences used for murine PEPCK-c and PEPCK-m qRT-PCR

Gene Target	Nucleotide Sequence	Product Size (base pairs)
PEPCK-c Forward	5'-AAAACGCCTTGAACCTGAAA-3'	137
PEPCK-c Reverse	5'-GTAAGGGAGGTCGGTGTTGA-3'	
PEPCK-m Forward	5'-CCAGGAGCCACACCTGTTAT-3'	143
PEPCK-m Reverse	5'-CTGGCTTTGGTAGCACGATT-3'	

sequencer (Beckman Coulter, Fullerton, CA).

qRT-PCR

Reverse transcription (RT) reactions (20 µl) were done using 1000 ng of total RNA and the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA). Negative controls were also used which did not include reverse transcriptase to ensure no DNA contamination of samples. Two µl of each RT reaction was used for subsequent qRT-PCR. qRT-PCR was performed in a 25-µl reaction volume using SYBR Green iQ Supermix and an iCycler realtime detection system (Bio-Rad Laboratories, Inc.). Cycle parameters included denaturation at 95.0 ºC for 3 min, followed by 40 cycles of 94.0 ºC for 15 seconds, 56.8 ºC for 30 seconds, 72.0 ºC for 30 seconds, followed by melting curve analysis. A standard curve was generated using known quantities of gel-purified PCR products of the desired targets ranging from 10^2 to 10^6 copies. Gel purification of the products was done using the Qiagen gel purification kid according to kit instructions (Qiagen , Inc., Valencia, CA). Linearity and amplification efficiency were calculated by the iCycler software. A linearity of $\geq$ 0.995 and efficiency between 85 and 108% was deemed acceptable. Melting curve analysis was used to indicate amplification of a single product and absence of primer-dimers.

Statistical Analysis

Statistical analysis for the effect of time (tracer feeding) on enrichment of isotopes and isotopomers was done via repeated measures ANOVA. The PROC MIXED option of SAS (SAS Inc., Cary, NC), with the repeated option, was used.

Enrichments were treated as continuous, quantitative response variables and time of tracer feeding was the qualitative explanatory variable. In all cases, the compound symmetry covariance and variance structure in SAS was used.

Analysis for the determination of metabolic pools was completed using PROC TTEST option of SAS. Enrichment pools (i.e. blood glucose, lactose, etc.) were analyzed as qualitative explanatory variables with enrichments serving as continuous response variables. The following model was used:

$$\frac{\overline{X}_T - \overline{X}_C}{SE\left(\overline{X}_T - \overline{X}_C\right)}$$

Where:

$\overline{X}_T - \overline{X}_C$ is the difference in the group means and

$SE\left(\overline{X}_T - \overline{X}_C\right)$ is the standard error of the difference between the two group means.

Significance was set at $P \leq 0.05$ with $P \leq 0.10$ considered a trend.

Results

Growth and Production

Figure 4 shows pup growth rate over the five days of feeding [U-^{13}C]glucose. Average daily intake of the lactating dams over the period of tracer feeding was 8.09 (± 1.73) g. This corresponded to an average daily tracer intake of 0.59 (± 0.20) g. Dam weights also tended to increase over this time period (Figure 4). No health issues arose during the study and all dams and pups were in good health at the time of tissue sampling on day five.

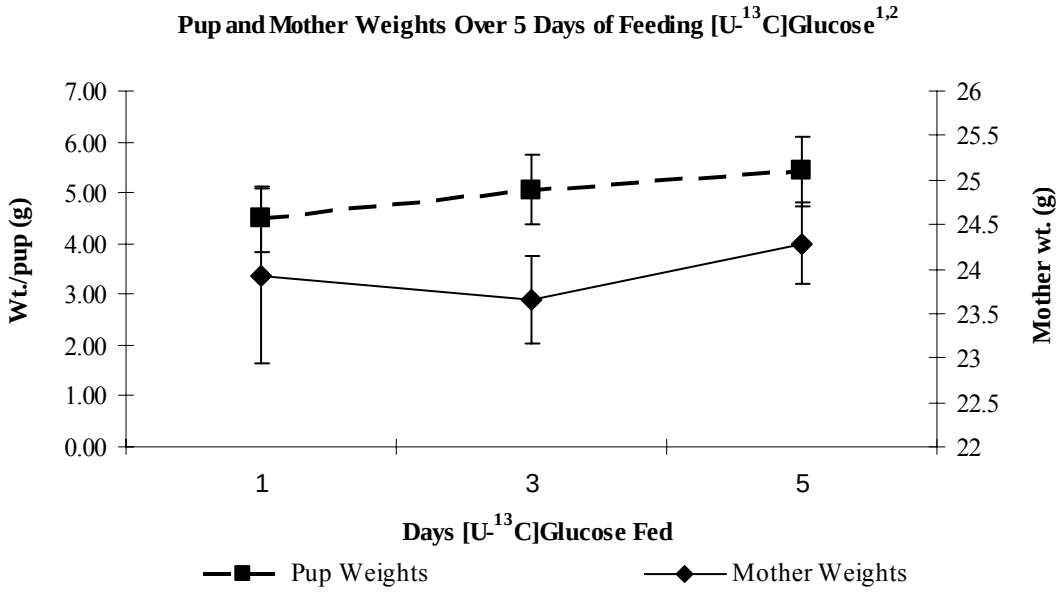


Figure 4. Pup and dam weights.

Weights were recorded at the time of milk collection after one, three, and five days of feeding [U-^{13}C]glucose as 10% of the dietary carbohydrate

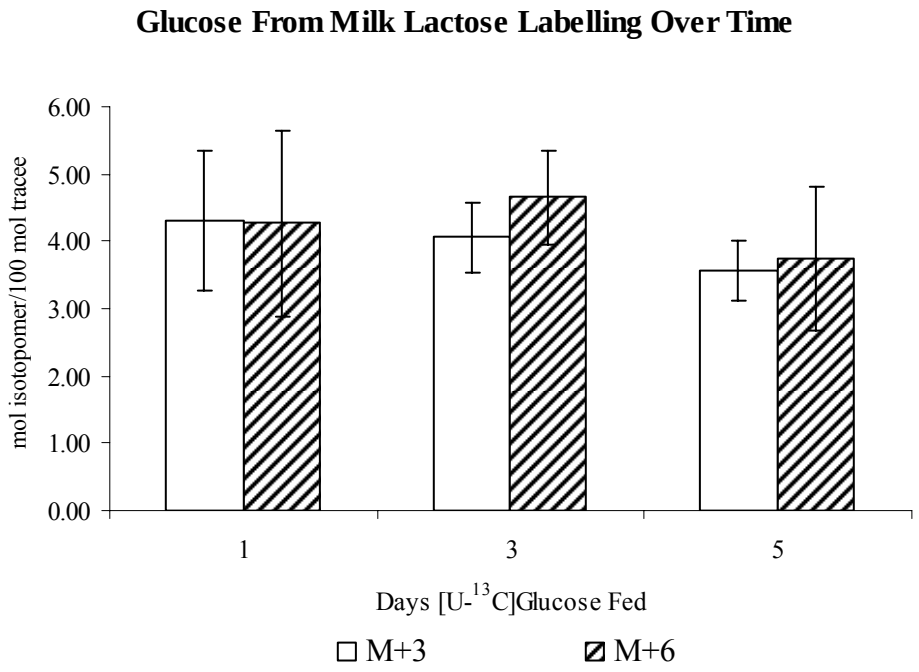
[1]Pup weight was recorded as the total litter weight (g)/number of pups and an average taken for all four litters on each day
[2]Dam weights are the average weight for all four dams on each day of sampling

Milk production is difficult to monitor in lactating mice. However, given that we saw an increase in average pup weight over the five days, as well as a trend for an increase in dam weights, we can assume good nutritional status for the mice on this study. Pup weights were similar to results seen in previous studies (Teter et al., 1992). The diet seemed to be meeting or exceeding all needs for growth and lactation.

Labeling of Blood Glucose and Milk Lactose and Casein

During the feeding of [U-^{13}C]glucose, isotopic (i.e. [M+6]) and isotopomer (i.e. [M+3]:[M+6]) plateau were determined in blood glucose and in milk lactose-derived glucose and galactose. Enrichments (TTR) were monitored over days one through five of tracer feeding. Using the MIDA approach explained in the previous section, the portion of fully ^{13}C-labeled ([M+6]) glucose and galactose in milk lactose was monitored, as well as the ratio [M+3]:[M+6] in each (Figure 5 and Figure 6, respectively).

In addition, NEAA from milk casein were monitored for changes in [M+3] for glutamate, alanine, and serine (Figure 7). The ratio of [M+2]/[M+3] in glutamate from casein was also monitored (Figure 8). No significant differences based on time of feeding tracer were found for any of the above mentioned measurements (P>0.10). [M+3] demonstrates isotopic plateau and [M+2]/[M+3] demonstrates the isotopomer plateaus. For both the hexoses and the NEAA, the demonstration of isotopomer steady state indicates equilibrium of ^{13}C skeletons throughout the major metabolic pathways.



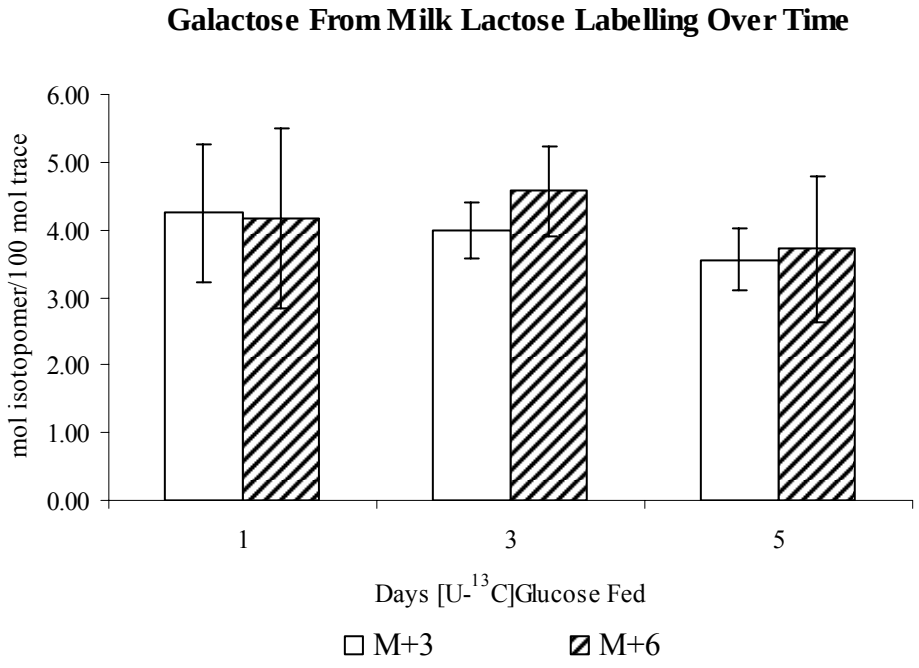


Figure 5. Enrichments of [M+3] and [M+6] in glucose and galactose from milk lactose.

Bars represent averages from milk of four mice; samples taken after one, three, and five days of feeding [U-^{13}C]glucose as 10% of the dietary carbohydrate. Error bars represent ± 1 standard deviation from the mean. No significant difference based on time of tracer feeding was found for any of the enrichments ($P > 0.1$).

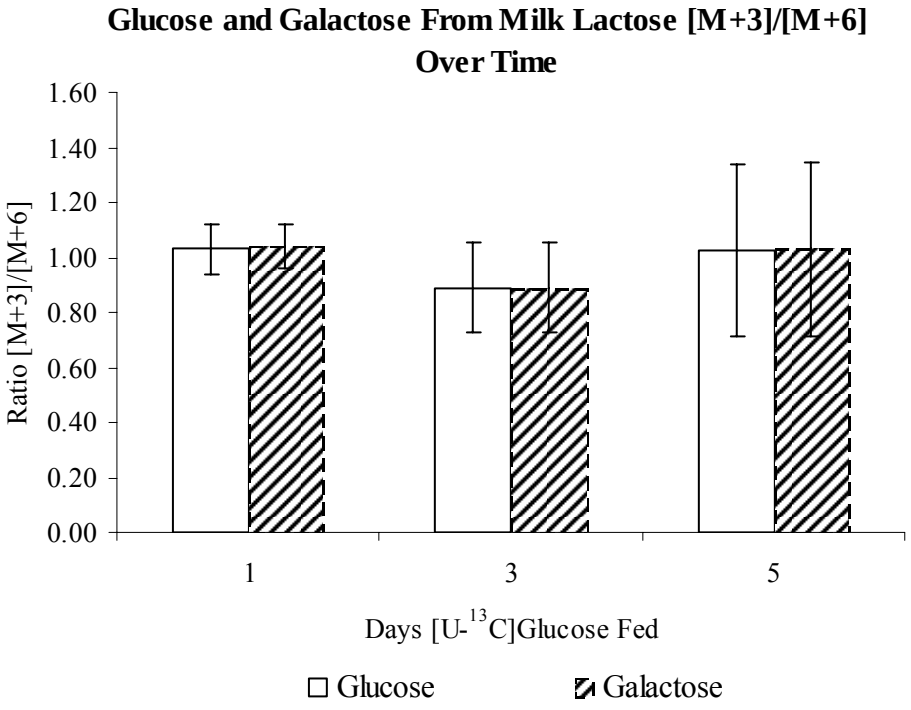


Figure 6. Relative enrichments of [M+3] and [M+6] glucose and galactose in lactose

Milk was collected after one, three, and five days of feeding [U-^{13}C]glucose at 10% of the dietary carbohydrate. Each bar represents the averages from milk of four mice. Error bars represent ± 1 standard deviation from the mean. No significant differences between days were observed (P>0.1).

