ABSTRACT

Title of Thesis: CAN CHOLINE SPARE METHIOININE FROM CATABOLISM IN LACTATING MICE AND DAIRY COWS?

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Several studies have demonstrated that supplementation of rumen-protected choline (RPC) improves milk production in the lactating dairy cow; however, there are an equal number of studies failing to observe production responses. To date, there are only three studies that provide quantitative information in ruminants on the metabolic fates of methyl groups derived from choline and Methionine (Met). This has limited the ability to predict when, and under what conditions, RPC supplementation will be beneficial. The objectives of this thesis were to determine the interaction of choline and Met methyl group metabolism and the extent of methyl group transfer during lactation, and define what role, if any, is there for RPC in remethylation of homocysteine and in the sparing of Met in lactating animals.

A preliminary study with lactating mice consuming a low protein basal diet (10%) was conducted. From 11 to 15 d postpartum, stable isotopes of [methyl-$^{2}H_3$] choline and [methyl,$^{2}H_3$]Met replaced the unlabeled choline and Met in the basal diet to measure the metabolic fates of choline and Met including Met remethylation and sources of Met

methyl in the mammary gland. Isotopic analysis revealed that the liver is a major site of Met remethylation from dietary choline with a minimum choline methyl group contribution to Met remethylation of 35%. Mammary tissue was not a major site of Met remethylation from dietary choline (< 10% of Met methyl) as measure by Met methyl in mammary tissue and milk casein. However, there was a significant unlabeled source of methyl groups contributing at a minimum of 45% Met remethylation in the mammary tissue, presumably by *de novo* synthesis. This suggested that in addition to the liver, the mammary gland is an active site of methyl group transactions.

In a subsequent study, lactating dairy cows were fed a total mixed ration formulated to meet the nutrient requirements with exception of metabolizable Met that was restricted to 1.49 % of metabolizable protein. Treatments included a Control (basal diet) and RPC supplemented diet where the basal diet was top dressed with 15g/d RPC, diets were fed in a single reversal design with 2 week experimental periods. Stable isotopes of Met, [1-^{13}C] Met, [$^{13}CH_3$] Met, and [methyl-$^{2}H_3$] choline were continuously infused on d 14 of each period to determine the metabolic fate and methyl transactions of Met methyl as measured in blood and milk casein.

Treatment had no effect on milk production or composition. However, plasma free Met from choline transmethylation was shown to act as a significant contributor to casein synthesis. Fractional Met remethylation rates in the control and RPC treatments were 26 and 23%, respectively.

Methionine Met methyl loss within the mammary tissue appears to be minimal. Based on casein Met enrichment, upwards of 40% of Met present in casein had undergone transmethylation with choline serving as the ultimate methyl donor. Furthermore, plasma versus casein Met methyl enrichment data suggested that a significant amount of *de novo* methyl group synthesis occurs in the dairy cow's mammary gland with choline serving as a major methyl donor.

CAN CHOLINE SPARE METHIOININE FROM CATABOLISM IN LACTATING MICE AND DAIRY COWS?

By

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Dedication

To my Mom and Dad.

With your support anything is possible.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

2AMP	2-Amino-2Methyl-1-Propanol
APE	Atoms Percent Excess
BHBA	ß –hydroxybutyrate
CHOL	Choline
CYS	Cysteine
GC-MS	Gas Chromatography-Mass Spectrometry
GAMT	guanidinoacetate methyltransferase
GNMT	Glycine N-methyltransferase
MET	Methionine
MTHF	Methyl Tetrahydrofolate
N^5-MTHF	5-Methyltetrahydrofolate
NEFA	Nonesterified Fatty Acid
PC	Phosphatidylcholine
PEMT	Phosphatidylethanolamine N-Methyltransferase
RPC	Rumen Protected Choline
RUP	Rumen Undegradable Protein
SAHC	S-adenosylhomocysteine
SAM	S-adenosylmethionine
TAG	Triacylglycerol
TMR	Total mixed ration
TTR	Tracer:Tracee Ratio
VFA	Volatile Fatty Acids
VLDL	Very Low Density Lipoproteins

CHAPTER I

Review of Literature

Dairy Cattle Nutrition Overview

Physiologically, lactation can be considered the most demanding phase of the mammalian life cycle (Bauman and Currie, 1980) where at peak lactation, the cow's total energy requirements can be