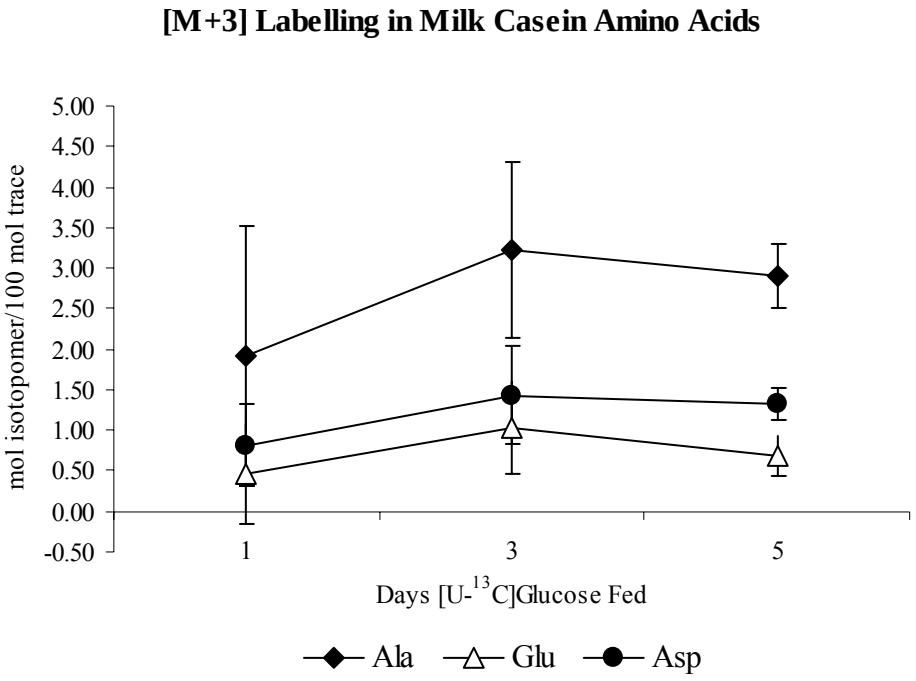


Figure 7. Enrichments of [M+3] in Alanine (Ala), Glutamate (Glu), and Aspartate (Asp) in milk casein

Milk was sampled after one, three, and five days of feeding [U-^{13}C]glucose at 10% of the dietary carbohydrate. Each line represents the averages from milk of four mice. Error bars represent ± 1 standard deviation from the mean. No significant difference based on time of tracer feeding was found for any of the enrichments ($P > 0.1$).

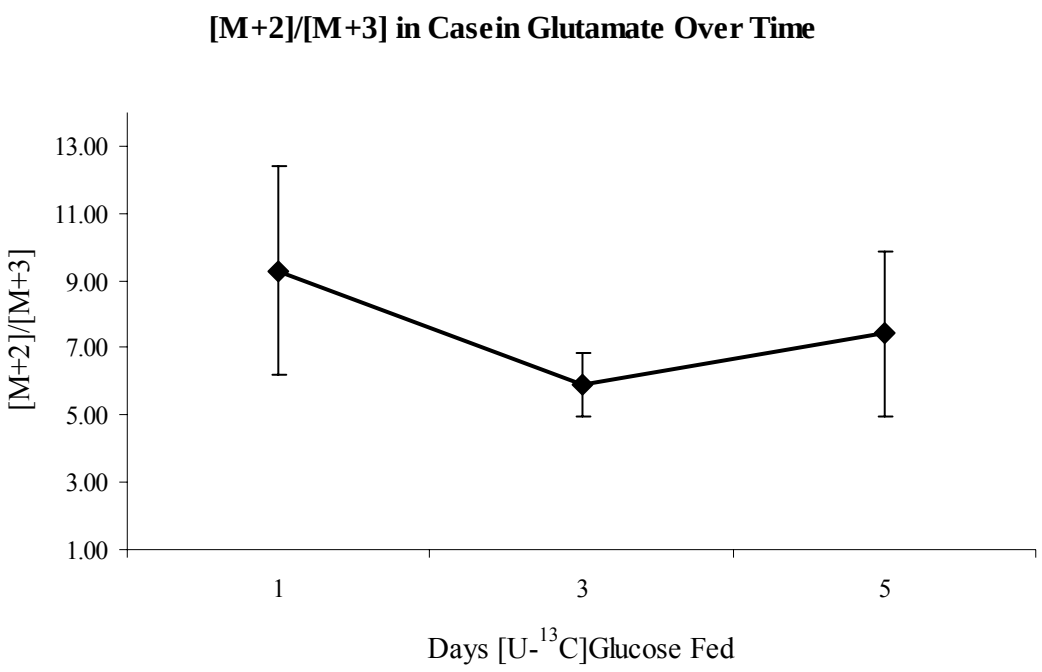


Figure 8. Ratios of enrichment of [M+2]/[M+3] Glutamate (Glu) in milk casein

Milk was sampled after one, three, and five days of feeding [U-^{13}C]glucose at 10% of the dietary carbohydrate. The line represents the averages from milk of four mice. Error bars represent ± 1 standard deviation from the mean. No significant differences between days were observed
($P > 0.1$).

Proportion of lactose-glucose from blood glucose

The proportion of lactose-glucose derived from blood glucose was determined by comparing [M+6] glucose in lactose to that in blood [M+6] glucose (Figure 9). On average, the enrichment of [M+6] glucose in lactose was 3.75 (± 1.07) TTR compared to 4.89 (± 0.84) TTR in blood glucose. Therefore, 76.7% (3.75/4.89) of glucose in lactose derived from blood glucose, or 23.3% of glucose in lactose was synthesized from carbon substrates other than blood glucose.

Metabolic pools for glucose and galactose in milk lactose

By comparing [M+6] in glucose and galactose from milk lactose, we can determine whether glucose and galactose were derived from the same metabolic pool within the mammary gland. The enrichment of [M+6]galactose (3.72 ± 1.07 TTR) did not differ from [M+6]glucose (3.75 ± 1.07 TTR) ($P = 0.8779$), indicating that synthesis of galactose in the mammary gland derives from the same metabolic pool as glucose in lactose (Figure 9).

Preliminary Data on Expression of PEPCK in Bovine Mammary Gland and Liver Tissues by qRT-PCR

Our preliminary work confirmed the presence of both isoforms of PEPCK in the bovine mammary gland and liver (Figure 10). PEPCK-c in the liver appears to be the primary form, while PEPCK-m seems to dominate expression in the mammary gland of the bovine. It is noted that mRNA expression of PEPCK-c in the bovine mammary was at levels (69 molecules mRNA/100 ng total RNA) below the lowest expression measured on the linear standard curve (100 molecules mRNA/100 ng total

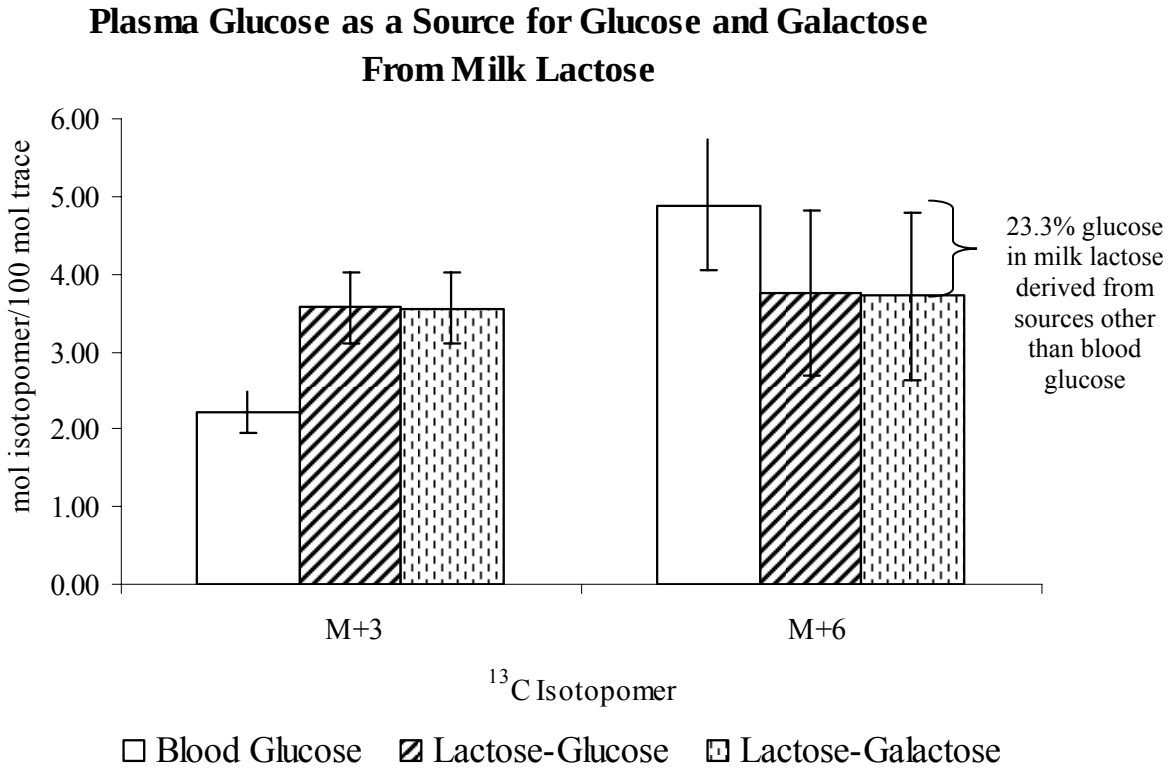


Figure 9. Enrichments of [M+3] and [M+6] in glucose and galactose from milk lactose and blood glucose.

Samples were taken after five days of feeding [U-^{13}C]glucose as 10% of the dietary carbohydrate. Bars represent averages from milk of four mice. Error bars represent ± 1 standard deviation from the mean. The [M+3] and [M+6] labeling of glucose and galactose from milk lactose were not statistically different ($P > 0.1$).

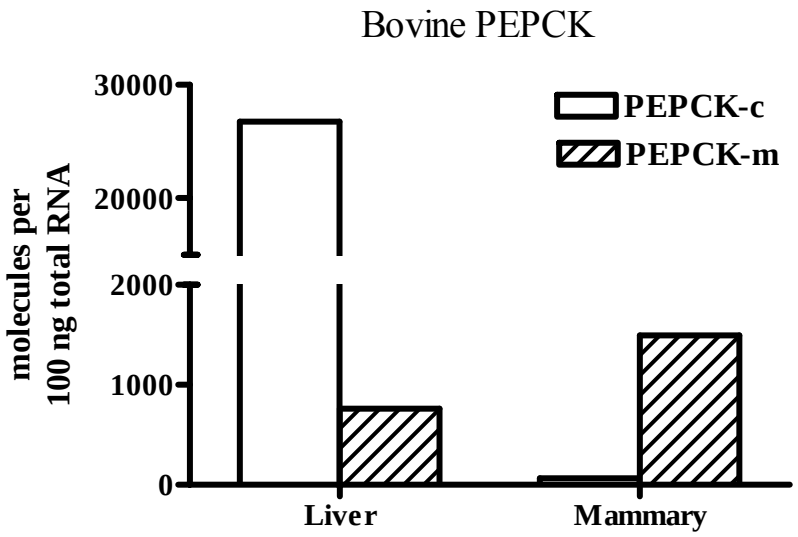


Figure 10. Preliminary data for the presence of PEPCK-c and PEPCK-m in the bovine mammary gland and liver.

Data shown for the two isoforms isolated from liver and mammary tissue of a lactating dairy cow. (Bequette et al., unpub.)

RNA). qRT-PCR efficiency for PEPCK-c analysis was 102.9% with an r^2 of 0.999. Efficiency for PEPCK-m was 101.1% with an r^2 of 0.996.

qRT-PCR of two PEPCK isoforms in murine liver and mammary gland

Despite individual variation, PEPCK-c gene expression in the murine liver was over 5,000-fold greater than PEPCK-m (n = 3 mice). By contrast, PEPCK isoform gene expressions in the mammary glands were similar (Figure 11) and significantly lower than in the liver.

Analysis confirmed amplification of a single product, as well as a lack of amplification of genes in reactions not including a reverse transcriptase. qRT-PCR amplification efficiency for PEPCK-c analysis was 107.1% with an r^2 of 0.995. Efficiency for PEPCK-m was 107.6% with an r^2 of 0.997.

Discussion

Time-course for isotopic enrichment

There are four assumptions that must be met when performing studies involving universally labeled stable isotopes. First, the tracer must be indistinguishable from the tracee during metabolism. Herein, [U-^{13}C]glucose was fed in the diet, and at a level (10% of diet dextrose) that will not lead to metabolic discrimination between labeled and unlabelled (natural) glucose. An exchange of 10% of the dietary hexose was also successfully utilized by Pascual et al. (1997).

Second, the stable isotope must be delivered in amounts that do not lead to a

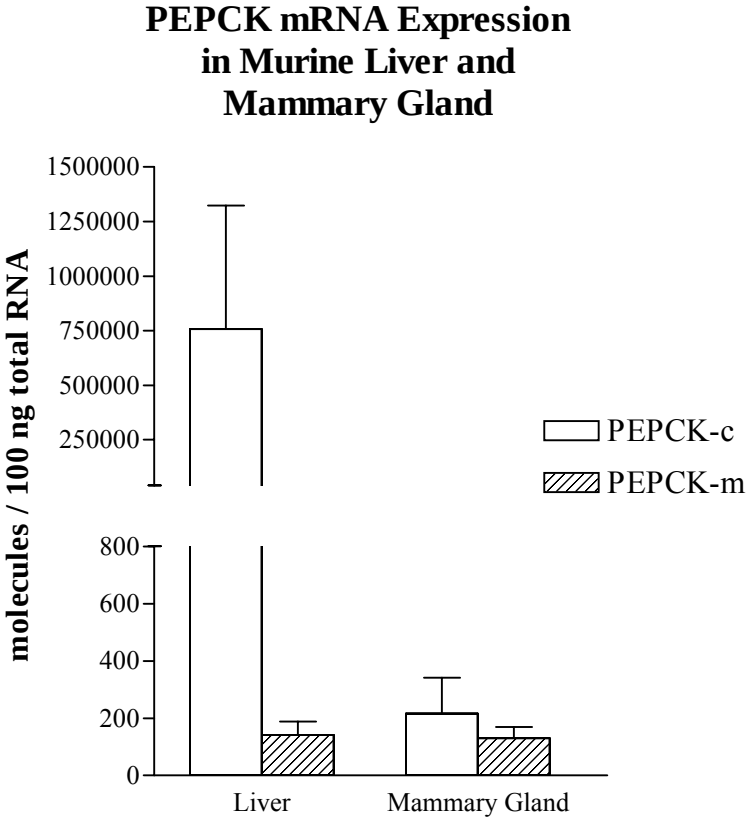


Figure 11. Gene expression for PEPCK-c and PEPCK-m in the murine liver and mammary gland determined by qRT-PCR

Values are the means from 3 lactating (day 11 of lactation, on average). Error bars represent ± 1 standard deviation from the mean.

substrate effect. This assumption was satisfied in the current study as the [U-^{13}C]glucose was added to the diet, replacing a portion of the natural dietary dextrose. Third, measurements of flux and nutrient turnover require that isotopic steady state or plateau in isotope enrichment is achieved. Based on previous studies, we hypothesized that isotopic plateau upon feeding of tracer in the diet would be reached after one day of tracer feeding. Pasqual et al. (1997) reached isotopic steady state in mouse blood isotopes within 12 h of feeding [U^{13}C]glucose. In the current study, isotopes (i.e. [M+6]glucose in blood and lactose) attained plateau by day three of feeding the labeled diet.

However, although steady state isotopic enrichment in [M+*n*] molecules (*n* = number of carbon atoms), the entire metabolic system may not be in equilibrium. The fourth requirement, if measurements of metabolic cycling (glucose carbon recycling, TCA cycle metabolism) are required, is that steady state must also be achieved for lower mass isotopomers (e.g. M+(*n*-1)) relative to the fully labeled molecule. In other words, as recycling occurs (via the TCA cycle, for example), shifts in the relative enrichments of [M+2], [M+3], etc. can occur. For example, during the administration of [U-^{13}C]glucose, the relationship [M+3]:[M+6]glucose must achieve a steady state to avoid underestimated glucose carbon recycling and in consequence underestimation of gluconeogenesis. Similarly, isotopomer steady state is also necessary for measurement of substrate fluxes into and out of the TCA cycle. Indeed, Pascual et. al. (1997) found that blood [M+1] glucose and [M+3] alanine did not reach plateau until after one day of feeding labeled glucose. Thus, sufficient time must be given for ^{13}C-carbon skeletons to cycle through the metabolic intermediates

of the TCA cycle and other connected pathways (i.e. glycolysis, NEAA synthesis, and catabolism). It is important, therefore, to also examine ratios for steady state as well.

In the current study, isotopomer steady state was achieved by day three for whole body and mammary tissue glucose (lactose) metabolism as well as NEAA metabolism in the mammary gland. For example, the ratio [M+2]:[M+3] in aspartate, alanine and glutamate in casein all achieved steady state by day three of feeding labeled glucose.

Since no significant differences in either isotopic enrichments or ratios of molecules derived from recycling were observed, we are confident that complete steady state had been achieved. That glucose, and also NEAA, had reached steady state was confirmed. That this is attained means that these data can be used to quantify the fluxes into and out of the major pathways by comparison of the labeling patterns. To ensure steady state in future studies, and to allow for adequate labeling of metabolites for measurement via GC-MS, it was determined that a tracer feeding schedule of three days would be adequate, and would facilitate accurate calculation of subsequent metabolic cycling relationships and(or) nutrient turnover rates. In this regard, all four requirements for the use of stable isotope tracers in predicting precursor:product relationships were met.

Mammary gland metabolism

The first question regarding general mammary gland metabolism that we sought to answer was how much of the glucose in lactose is derived from blood glucose versus other sources. Using the approach of Sunehag et al. (2002), the unequal [M+6] labeling in glucose from lactose compared to blood glucose suggests

that not all glucose in lactose is derived from blood glucose (Figure 9). Since [M+6] glucose in lactose can realistically only derive from [M+6] in the diet, the source of this glucose for the mammary gland must be from the diet via blood uptake. In the fed state in lactating women, Sunehag et al. (2002) found that a majority (almost 100% in the fed state) of the glucose in lactose derived from blood glucose. In the current study, blood [M+6] glucose was higher (by 30%, though not significantly so ($P = 0.1585$)) than glucose found from hydrolysis of lactose glucose. Although not statistically significant, a greater sampling size would be needed to substantiate this numerically larger value as significant. The difference in enrichment between blood glucose and lactose-glucose suggests that 23.3% of glucose in lactose is derived from sources other than blood glucose. In addition, since the enrichment was lower in lactose, the additional sources must be unlabelled (dilution). Furthermore, these conclusions hold true for galactose from lactose because it is derived from the same metabolic pool as lactose glucose in the mammary gland. A potential unlabelled source for mammary gland hexogenesis found in previous studies includes glycerol in the case of humans (Sunehag et al., 2002) and cows (Wood et al., 1958). Although not yet validated, other unlabelled sources could include amino acids and lactate.

By comparison of the enrichments observed in glucose and galactose from milk lactose, it is possible to determine their metabolic precursor pools. For example, if the enrichments (M+3, M+6) are the same for the two hexoses, then they must be derived from the same metabolic pool. In the present study, the labeling patterns of glucose and galactose in lactose were identical (Figure 9). That means then that the

unlabelled pool, separate from blood glucose, is contributing equally to glucose and galactose in milk lactose.

Presence of PEPCK Isoforms

In the present study, we also assayed gene expression of the PEPCK isoforms in the bovine and mouse liver and mammary gland to further verify the similarities of their metabolism. In addition, verifying the gene expression of the PEPCK isoforms was important to demonstrating the roles of these pivotal enzymes in mammary gland hexoneogenesis versus TCA cycle flux. Though activities of both forms have been suggested in the bovine mammary gland (Scott et al., 1975), no one to date has measured PEPCK at the level of gene expression in this tissue. Our preliminary findings in the bovine appear to be similar to the rat in that, as far as the liver is concerned, the cytosolic form of PEPCK is dominant (97% of total expression) (Figure 10). We can assume that this relative hepatic expression should correlate closely with actual activity based on the work of Agca et al. (2002), but questions about potential differences in translational efficiency and mRNA stability of the two isoforms weaken these assumptions. Possibilities do exist, however, to make parallel comparisons between the two species with regards to relative PEPCK gene expression in the liver. We can also combine the gene expression data with fluxes seen in metabolism based on MIDA results to further quantify changes in PEPCK activity.

On the other hand, gene expression of PEPCK-m seems to dominate PEPCK-c in the bovine mammary gland (Figure 11). We found that PEPCK-m in the bovine mammary gland was 96% of total PEPCK gene expression, comparing well to the

activity measurements of Scott et al. (1975) where PEPCK-m accounted for 88% of total activity in bovine mammary tissue. The low level of mRNA expression for bovine PEPCK-c requires further investigation into the presence of this isoform in the bovine mammary gland. Above all it seems that the relative abundance of the two PEPCK isoforms are opposite in the bovine liver versus mammary gland (the same isoform does not dominate in both tissues). Jones et al. (1989) saw similar results in guinea pig mammary gland PEPCK activity; as the mitochondrial form dominated (and was highly regulated during lactation). What this might mean metabolically is not clear. It is interesting to note that if indeed the bovine and guinea pig show different proportions of PEPCK isoform gene expression (or activity) in the mammary gland versus liver, this contradicts previous suggestions that the proportion of activity is generally the same in all tissues in a given species (Hanson and Patel, 1997). One must also be cautious to compare enzyme gene expression and activity as regulation could be occurring (though not yet proven for PEPCK) beyond the level of gene expression.

The relative gene expression of the PEPCK isoforms in the lactating mouse liver was similar to those that we observed in the bovine liver. Agca et al. (2002) found greater proportions of PEPCK-c:PEPCK-m gene expression in bovine liver tissue at various stages of lactation. Unlike in the bovine, PEPCK-c is the dominant form in the liver in mouse and PEPCK-c and PEPCK-m seem to have similar gene expression patterns in the murine mammary gland (Figure 11). García-Ruíz et al. (1983) measured PEPCK-c (only) in rat mammary gland and liver and found that activity developed rapidly in the mammary gland and liver at the onset of lactation.

Unfortunately, in our case, we have no base measurement (pre-partum) to compare increases in PEPCK that occur due to lactation. Our data does corroborate with the García-Ruíz et al. (1983) work in that they also saw a much larger relative activity of PEPCK-c in the liver versus mammary gland. It is still unclear what this may mean in relation to mammary gland metabolism, and how different responses amongst species (bovine, guinea pig, murine) and tissues (liver versus mammary gland) may relate to metabolic function.

Conclusions and Future Implications

In conclusion, we have substantiated the use of C57BL/6 mice as a model for mammary gland metabolism. Furthermore, we confirmed that the use of a [U-^{13}C]glucose tracer in the diet, at a level of 10% of the dietary carbohydrate, allows for accurate measurement of various aspects of metabolism via the MIDA approach. Three days of feeding the [U-^{13}C]glucose tracer was adequate to reach steady state in isotopes and isotopomers measured in milk and tissues, as well as to label intermediates in proportions large enough to be accurately measured via GC-MS. We also confirmed the presence of the two PEPCK isoforms in the bovine (although PEPCK-c requires further examination) and murine mammary gland and liver, allowing for future investigation of these isoforms and their role in mammary gland metabolism.

This study also provided opportunities to make estimates directly related to milk synthesis. The discovery that 23% of glucose in milk lactose is derived from sources other than blood glucose is interesting, as is the finding that glucose and galactose in milk lactose seem to be derived from the same precursor pool. These

results lead to several follow-up questions: What are the sources, other than blood glucose, that are contributing to milk lactose synthesis? How are glucose and galactose synthesis regulated if they are derived from the same precursor pool? What is the role of PEPCK in the regulation of these and other metabolic pathways in the mammary gland? In addition, questions also remain regarding the contribution of NEAA to glucose carbon in the mammary gland.

This study has laid the groundwork for the work to be discussed in Chapter 3, as well as countless other possibilities. Chapter 3 will test similar hypotheses as mentioned in this study, as well as examine the effects of altered dietary protein level on mammary gland metabolism and milk synthesis. Since nutritional manipulation of milk components is a viable option commercially, the effects of nutrition will first be tested using the mouse and MIDA techniques developed here. Besides nutritional regulation, the effects of hormone regulation could be examined using similar approaches. More intricate gene technologies such as the incorporation of gene knock-out mice or small interfering RNA will provide greater opportunities to examine the role of PEPCK in the future.

Chapter 3: Effect of dietary protein and carbohydrate level on mammary gland metabolism in C57BL/6 mice

Introduction

The mammary gland is a unique model for the study of the intersecting pathways of metabolism. Milk synthesis requires complex regulation of the pathways involving glucose, protein, and fat metabolism. In addition, the precursors supplied, and their relationship to output demands, is not only constantly changing, but often limiting. Therefore, further knowledge of these complex relationships is required.

A few key observations illustrate that the coordination of milk synthesis is not fully understood. First, though it seems logical that most, if not all, glucose in milk lactose comes from blood glucose, evidence suggests that other precursors may be contributing to milk lactose. These "other" precursors have been shown in part to be glycerol in the case of the humans (Tigas et al., 2002; Sunehag et al., 2003) and dairy cows (Scott et al., 1975). This requires that the mammary gland synthesize lactose *de novo* from sources other than blood glucose, and we suggest that amino acids may serve as an additional source of glucose carbon. Our hypothesis is that this *de novo* synthesis also occurs in the murine mammary gland, and the use of non-glucose precursors for lactose synthesis will vary with dietary protein (amino acid) treatment. Our mouse model, along with the application of the MIDA techniques described in detail in Chapter 2, allow us to quantify the amount of *de novo* mammary gland synthesis of lactose. We hypothesize that under conditions of lower dietary protein, a reduction in amino acid supply will result in impaired ability of the mammary gland to support casein and lactose synthesis.

Use of non-glucose precursors (i.e. amino acids and glycerol) for lactose synthesis would require greater relative activity of PEPCK-c versus PEPCK-m. PEPCK-c promotes the channeling of amino acids out of the mitochondria towards gluconeogenesis (or glyceroneogenesis) (Hanson and Reshef, 2003). Thus, PEPCK activity is likely to be associated with the regulation of the use of amino acids for lactose synthesis, and it is important to quantify the relative expression of the two isoforms in the murine mammary gland and liver. With reduced amino acid supply from the diet, PEPCK-m should promote the use of amino acids for casein synthesis.

Our second observation of mammary gland metabolism is that several NEAA (especially glutamate/glutamine, aspartate/asparagine, serine and proline) are taken up by the mammary gland in quantities less than what is required for milk synthesis (Fleet and Mepham, 1985). The logical source for these amino acids is *de novo* synthesis in the mammary gland with EAA serving as the main precursors. However, as has been discussed already in more detail in Chapter 2, the interconnectivity of glucose and amino acid metabolism requires intricate regulation of several pathways involved in milk synthesis. There is potential for involvement of the use of glucose for NEAA synthesis. In this case, PEPCK-m activity would exceed PEPCK-c and regulate the recycling of amino acid carbon within the mitochondria and towards amino acid synthesis (Hanson and Reshef, 2003). This scenario would hold when dietary amino acids are limiting.

We were able to substantiate the use of the mouse whole animal model for investigating mammary gland metabolism in Chapter 2, and the [U-^{13}C]glucose tracer approach was validated. The following experiment will test the hypothesis that not

all lactose is derived from blood glucose, and that glucose carbon may also contribute to NEAA within the mammary gland. The effect of dietary protein on these relationships will be examined, and the relationship of this regulation to relative PEPCK isoform gene expression will also be investigated.

Materials and Methods

Animal Care

The details of the animal care protocol were reviewed and accepted by the University of Maryland Institutional Animal Care and Use Committee (Protocol #R-05-57). All procedures were followed as in Chapter 2, with a few exceptions detailed below. A total of 12 C57BL/6 mice, obtained from the University of Maryland Department of Animal and Avian Sciences breeding colony, were used for this experiment. At the time of milk sampling, mice ranged in age from three to seven months and weighed 27.14 (± 2.46) g (n = 12). For breeding, three females at a time were placed in a cage with one male. Several days prior to predicted parturition date, females were placed into individual plastic shoebox cages. Mice were checked daily for food, water, and health status. Cages were cleaned once weekly.

Diets and Feeding

Prior to parturition, mice were fed a standard lab animal diet in pelleted form (Lab Diet 5001 Rodent Diet, PMI Nutrition International, LLC, Brentwood, MO). Mice were placed on experimental diets within four days of parturition. In all cases, the diets were based on recommendations of the National Research Council Dietary Recommendations for Laboratory Animals (NRC, 1995). Each mouse was fed either a normal (20%) or low (10%) protein diet. Compositions of diets are shown in Table

5. Diet ingredients were mixed as described in Chapter 2. During the three days of tracer feeding (three days prior to sampling), [U-^{13}C]glucose replaced 10% of the total dietary dextrose.

Experimental Design and Sample Collection

A randomized paired experimental design was used to test the effect of dietary protein (categorical explanatory variable with two levels, normal (N) and low (L)) on metabolic responses measured via [U-^{13}C]glucose tracer methodology employing GC-MS, and PEPCK isoform gene expression using qRT-PCR.

Dams were monitored twice daily for parturition, and the day that pups were first present in the nest was considered day one of lactation. Within four days of parturition, dams were assigned randomly to either the low (L) or normal (N) protein groups. In addition, dams on the N diet were paired with an L counterpart such that the N dam was offered the amount of feed consumed by the L individual the previous day (within approximately 0.3 g). The L mice were fed *ad libitum*. Pairs were also adjusted for number of pups, and additional pups were euthanized via submersion in isoflurane gas so that each individual in the pair had the same number of pups. Daily intakes were recorded.

Mice averaged day 15 (day 13-16) of lactation at sampling. The unlabelled diets (both normal and low protein levels) were exchanged with diets containing [U-

Table 5. Composition of Experimental Diets

Ingredient	Normal Protein (20%) Unlabelled Diet g/100g	Normal Protein (20%) Labelled Diet g/100g	Low Protein (10%) Unlabelled Diet g/100g	Low Protein (10%) Labelled Diet g/100g
Dextrose[1]	60.00	54.00	70.00	63.00
[U-^{13}C]Glucose[5]	-	6.00	-	7.00
Vit. Free Casein[1]	20.00	20.00	10.00	10.00
Solka-Floc[2]	5.00	5.00	5.00	5.00
Soybean Oil[1]	9.00	9.00	9.00	9.00
AIN 93 Mineral Mix[3]	4.00	4.00	4.00	4.00
AIN 76 Vitamin Mix[4]	1.50	1.50	1.50	1.50
Methonine[1]	0.30	0.30	0.30	0.30
Choline Bitartrate[1]	0.20	0.20	0.20	0.20

[1]Source: Dyets, Inc.

[2]Source: International Fiber Corporation

[3]Source: MPBio

[4]Source: ICN Biomedicals, Inc.

[5]Source: Cambridge Isotope Labratories, Inc.

^{13}C]glucose three days prior to sampling, based on previous results (Chapter 2). Collections proceeded as described in Chapter 2.

Sample Preparation and Analysis via GC-MS

GC-MS analysis was performed on galactose and glucose hydrolyzed from milk lactose, blood glucose, amino acids derived from milk casein, liver and mammary tissue, blood amino acids, and TCA cycle intermediates from liver, mammary gland, muscle and intestinal tissue as described in Chapter 2.

Calculations and MIDA

Calculations were as described in Chapter 2 with the following additions:

Glu = Glutamate
Ala = Alanine
Glc = Glucose

Proportion of milk casein NEAA derived from blood NEAA:

[M+3]NEAA from blood / [M+3]NEAA from casein (eq. b)

Proportion of milk casein NEAA derived from sources other than blood NEAA (theoretical maximum for mammary gland *de novo* synthesis):

(1 – eq. b) (eq. c)

***De novo* synthesis in the mammary gland, assuming no contribution of blood NEAA** (Glu or Ala):

Glu:
(2 × [M+3]Glu) / ((0.5 × [M+3]Glc) + [M+6]Glc)) (eq. d)

Ala:
[M+3]Ala / ((0.5 × [M+3]Glc) + [M+6]Glc)) (eq. e)

***De novo* NEAA synthesis in the mammary gland, correcting for the potential contribution of blood NEAA**:

$$\frac{([M+3]\text{NEAA in casein} - [M+3]\text{NEAA in blood})}{((0.5 \times [M+3]\text{Glc}) + [M+6]\text{Glc}))} \quad \text{(eq. f)}$$

NEAA synthesized *de novo* from glucose:

(eq. c × eq. f) (eq. g)

NEAA synthesized *de novo* from other amino acids:

eq. c × (1 – eq. f) (eq. h)

Minimum fractional gluconeogenesis, for either liver (whole-body) or mammary gland pools (Pascual et al., 1997) :

$$\frac{([M+1]\text{Glc} + (2 \times [M+2]\text{Glc}) + (3 \times [M+3]\text{Glc}))}{([M+1]\text{Glc} + (2 \times [M+2]\text{Glc}) + (3\times [M+3]\text{Glc}) + (6 \times [M+6]\text{Glc})} \quad \text{(eq. i)}$$

3-carbon dilution factor (Pascual et al., 1997):

$$\frac{([M+1]\text{Glc} + (2 \times [M+2]\text{Glc}) + (3 \times [M+3]\text{Glc}) + (6 \times [M+6]\text{Glc})}{2 \times ([M+1]\text{Ala} + (2 \times [M+2]\text{Ala}) + (3 \times [M+3]\text{Ala}))} \quad \text{(eq. j)}$$

TCA cycle dilution factor (Pascual et al., 1997):

$$\frac{3 \times ([M+1]\ \text{Glc} + [M+2]\text{Glc} + [M+3]\text{Glc})}{([M+1]\text{Glc} + (2 \times [M+2]\text{Glc}) + (3 \times [M+3]\text{Glc}))} \quad \text{(eq. k)}$$

Fractional gluconeogenesis (Pascual et al., 1997):

(eq.i × eq. j × eq. k) (eq. l)

Acetyl-CoA from pyruvate (Pascual et al., 1998):

$$\frac{[M+2]Glu - (2 \times [M+3]Glu)}{[M+3]Ala + (0.5 \times [M+2]Ala)} \quad \text{(eq. m)}$$

Acetyl-CoA from glucose (Pascual et al., 1998):

$$\frac{[M+3]Ala}{[M+6]Glc + (0.5 \times [M+3]Glc)} \times \text{eq. m} \quad \text{(eq. n)}$$

Glycolysis versus OAA to PEP:

[M+3]Ala / [M+2]Ala (eq. o)

Pyruvate recycling:

$$\frac{[M+1]Ala + [M+2]Ala}{[M+1]Ala + [M+2]Ala + [M+3]Ala} \quad \text{(eq. p)}$$

Glutamate from Alanine (Pascual et al., 1998):

$$\frac{2 \times [M+3]Glu}{[M+3]Ala} \quad \text{(eq. q)}$$

Glutamate from Pyruvate (Pascual et al., 1998)

$$\frac{[M+1]Glu + [M+2]Glu + [M+3]Glu}{[M+1]Ala + [M+2]Ala + [M+3]Ala} \quad \text{(eq. r)}$$

Pyruvate dehydrogenase activity verssu pyruvate carboxylase activity:

$$\frac{[M+2]Glu}{[M+3]Glu} \quad \text{(eq. s)}$$

Sample Preparation and Analysis via qRT-PCR

RNA Extraction

RNA was extracted using the RNAeasy Lipid Tissue Midi Kit (Qiagen , Inc.) according to kit instructions. On average, 0.151 (+/- 0.01) g of tissue was placed in 3.0 ml of Qiazol. Tissue samples were homogenized for one min each using a conventional Rotor-Stator homogenizer and left to sit at room temperature for five min. Between samples, the probe was washed three times each with molecular grade H_20 and 70% ethanol, followed by a final wash with Qiazol. Following homogenization, 600 µl chloroform was added, the sample shaken vigorously by hand for 15 seconds, and left to sit at room temperature for three min. Next, samples were centrifuged for 15 min at 4ºC and 5,000 × g. The top aqueous layer (RNA-containing) was then placed into a new collection tube and one volume (approximately 1300 µl) of 70% ethanol was added. Samples were then transferred to an RNAeasy Midi Spin Column placed in a 15-mL collection tube. The column and tube were centrifuged for five min at 25 ºC (5,000 x g) and the flow through in the collection tube was discarded. The buffer RW1 (2.0 ml) (supplied) was added to the top of the column and the column was once again centrifuged for 5 min at 25 ºC (5000 x g). Flow through was discarded.

In order to DNAse treat the samples for qRT-PCR, an additional DNAse treatment was employed (RNase-Free DNase Set, Qiagen, Inc.). For preparation, 20 µl DNase 1 stock solution was added to 140 µl buffer RDD and gently mixed. The 160 µl of solution was applied directly to the column and left to sit for 15 min at room temperature. Two ml of buffer RW1 was applied to the column and again centrifuged at 5,000 × g for five min at 25 ºC. Next, 2.5 ml of RPE buffer was added to the column and centrifuged at 5,000 × g for two min at 25 ºC. The effluent flowing through the column was discarded. Again, 2.5 ml of RPE buffer was added to the column and centrifuged as before. Flow through was once again discarded and the column spun dry for two min at 25 ºC (5000 × g). Finally, a fresh 15-ml collection tube was used and 150µl of RNase-free water was added directly to the column and left to stand at room temperature for one min. The column was then centrifuged for three min at 25 ºC (5000 x g). The sample, containing RNA, was then transferred to a clean nuclease-free microcentrifuge tube and placed in a wet bath at 65ºC for five min to remove RNA secondary structure. RNA concentrations and purity of the samples were then determined using a NanoDrop spectrophotometer.

Primer Design

Primer design was as described in Chapter 2.

qRT-PCR

qRT-PCR continued essentially as described in Chapter 2. A standard curve was generated using known quantities of gel-purified PCR products of the desired targets ranging from 10^1 to 10^6 copies. Gel purification of the products was done using the Qiagen gel purification kid according to kit instructions (Qiagen , Inc.).

Linearity and amplification efficiency were calculated by the iCycler software. A linearity of $\geq$ 0.995 and efficiency between 85 and 109% was deemed acceptable. Melting curve analysis was used to indicate amplification of a single product and absence of primer-dimers.

Statistical Analysis

Statistical analysis for the effect of dietary protein on continuous response variables (enrichment, fractional gluconeogenesis, etc.) was conducted using the PROC MEANS option of SAS (SAS Inc., Cary, NC) with the test of difference between paired individuals. Dietary protein was treated as a qualitative explanatory variable, with two levels. The following model was used:

$$\frac{\overline{X}_T - \overline{X}_C}{SE\left(\overline{X}_T - \overline{X}_C\right)}$$

Where:

$\overline{X}_T - \overline{X}_C$ is the difference in the group means and

$SE\left(\overline{X}_T - \overline{X}_C\right)$ is the standard error of the difference between the two group means.

Significance was set at $P \leq 0.05$ with $P \leq 0.10$ considered a trend.

Results

Growth and Production

Pup numbers for each pair-fed set of dams remained equal throughout the study. Dams fed the L diet consumed the same amount of diet as their pair-fed N diet

counterpart (L diet: 9.48 ± 1.63 g/d, N diet: 9.33 ± 1.63 g/d). On the day of milk sampling and tissue collection (d 13-16 of lactation) dams on the N diet (n = 6) weighed 28.67 (± 2.17) g while dams fed the L diet (n = 6) weighed significantly (P = .0007) less at 25.3 (± 1.22) g. Weight per pup was also significantly (P = 0.018) lower for the L diet-fed litters (5.86 ± 0.66 g/pup) compared to the N diet-fed litter (7.27 ± 0.73 g/pup). Dam weight and pup weight average were only available for five mice on the L diet; values were not recorded for one mouse.

Mammary Gland Metabolic Pools for Lactose Synthesis

The [M+6] in glucose and galactose from milk lactose were compared to determine the similarities in their metabolic precursor pools. The results of this study were in agreement with those in Chapter 2 in that glucose and galactose had the same [M+6]-labeling patterns (Figure 12). No significant differences were observed between the [M+3] and [M+6] enrichments in glucose and galactose. The ratio [M+6]glucose:[M+6]galactose in lactose tended (P = 0.0662) to be higher for dams fed the N diet (1.04 ± 0.05) compared to those fed the L diet (0.99 ± 0.05). The [M+6] in glucose and galactose from lactose had a lower enrichment for the N group (N: 4.21 ± 0.27 TTR versus L: 5.66 ± 0.77 TTR; P = 0.0065).

Labeling in Glucose from Lactose as a measure of glucose recycling

Since all of the [M+6] in glucose from milk lactose must be derived from the diet (via blood glucose taken up by the mammary gland), the ratio of [M+3]:[M+6] in glucose from lactose is a rough measure of the amount of glucose carbon recycling.

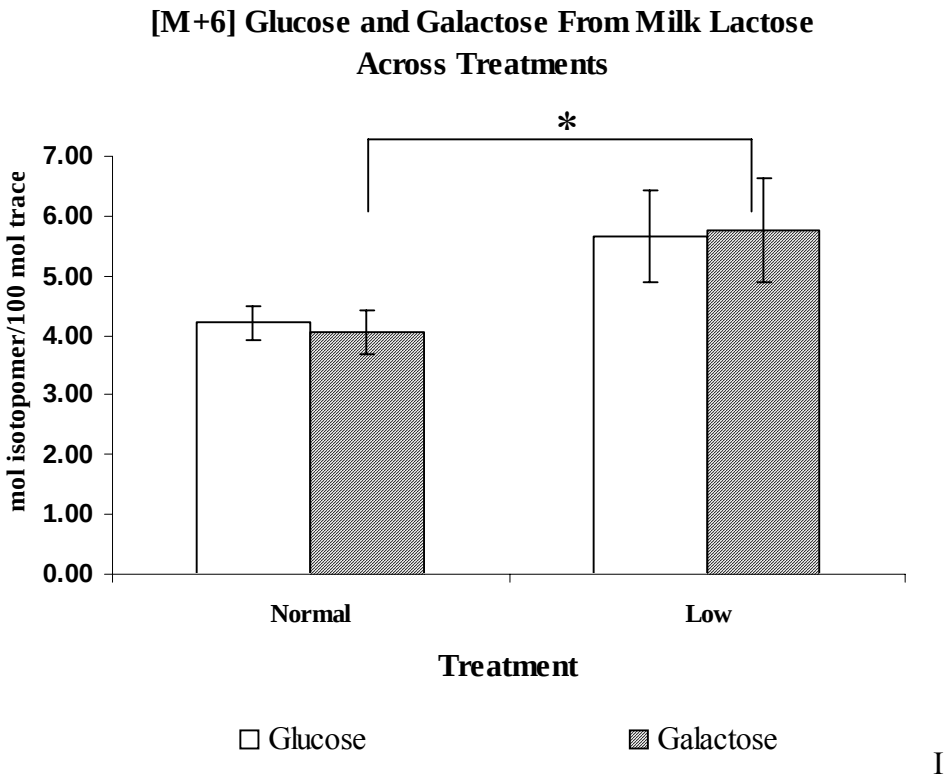


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Figure 12. The enrichments of [M+6] in glucose and galactose from milk lactose.

Bars represent averages from pair-fed mice (n=5 for Low group, 10% dietary protein, n=6 for Normal group, 20% dietary protein); samples taken after 3 days of feeding [^{13}C]glucose at 10% of the dietary carbohydrate. Normal group was fed 20% dietary protein; Error bars represent ± 1 standard deviation from the mean.
*Indicates a significant difference (P= 0.0065).

This ratio was higher for the N group (0.91 ± 0.1) than the L group (0.64 ± 0.13; P = 0.0052; Figure 13). In addition, [M+3] in glucose from lactose was higher overall than the [M+3] in blood glucose for both treatments (Lactose-Glucose: 3.69 ± 0.29 TTR; Blood Glucose: 2.00 ± 0.71 TTR; P = <.0001). There was a trend for [M+3] in lactose-glucose to be higher for the N group than the L group (N: 3.82 ± 0.32 TTR; L: 3.53 ± 0.31 TTR; P = 0.0989, Figure 14), but no significant difference in [M+3] in blood.

Potential for mammary gland de novo synthesis of NEAA

Comparing the [M+3] in NEAA from blood and milk casein allows us to estimate the total potential casein NEAA synthesis from blood or other sources. Approximately 50% of casein glutamate appears to be derived from blood glutamate for both treatments (P = 0.8791), while 27-32% appears to be derived from sources other than the blood for alanine (P = 0.7400). A treatment effect exists for serine in that 82% of casein serine is derived from blood serine for the N group, while 62% is derived from blood serine for the L group (P = 0.0332) (Figure 16). Further calculations allow us to determine what proportion of the NEAA synthesized within the mammary gland (*de novo*, or not derived from blood NEAA) is derived from glucose (Table 6).

Gluconeogenesis

Potential gluconeogenesis for various pools (liver, mammary tissue) was calculated. Acetyl-CoA from glucose, acetyl-CoA from pyruvate, the balance of glycolysis versus conversion of oxaloacetate to phosphoenolpyruvate, a measure of

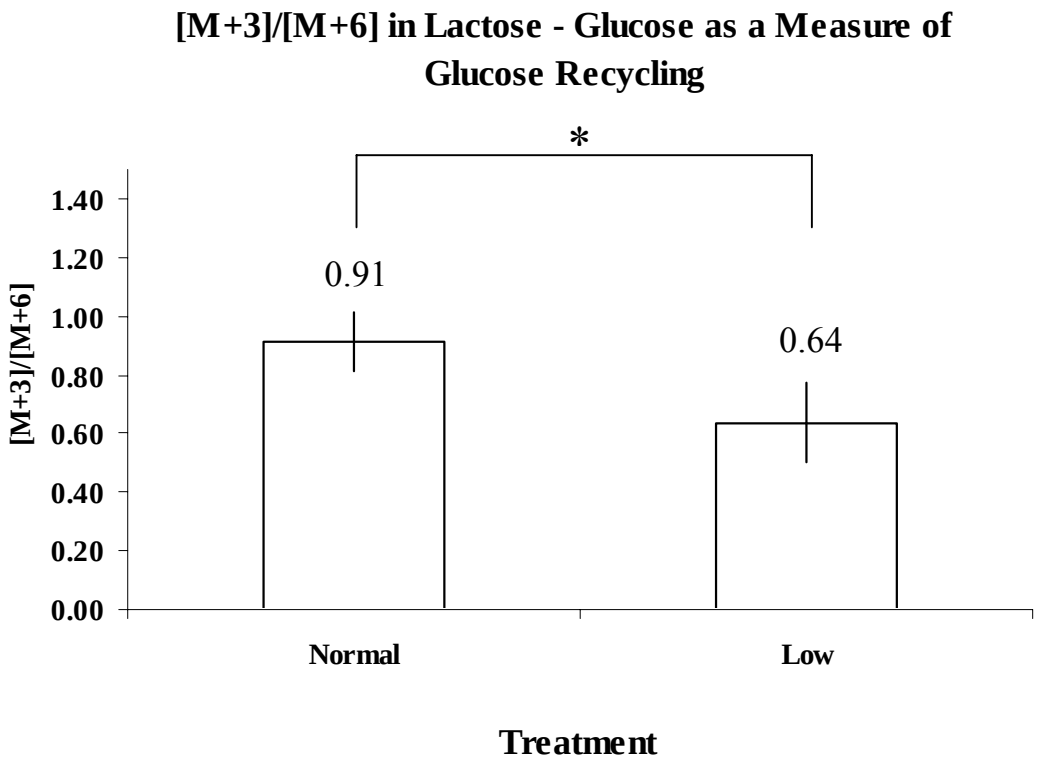


Figure 13. The ratios of enrichments of [M+3]/[M+6] in glucose from milk lactose.

Bars represent averages from pair-fed mice (n=5 for Low group, n=6 for Normal group); samples taken after three days of feeding [U-^{13}C]glucose as 10% of the dietary carbohydrate. Normal group was fed 20% dietary protein; Low group was fed 10% dietary protein. Error bars represent ± 1 standard deviation from the mean.
*Indicates a significant difference (P = 0.0052).

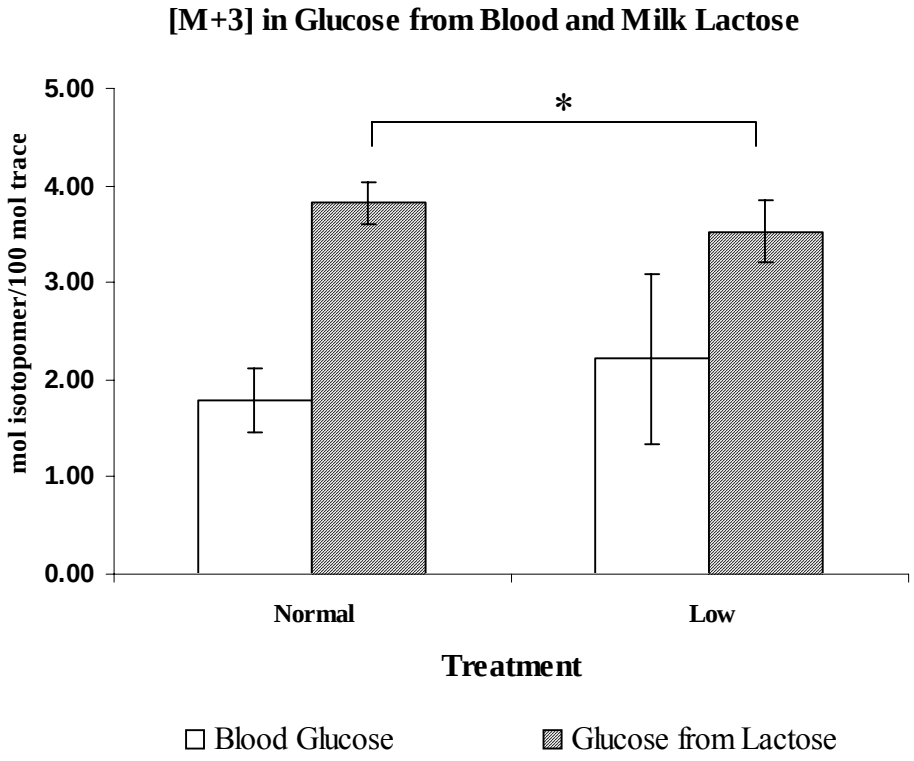


Figure 14. Enrichments of [M+3]glucose in blood and in milk lactose.

Bars represent averages from pair-fed mice (n=5 for Low group, n=6 for Normal group); samples taken after three days of feeding [U-^{13}C]glucose as 10% of the dietary carbohydrate. Normal group was fed 20% dietary protein; Low group was fed 10% dietary protein. Error bars represent ± 1 standard deviation from the mean.

*Indicates a trend (P = .0989).

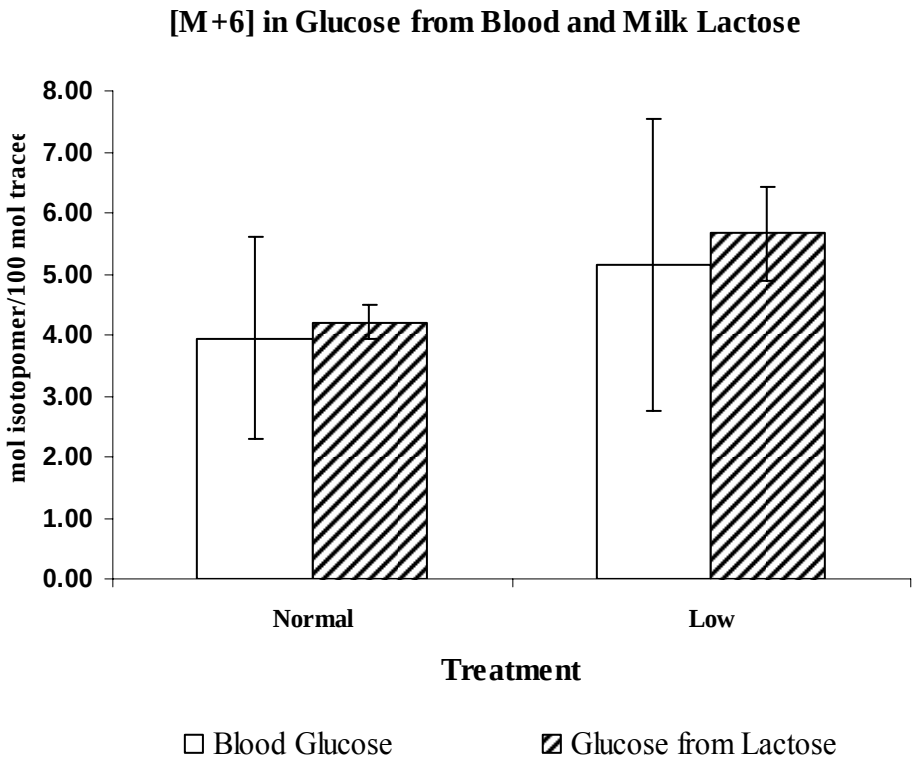


Figure 15. Enrichments of [M+6]glucose in blood and in milk lactose.

Bars represent averages from pair-fed mice (n=5 for Low group, n=6 for Normal group); samples taken after three days of feeding [U-^{13}C]glucose as 10% of the dietary carbohydrate. Normal group was fed 20% dietary protein; Low group was fed 10% dietary protein. Error bars represent ± 1 standard deviation from the mean.

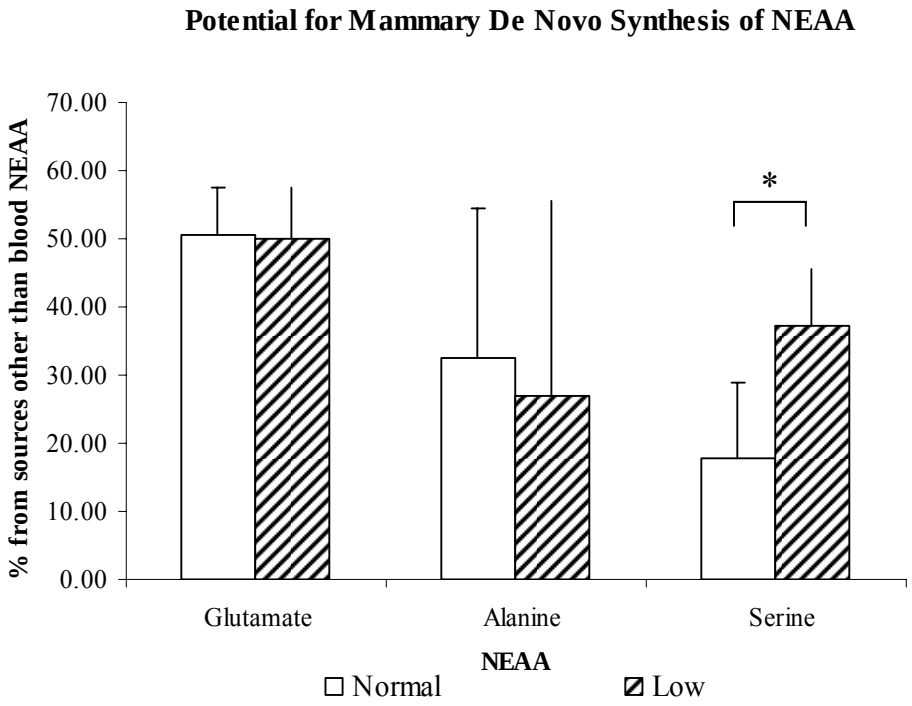


Figure 16. Minimum proportions of Alanine (Ala), Glutamate (Glu), and Serine (Ser) synthesized in the mammary gland.

Calculated as:

$$1 - \left\{ \frac{\text{[M+3] enrichment (mol isotopomer/100mol tracee) of NEAA in blood}}{\text{[M+3] enrichment (mol isotopomer/100mol tracee) of NEAA in milk casein}} \right\} \times 100$$

Bars represent averages from pair-fed mice (n=6 for both Low and Normal group); samples taken after three days of feeding [U-^{13}C]glucose as 10% of the dietary carbohydrate. Normal group was fed 20% dietary protein; Low group was fed 10% dietary protein. Error bars represent + 1 standard deviation from the mean.
*Indicates a significant difference (P = 0.0332).

Table 6. Estimates of *de novo* synthesis by the mammary gland of glutamate and alanine for milk casein for mice fed normal (20%) and low (10%) dietary protein[1]

	Glu			Ala		
	Normal	Low	Protein Effect	Normal	Low	Protein Effect
Total potential *de novo* synthesis[2]	0.51 ± 0.07	0.50 ± 0.08	NS	0.32 ± 0.22	0.27 ± 0.29	NS
De novo synthesis assuming no blood Glu[3]	0.49 ± 0.05	0.61 ± 0.08	$P = 0.0249$	0.84 ± 0.04	0.82 ± 0.06	NS
Corrected *de novo* synthesis within the MG[4,5]	0.25 ± 0.06	0.30 ± 0.09	NS	0.55 ± 0.36	0.31 ± 0.37	NS

[1]Values are means within treatment (n=6) for the pair-fed mice ± 1 standard deviation; NS = not significant, $P > 0.05$

[2]Calculated from equation b

[3]Calculated from equation d, e

[4]Calculated from equation f

[5]MG =Mammary gland

pyruvate recycling, the proportion of glutamate synthesized from alanine, the proportion of glutamate from pyruvate, and the relative activities of pyruvate dehydrogenase (PDH) versus pyruvate carboxylase (PC) were also calculated (Table 7). Data for all six mice was available for the N group, and data for one mouse in the L group was not used due to inability to measure blood glucose and lactose-derived glucose. Of note is the significantly higher proportion of glutamate from OAA (Ala) in the mammary gland for the L group (0.74 versus 0.59 for N; P=0.0038), and the higher proportion of acetyl-CoA derived from pyruvate for the L group (0.98 versus for N 0.88; P=.0027). Pyruvate recycling was higher for the N group (0.60 vs. 0.55 for L; P=0.005), as was the relative activities of PDH versus PC (5.85 for N, 5.3 for L; P=.027).

PEPCK Gene Expression

The relative expression of the two PEPCK isoforms showed similar patterns as in Study 1 (Figure 17). Liver gene expression of PEPCK-c was significantly higher than for PEPCK-m, and the relative expression was not different in dams for the L diet versus those fed the N diet. As observed previously, expression of the two isoforms in the mammary gland were not different (P = 0.2226). By contrast, in the liver, gene expression of PEPCK-c was considerably higher than PEPCK-m (P < 0.0001). For both tissues, dietary protein did not affect PEPCK isoform gene expression. Though this indicates no treatment effect, large individual variation was noted which may explain the inability to detect a difference. qRT-PCR efficiency for PEPCK-c analysis was 107.3% with an r^2 of 0.999. Efficiency for PEPCK-m was 103.8% with an r^2 of 0.997.

Table 7. Estimates of fractional gluconeogenesis and other various entry rates of metabolism from mammary tissue, liver and blood pools of mice fed normal (20%) and low (10%) levels of dietary protein[1].

	Mammary			Liver			Blood		
	Normal	Low	Protein Effect	Normal	Low	Protein Effect	Normal	Low	Protein Effect
Fractional Gluconeogenesis[2]	0.95 ± 0.11	0.69 ± 0.10	$P = 0.0019$	0.96 ± 0.44	0.68 ± 0.39	NS	0.81 ± 0.35	0.58 ± 0.30	NS
Acetyl-CoA from Glucose[3]	0.50 ± 0.16	0.65 ± 0.13	NS	0.59 ± 0.20	0.76 ± 0.37	NS	0.69 ± 0.23	0.75 ± 0.36	NS
Acetyl-CoA from Pyruvate[4]	0.88 ± 0.03	0.98 ± 0.03	$P = 0.0027$	0.90 ± 0.23	1.05 ± 0.57	NS	0.96 ± 0.30	1.05 ± 0.49	NS
Glycolysis vs. OAA to PEP[5]	1.83 ± 0.08	2.05 ± 0.14	$P = 0.0132$	2.13 ± 0.74	3.30 ± 1.14	$P = 0.0743$	1.82 ± 0.52	2.20 ± 0.70	NS
Pyruvate Recycling[6]	0.60 ± 0.02	0.55 ± 0.02	$P = 0.0050$	0.62 ± 0.10	0.49 ± 0.12	NS	0.65 ± 0.11	0.60 ± 0.10	NS
Glu from Ala[7]	0.59 ± 0.05	0.74 ± 0.06	$P = 0.0038$	0.36 ± 0.08	0.52 ± 0.26	NS	0.47 ± 0.15	0.60 ± 0.32	NS
Glu from Pyruvate[8]	1.47 ± 0.06	1.84 ± 0.10	$P = 0.0005$	1.32 ± 0.12	1.98 ± 0.58	$P = 0.0456$	1.45 ± 0.08	1.76 ± 0.31	$P = 0.0476$
PDH vs. PC[9]	5.85 ± 0.35	5.3 ± 0.28	$P = 0.0270$	8.20 ± 0.81	6.72 ± 0.58	$P = 0.0376$	7.38 ± 0.41	6.50 ± 0.45	$P = 0.0418$

[1]Values are means within treatment (L, n=5; N, n=6) for the pair-fed mice ± 1 standard deviation; NS = not significant, $P > 0.05$

[2]Calculated from equation l

[3]Calculated from equation n

[4]Calculated from equation m

[5]Calculated from equation o

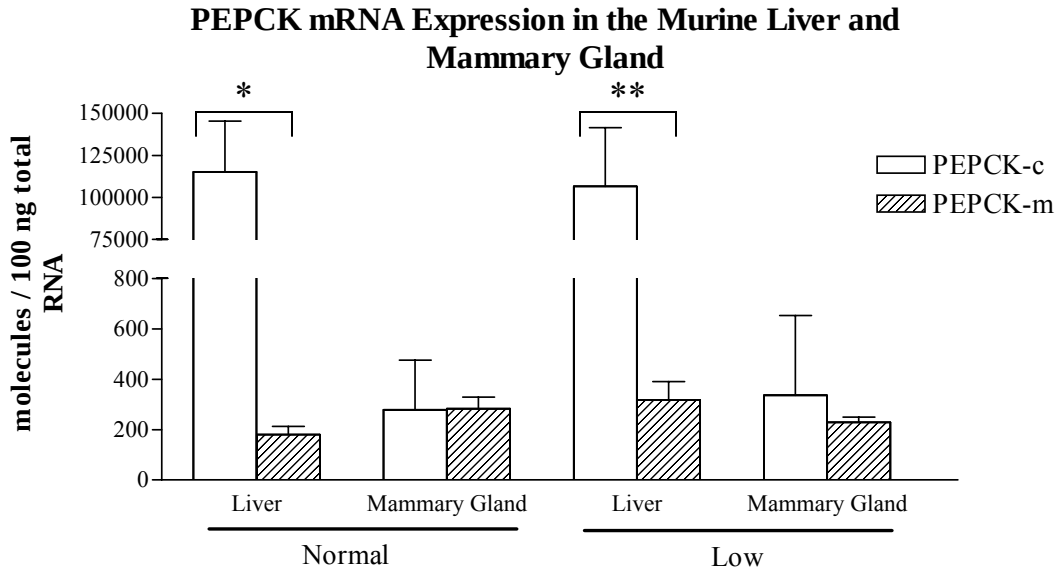
[6]Calculated from equation p

[7]Calculated from equation q

[8]Calculated from equation r

[9]Calculated from equation s

Figure 17. Gene expression level for both PEPCK isoforms in the murine liver and mammary gland.



Gene expression was determined by qRT-PCR for mice fed normal (20%) or low (10%) protein diets. Values are molecules /100 ng total RNA, averaged for each treatment (n=5 in each treatment for liver, n=6 in each treatment for mammary tissue). Error bars represent ± 1 standard deviation from the mean.
*Indicates a significant difference ($P < 0.0001$).
**Indicates a significant difference ($P < 0.0001$).

Discussion

Milk Production

Since milk production and composition data are unavailable, we used growth (in the form of live-weight) data to assess milk production by the dams on the L and N treatments. That pups nursing from L dams weighed less, even though their dam counterpart was eating the same amount as the other in the pair, suggests that either milk production or quality was compromised on the L diet. No correlation with the amount of milk available to collect was noticed at the time of sampling (the L group did not seem to be producing less milk), but it cannot be assumed that there were no differences in milk production. It is possible, however, that mice on the L diet were producing milk of lower milk protein content. Future analysis of milk samples from these experiments will shed more light on these conclusions.

Finally, since the dams on the L diet also weighed less, these dams partitioned more dietary nutrients towards lactation rather than growth. Homeorhetic adjustments are occurring to support lactation, especially for those dams who are dealing metabolically with a lower protein diet (Bauman and Currie, 1980). The decrease in dietary protein of 10% was adequate to illicit a response. At the expense of their own body reserves, the L treatment dams are supporting lactation; but as evidenced by the difference in pup and dam weights, the N group is able to support lactation and growth to a greater extent.

Sources of Glucose in Milk Lactose

Results from Chapter 2 indicated that 23% of glucose and galactose in milk lactose are derived from a source other than blood glucose. Chapter 2 results suggested that the mammary gland of the lactating mouse was capable of synthesizing both glucose and galactose, which was in agreement with previous observations in lactating women (Sunehag et al., 2002). Sources of carbon for *de novo* mammary gland synthesis of these hexoses could be glycerol, as demonstrated by Sunehag et al. (2002), and/or amino acids taken up by the mammary gland from the blood. In the current study, lactating dams were fed either a low or normal protein diet to test whether amino acids serve as a source for mammary gland *de novo* hexoneogenesis. Moreover, if there is an obligate requirement for use of amino acids for mammary gland synthesis of lactose, we expected to observe reduced lactose synthesis in addition to reduced protein synthesis in mice fed the L diet. Since lactose is the major osmotic regulator of milk, and subsequently determines overall milk yield, decreased lactose synthesis would be expected to affect total milk production. The reduced pup and dam weights observed support this conclusion. Additionally, if the mammary gland metabolizes amino acids for milk lactose synthesis we should observe dilution of the enrichment in lactose-glucose due to the introduction of unlabelled carbon skeletons arising from essential amino acids. Therefore, we would expect to see reduced enrichment of lactose-glucose from mice fed the L diet as well.

Lower [M+6] in lactose glucose (and galactose) in the L group indicates that dilution of fully labeled isotopomer is occurring. Higher [M+6] in lactose-glucose for the L group indicates that less dilution from unlabelled sources, or rather, decreased

synthesis from unlabelled (not from [U-^{13}C]glucose) is occurring. The [M+6] may be diluted by amino acids, glycerol, or contributions from the pentose phosphate pathway (Katz and Wals, 1972; Tigas et al., 2002). The low protein diet could also be described as a higher carbohydrate diet since dietary protein was replaced with carbohydrate. For both diets, tracer was exchanged for 10% of the dietary carbohydrate, Therefore, even though mice consuming the L diet (high carbohydrate, low protein) absorbed greater amounts of [^{13}C]glucose (and thus [M+6]glucose), the proportion of labeled to unlabelled remained the same for the two diets. Thus, since the only measurable source of [M+6] is from the label in the diet, then the [M+6] in blood and lactose glucose (or galactose) in lactose is being diluted by metabolism of other unlabelled compounds (amino acids). This confirms our hypothesis that plasma glucose is not the single contributor to lactose synthesis.

As the [M+6]glucose from the tracer is absorbed and metabolized, it gets broken down into [M+3]-containing compounds. Specifically, [M+6]glucose produces [M+3]pyruvate. This [M+3]pyruvate then has four fates: reduction to lactate, transamination to alanine, decarboxylation to acetyl-CoA and carboxylation to OAA (Pascual et al., 1997). Eventually, [M+*n*]glucose is resynthesized as the result of the breakdown of the [M+6]glucose and eventual recycling of [M+1]-[M+3] metabolites through PEP. This also accounts for PEPCK-c activity. [M+3]glucose gives us a minimum measurement for glucose that has been recycled from OAA via pyruvate (Pascual et al., 1997). The chance that two full labeled [M+3]PEP molecules find each other and are resynthesized to a "new" [M+6]glucose molecule is very rare, and this chance is limited by the rate of tracer feeding. Therefore,

[M+6]glucose enrichment is derived directly from the diet (tracer) via digestion and absorption.

If we compare the [M+3]/[M+6] ratios in glucose (or galactose) from lactose, we can get a rough measure of the amount of glucose recycling occurring. It makes sense, then, that the amount of recycling occurring was higher for the N group than the L group. Again, since the low protein diet has more carbohydrate, there is less glucose recycling required and the contribution of amino acids to lactose synthesis will be less. The N group has less total glucose available and so you will see more recycling to support glucose metabolism. It is worth mentioning that this [M+3]/[M+6] is a crude ratio of glucose recycling (or gluconeogenesis from pyruvate) and actually underestimates the contribution of this recycling (Reeds et al., 1997). Calculated fractional gluconeogenesis within the mammary gland also was 38% higher for the mice on the N diet.

The fact that the [M+3] in glucose from lactose is higher than the [M+3] in blood also suggests glucose metabolism and recycling. Again, this means that there are additional contributions to glucose in lactose besides direct incorporation from blood glucose. We calculated a very wide and variable range of lactose glucose derived from blood glucose, including values greater than 100%. This suggests to us that sampling of blood glucose in Study 2 was not consistent, most like due to collection of mixed proportions of arterial and venous blood. In the future, more controlled collection of blood, perhaps via the tail vein prior to tissue collection, would help to substantiate the results found in Chapter 2. Regardless, the [M+3] ratios shown here lend further evidence that not all glucose in lactose is derived

directly from blood glucose, and that complex coordination of carbon metabolism is occurring.

The above conclusions (that not all lactose is derived from blood glucose, and that there is extensive recycling of glucose occurring) confirms that the substrates for lactose synthesis are somehow coordinated with dietary protein. More glucose recycling is occurring in the N group, and the L group is seeing less dilution of glucose in lactose by sources other than dietary glucose. This means the L group is seeing less contribution of unlabelled sources (potentially amino acids) to glucose in lactose. By examining potential *de novo* synthesis of NEAA in the mammary gland, we can begin to understand whether one source of this "other" glucose may be EAA (which must generate NEAA), and to what extent shifts in NEAA synthesis may be directing metabolism in the gland.

Mammary gland de novo synthesis of NEAA

Since the extraction of NEAA across the mammary gland does not meet output demands in milk casein (Fleet and Mepham, 1985), this deficit must be met via *de novo* synthesis by the mammary gland. As has been suggested, this *de novo* synthesis potentially involves glucose as a precursor. The [M+3] of a NEAA in blood over the [M+3] of the same NEAA from milk casein estimates the amount of that NEAA derived directly from the blood via absorption. Therefore, one minus this ratio is the amount that is derived from sources other than blood, or potential *de novo* synthesis within the gland. We can then make some assumptions about the amount of the "other" pool that comes from glucose or other amino acids. See Materials and

Methods for the calculations of these estimates. In summary, approximately 50% of glutamate, 70% of alanine, and 83% (for N group) or 63% (for L group) of serine seems to be derived directly from blood uptake. These results are almost identical to the percentage of amino acid uptake versus output in milk from dairy cows reported by Fleet and Mepham (1985). This suggests that, at least in the case of amino acid metabolism, the mouse mammary gland is acting similarly to the bovine, further confirming the use of our animal model for the study of milk protein and lactose synthesis.

By examining these relationships more closely in mice, we see that 49% of glutamate in casein derived directly from blood NEAA while 13% derived from *de novo* synthesis from glucose and approximately 38% derived from other amino acids (Figure 18). Trottier et al. (1996) observed large retention rates for glutamate and alanine across the mammary gland of sows, and suggested that this might lead to significant transamination limiting milk protein synthesis. It would be interesting to observe these retention rates in mice since 38% of glutamate seems to be derived from other amino acids. If similar retention was seen in mice, it is not know how or why a large portion of glutamate would be derived from other amino acids, but in our case it seems as though it is. Again, we hypothesize that one fate of these amino acids not taken up in amounts sufficient to account for milk casein could be hexogenesis in the mammary gland.

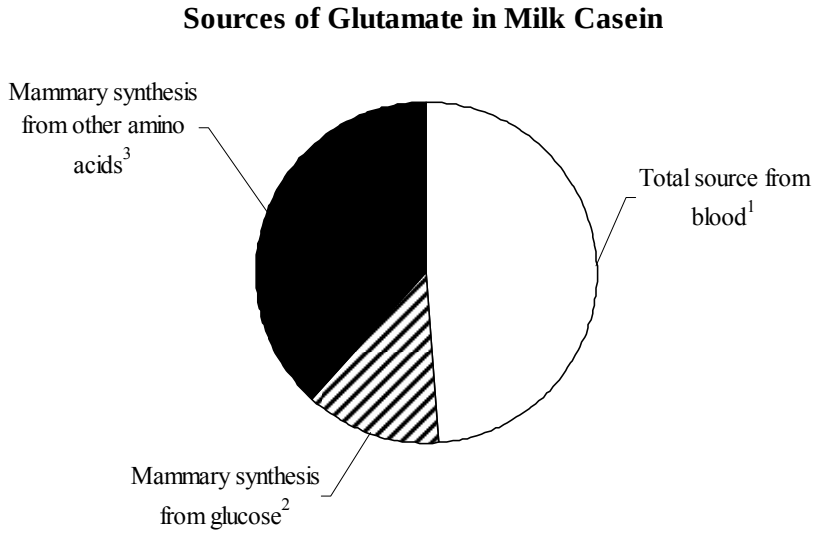


Figure 18. Proportions of glutamate (Glu) in milk casein derived from blood, and *de novo* synthesis from glucose or other amino acids.

Pie sections are averages for 6 mice on the normal (20%) protein diet.

$$\frac{[M+3]Glu \text{ from blood}}{[M+3]Glu \text{ from casein}} = A$$

$$\frac{[M+3]Glu \text{ from blood}}{(([M+3]Glu \text{ from casein})-([M+3]Glu \text{ from blood}))} = B$$

[1]Calculated as A
[2]Calculated as (1 – A) x B
[3]Calculated as (1 – A) x (1 – B)

Metabolism of glucose and gluconeogenesis

Greater [M+3]:[M+6] for the N group in and of itself suggests greater contribution of glucose recycling. Further calculations allow for quantification of contributions of various TCA intermediates to metabolism. We employed the calculations of Pascual et al. (1997) and others (Wykes et al., 1998; Haymond et al., 2000) and applied them to our own precursor: product relationships involving the metabolic pools of the mammary gland and liver. Two approaches to the "whole body" calculations were used. In one ("blood") blood amino acids were used as the pool. In the other ("liver"), liver amino acids were used. In both of these cases, blood glucose was used as the pool. For the mammary gland, the glucose pool used was glucose from lactose. This pool is much steadier and slower to respond to metabolic perturbations, unlike the blood values, which could vary wildly based on time of last feeding, and other variables including the proportion of the blood from venous, arterial, or mixed sources.

The proportion of glutamate derived from the three-carbon pool (via OAA (Ala)) is higher for the L group, though only statistically significant for the mammary gland (Table 7). These results are parallel with Pasqual et al. (1997) who saw a higher proportion derived from OAA when feeding a high carbohydrate diet. Again, our low protein diet can also be considered a higher carbohydrate diet. Similar results also hold true for the proportion of acetyl-CoA derived from three-carbon precursors (pyruvate) (Table 7). The proportion of acetyl-CoA derived from this pool is higher for mice on the L diet (or in the case of Pascual et al. (1997), high carbohydrate).

The measure of a metabolite's contribution to energy production can be measured via its contribution to acetyl-CoA (Pasqual et al., 1997). Glucose made a numerically larger, though not statistically significant, contribution to acetyl-CoA in the L group across all pools (mammary gland (Figure 19), liver, and blood (Table 7)). A larger sample size may be needed to detect a significant difference. It makes sense that as the proportion of carbohydrate increases (and amino acids decrease), more glucose will be utilized metabolically. This is also seen in the fractional gluconeogenesis rates, which are higher for the animals on the N diet. Bizeau et al. (2001) also observed increased gluconeogenesis in hepatic incubations from rats fed a high sucrose diet. Less glucose available means that these animals are synthesizing more glucose from recycled intermediates from the TCA cycle and/or amino acids. In addition, this means that more amino acids are available for potential gluconeogenesis from these sources. The mammary gland is the only pool in which there was a significant difference in gluconeogenesis (hexoneogenesis of glucose and galactose). This suggests, as we suspected, that the mammary gland is adjusting glucose synthesis based on available substrates and potentially using amino acids as precursors for lactose synthesis.

Of all of these measures, the ones most closely related to PEPCK activity would be pyruvate recycling and the balance of glycolysis versus conversion of OAA to PEP. These values exhibit an inverse relationship: pyruvate recycling is lower for the L group and relative glycolysis is higher for the L group. Pyruvate recycling is higher for the N group because amino acids are not limiting and thus there is potential for greater shuttling out of the mitochondria through PEPCK-c (Figure 2). In a sense,

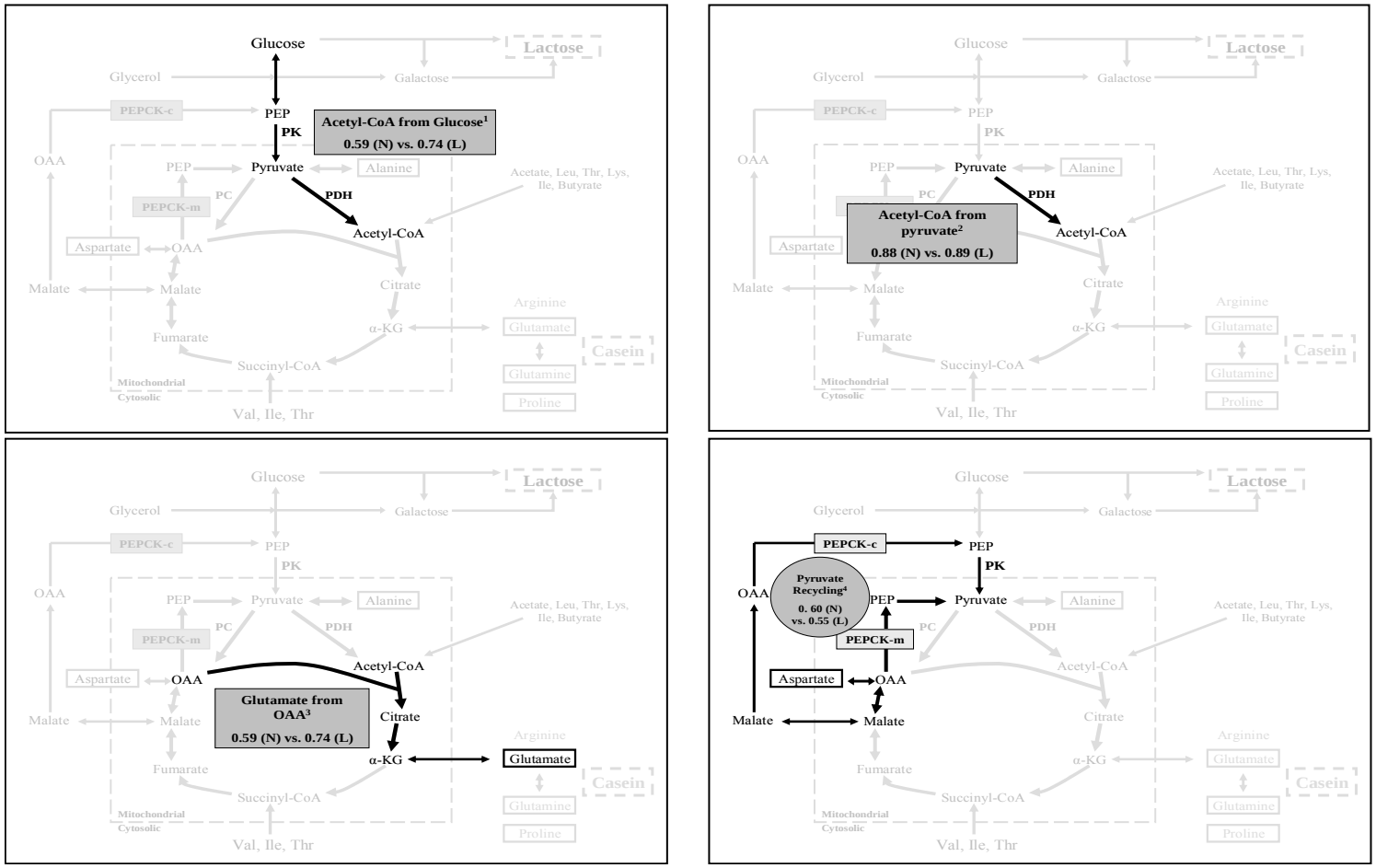


Figure 19. Estimates for entry rates of metabolism in the murine mammary gland.

Values are averages for mice fed a 20% (N, n=6) protein diet or 10% (L, n=5) protein diet for 3 days with 10% of the dietary carbohydrate exchanged with [U-^{13}C]glucose. [1]Calculated from equation n; [2]Calculated from equation m; [3]Calculated from equation q;[4]Calculated from equation r

pyruvate recycling would serve to counteract glycolysis via PEP synthesis. PEPCK is involved in conversion of OAA to pyruvate via PEP in both the cytosol and mitochondria. Since there is no difference seen in the relative expression of PEPCK-c versus PEPCK-m in the mammary gland, we can assume based on these observations that these isoforms must contribute equally to pyruvate recycling. In the liver, however, the much greater expression of PEPCK-c leads us to believe that shuttling out of the mitochondria via malate and to OAA and eventually PEP plays a larger role. Though we did not see any treatment differences in PEPCK expression, it is possible that regulation is occurring beyond translation, that individual variation is obscuring a treatment effect, or that a larger sample size is needed to detect an effect.

There was a difference, however, in PDH versus PC activity as estimated by the crude entry rate of acetyl-CoA into the TCA cycle. The ratio of PHD activity to PC activity was larger for the N group, indicating a greater contribution of pyruvate to acetyl-CoA (versus OAA). These observations are further substantiated by the proportion of Glu derived from OAA. Less Glu is derived from OOA for the N group, and thus more is derived from the acetyl-CoA pool provided by pyruvate. This suggests that besides further investigation of PEPCK as a metabolic regulator in the mammary gland, there is potential for PDH and PC to be targets as well. This comes as no surprise considering all of the enzymes involved in the TCA cycle and all contributing metabolic pathways deserve mention as potential regulators. There certainly is great potential for unlocking the specifics behind this very complex

interlocking of carbohydrate and amino acid metabolism. One cannot simply mention one without involving the other.

Conclusions and Future Implications

In conclusion, we were correct in our assumption that not all glucose in milk lactose is derived from blood glucose. Of the 23% of lactose-glucose derived from sources other than blood glucose, amino acids have the potential to be one of these substrates. Above all, the additional contributor to lactose synthesis is unlabelled. It is possible that, like in humans, glycerol is serving as a contributor as well. Use of a glycerol tracer would expose whether glycerol is serving as a substrate for glucose synthesis in the murine mammary gland. In addition, it would be interesting to observe whether differences exist in the contribution of glycerol to glucose versus galactose. Sunehag et al. (2002) found a dissimilar contribution to the glucose and galactose moieties in lactose (glycerol contributed only to galactose synthesis). However, in our case, glucose and galactose appear to be derived from the same metabolic pool. It is hypothesized then that if glycerol contributes to lactose synthesis in the murine mammary gland, then equivalent labeling would be observed in glucose and galactose from milk lactose. Potential also exists for the use of labeled amino acids to further quantify the contribution of carbon skeletons derived from amino acids to glucose synthesis.

Decreasing dietary protein decreased fractional gluconeogenesis in the mammary gland, and increased the contribution of fully labeled glucose from the diet to blood glucose and glucose in lactose. Thus, dietary protein (and/or carbohydrate) does have an effect on mammary tissue metabolism and regulation of milk

component synthesis. Future plans involve analysis of milk samples from this study. The difficulty in quantifying milk protein and lactose is in the limited sample quantity for analysis. Development of the use of fully labeled lactose and amino acids to quantify milk protein and lactose in mouse milk via GC-MS techniques is currently underway in our laboratory. We suspect that this analysis will confirm that mice on the L diet were producing milk with lower levels of protein and lactose.

In the current study, the large individual variation observed in the quantification of PEPCK mRNA proved to be troubling. Though the experiment was tightly controlled and the pair-feeding setup limited the amount of individual variation, on the molecular level there seems to still be large individual variation. One way to circumvent this problem would be to take multiple samples from an individual over the time-course of a treatment in order to compare baseline levels with potential treatment effects. Obviously this would be difficult in the case of the mouse where the total mass and structure of the mammary gland makes multiple sampling from the same animal difficult. In an animal like the dairy cow, however, large and isolated mammary glands (as in the case of four single glands) would allow for easy sampling at multiples times and locations. Furthermore, it is also possible that significant treatment effects were not seen in PEPCK isoform expression because regulation is occurring beyond mRNA expression. Unlimited possibilities exist if one examines opportunities for the use of PEPCK isoform knockout mice in conjunction with MIDA techniques. She et al. (2003) and Burgess et al. (2004) have successfully used liver-specific PEPCK-c knockout mice as a model for diabetes research. In addition, small interfering RNA also provides opportunity for tissue-specific

regulation of PEPCK isoforms at the gene expression level. By employing these techniques, one can measure the effect of changing PEPCK activity versus the responses in activity based on outside modulations.

The combination of these studies has paved the way for future opportunities in this area. Many questions still remain to be answered, several of which involve confirming and improving the accuracy of the estimations of amino acid carbon used for lactose synthesis, and glucose carbon use for amino acids within the mammary gland. The synergistic approach of combining enzyme expression data with the quantification of metabolism via the MIDA approach offers an enticing avenue for further exploration of mammary gland metabolism. Future studies will no doubt allow for potential practical applications for the manipulation of milk components for the benefit of producers and the dairy industry. Cross species comparisons alluded to here also provide opportunity for further work. Differences in monogastric versus ruminant mammary gland metabolism can be further investigated. Additional research involving PEPCK will also have far reaching effects in whole body metabolism as the function of PEPCK in several tissues has yet to be fully explained.

Appendix A

Molar tracer:tracee ratios of amino acids obtained from blood, milk casein, and liver tissue of C57BL/6 mice; Study 1, Chapter 2[1]

Amino Acid	Source	mol isotopomer/100 mol tracee		
		M+1	M+2	M+3
Alanine	Blood	3.39 ± 0.11	1.62 ± 0.23	4.94 ± 1.14
	Casein	2.34 ± 0.47	1.44 ± 0.07	2.90 ± 0.39
	Liver	2.99 ± 0.22	1.42 ± 0.16	5.58 ± 0.99
Aspartate	Blood	4.55 ± 1.35	1.71 ± 0.55	0.47 ± 0.08
	Casein	3.30 ± 1.15	1.10 ± 0.10	1.32 ± 0.20
	Liver	6.43 ± 0.80	2.02 ± 0.36	0.69 ± 0.15
Glutamate	Blood	6.93 ± 0.52	4.96 ± 0.53	0.61 ± 0.08
	Casein	5.01 ± 0.66	4.72 ± 0.50	0.69 ± 0.24
	Liver	5.66 ± 0.60	4.30 ± 0.73	0.54 ± 0.17
Serine	Blood	2.68 ± 0.34	0.66 ± 0.10	1.04 ± 0.13
	Casein	1.28 ± 0.96	0.23 ± 0.16	1.12 ± 0.36
	Liver	1.67 ± 0.10	0.64 ± 0.00	0.14 ± 0.02

[1]Values are means ± standard deviation for 4 mice; casein values are for milk collected after 5 days of feeding tracer

Appendix B

Molar tracer:tracee ratios of TCA intermediates obtained from various tissues and blood of C57BL/6 mice; Study 1, Chapter 2[1]

	Tissue Source	mol isotopomer/100 mol tracee					
		M+1	M+2	M+3	M+4	M+5	M+6
α-ketoglutarate	Mammary	9.30 ± 0.86	7.09 ± 1.13	0.90 ± 0.20	0.16 ± 0.05	0.03 ± 0.01	-
	Liver	5.37 ± 2.91	3.72 ± 0.64	0.46 ± 0.15	0.20 ± 0.07	0.11 ± 0.04	-
Lactate	Mammary	4.71 ± 0.67	2.27 ± 0.39	4.27 ± 1.11	-	-	-
	Liver	2.69 ± 0.31	1.34 ± 0.19	4.39 ± 0.78	-	-	-
	Intestine	1.86 ± 1.25	0.93 ± 0.38	3.94 ± 0.99	-	-	-
	Muscle	1.61 ± 0.71	0.87 ± 0.18	4.83 ± 0.62	-	-	-
	Blood	2.61 ± 0.14	1.36 ± 0.08	4.29 ± 0.93	-	-	-
Malate	Mammary	3.24 ± 1.19	1.23 ± 0.56	0.76 ± 0.43	0.06 ± 0.02	-	-
	Liver	7.85 ± 1.13	2.46 ± 0.56	0.71 ± 0.19	0.07 ± 0.03	-	-
	Intestine	4.60 ± 0.52	2.18 ± 0.14	0.40 ± 0.12	-0.02 ± 0.03	-	-
	Muscle	6.67 ± 0.62	2.51 ± 0.55	0.54 ± 0.16	0.02 ± 0.02	-	-
Pyruvate	Mammary	4.55 ± 0.59	2.30 ± 0.36	4.23 ± 1.09	-	-	-
	Liver	2.51 ± 0.33	1.36 ± 0.31	4.07 ± 0.64	-	-	-
	Intestine	2.78 ± 0.35	0.88 ± 0.15	3.39 ± 1.15	-	-	-
	Muscle	2.09 ± 0.44	0.76 ± 0.15	4.89 ± 0.56	-	-	-
Succinate	Mammary	5.14 ± 0.94	4.33 ± 1.04	0.39 ± 0.15	0.10 ± 0.03	-	-
	Liver	4.32 ± 0.71	2.21 ± 0.56	0.45 ± 0.04	0.04 ± 0.03	-	-
	Intestine	3.61 ± 0.46	1.62 ± 0.21	0.21 ± 0.06	0.04 ± 0.02	-	-
	Muscle	4.18 ± 0.69	1.76 ± 0.37	0.41 ± 0.07	0.08 ± 0.04	-	-

[1]Values are means ± standard deviation for 4 mice after 5 days of feeding [U-^{13}C]glucose as 10% of the dietary carbohydrate

Appendix C

Molar tracer:tracee ratios of amino acids obtained from blood, milk casein, and liver tissue of C57BL/6 mice; Study 2; Chapter 3[1]

			mol isotopomer/100 mol tracee		
Amino Acid	Treatment[2] (Normal/Low)	Source	M+1	M+2	M+3
Alanine	N	Blood	4.52 ± 1.12	1.88 ± .016	3.45 ± 1.04
		Casein	4.94 ± 0.25	2.81 ± 0.06	5.13 ± 0.28
		Liver	3.61 ± 0.56	1.48 ± 0.14	3.11 ± 0.92
	L	Blood	4.38 ± 0.61	2.00 ± 0.32	4.48 ± 1.80
		Casein	4.62 ± 0.39	2.98 ± 0.18	6.12 ± 0.48
		Liver	2.80 ± 0.54	1.41 ± 0.35	4.85 ± 2.36
Aspartate	N	Casein	6.40 ± 0.31	2.26 ± 0.20	2.31 ± 0.12
		Liver	5.01 ± 0.78	1.73 ± 0.17	0.45 ± 0.11
	L	Casein	8.06 ± 0.49	2.84 ± 0.24	3.13 ± 0.26
		Liver	7.37 ± 1.60	2.54 ± 0.47	0.90 ± 0.28
Glutamate	N	Blood	8.08 ± 0.34	5.44 ± 0.16	0.74 ± 0.06
		Casein	8.58 ± 0.39	8.81 ± 0.51	1.51 ± 0.16
		Liver	5.88 ± 0.54	4.37 ± 0.27	0.54 ± 0.06
	L	Blood	10.17 ± 0.65	7.29 ± 0.55	1.13 ± 0.15
		Casein	11.03 ± 0.64	11.99 ± 0.84	2.27 ± 0.26
		Liver	8.87 ± 1.09	6.75 ± 0.88	1.01 ± 0.20
Proline	N	Casein	0.97 ± 0.42	0.56 ± 0.30	0.09 ± 0.05
		Liver	0.45 ± 0.12	0.16 ± 0.07	0.11 ± 0.05
	L	Casein	0.97 ± 0.56	0.79 ± 0.43	0.13 ± 0.07
		Liver	0.72 ± 0.17	0.45 ± 0.10	0.19 ± 0.05
Serine	N	Blood	4.53 ± 0.52	1.06 ± 0.38	1.82 ± 0.29
		Casein	3.51 ± 0.46	1.17 ± 0.16	2.21 ± 0.22
		Liver	1.06 ± 0.17	0.46 ± 0.07	0.13 ± 0.02
	L	Blood	4.85 ± 0.36	1.13 ± 0.39	1.78 ± 0.19
		Casein	4.01 ± 0.22	1.53 ± 0.07	2.86 ± 0.24
		Liver	1.76 ± 0.26	0.79 ± 0.07	0.21 ± 0.06

[1]Values are means ± standard deviation for each treatment group (n=6); mice were pair fed [U-^{13}C]glucose as 10% of the dietary carbohydrate for 3 days

[2]Normal (N) treatment was 20% dietary protein; Low (L) treatment was 10% dietary protein

Appendix D

Molar tracer:tracee ratios of TCA intermediates obtained from various tissues and blood of C57BL/6 mice; Study 2, Chapter 3[1]

			mol isotopomer/100 mol tracee			
	Treatment[2] (Normal/Low)	Tissue Source	M+1	M+2	M+3	M+4
Lactate	N	Mammary	4.68 ± 0.72	1.98 ± 0.24	3.41 ± 1.08	-
		Liver	3.33 ± 0.66	1.32 ± 0.14	2.55 ± 0.74	-
		Intestine	3.28 ± 1.07	1.25 ± 0.25	4.19 ± 1.38	-
		Muscle	2.19 ± 0.75	0.85 ± 0.24	2.72 ± 0.55	-
		Blood	3.93 ± 0.62	1.67 ± 0.15	3.07 ± 0.73	-
	L	Mammary	4.37 ± 0.58	2.00 ± 0.24	4.29 ± 1.60	-
		Liver	2.48 ± 0.58	1.19 ± 0.39	3.93 ± 2.08	-
		Intestine	3.36 ± 0.86	1.48 ± 0.35	5.14 ± 1.53	-
		Muscle	1.67 ± 0.46	0.69 ± 0.18	3.02 ± 0.71	-
		Blood	3.24 ± 0.59	1.55 ± 0.34	3.99 ± 1.57	-
Malate	N	Liver	4.76 ± 2.22	2.01 ± 0.93	0.38 ± 0.14	0.06 ± 0.02
		Intestine	6.62 ± 1.04	2.50 ± 0.58	0.41 ± 0.08	0.01 ± 0.03
		Muscle	7.49 ± 0.56	1.92 ± 0.45	0.54 ± 0.13	0.04 ± 0.02
	L	Liver	9.23 ± 1.86	4.13 ± 0.59	0.99 ± 0.43	0.10 ± 0.03
		Intestine	9.79 ± 1.40	3.89 ± 0.54	0.54 ± 0.09	0.05 ± 0.03
		Muscle	8.66 ± 2.53	2.26 ± 0.52	0.48 ± 0.11	0.04 ± 0.02
Pyruvate	N	Mammary	3.95 ± 0.44	1.93 ± 0.19	3.41 ± 1.04	-
		Liver	1.89 ± 0.36	0.91 ± 0.08	2.28 ± 0.53	-
		Intestine	3.20 ± 0.51	1.08 ± 0.13	3.60 ± 1.21	-
		Muscle	4.95 ± 7.13	1.26 ± 1.40	2.84 ± 1.01	-
	L	Mammary	3.44 ± 0.56	1.69 ± 0.22	3.80 ± 1.34	-
		Liver	1.76 ± 0.57	0.97 ± 0.34	3.40 ± 1.52	-
		Intestine	3.73 ± 0.57	1.31 ± 0.28	4.36 ± 1.29	-
		Muscle	4.37 ± 5.81	1.11 ± 1.23	3.12 ± 1.30	-
Succinate	N	Mammary	4.60 ± 1.50	3.81 ± 0.64	0.49 ± 0.17	0.05 ± 0.02
		Liver	5.48 ± 0.96	2.89 ± 0.39	0.47 ± 0.19	0.04 ± 0.01
		Intestine	5.04 ± 0.68	2.55 ± 0.33	0.27 ± 0.06	0.01 ± 0.01
		Muscle	4.28 ± 0.56	2.35 ± 0.44	0.29 ± 0.14	0.01 ± 0.01
	L	Mammary	5.02 ± 2.37	3.58 ± 1.54	0.67 ± 0.61	0.07 ± 0.04
		Liver	7.87 ± 2.26	4.13 ± 1.06	0.89 ± 0.47	0.07 ± 0.03
		Intestine	8.15 ± 1.04	3.94 ± 0.54	0.45 ± 0.07	0.03 ± 0.03
		Muscle	5.44 ± 1.31	2.55 ± 0.56	0.36 ± 0.15	0.02 ± 0.01

[1]Values are means ± standard deviation for each treatment group (n=6); mice were pair fed [U-^{13}C]glucose as 10% of the dietary carbohydrate for 3 days

[2]Normal (N) treatment was 20% dietary protein; Low (L) treatment was 10% dietary protein

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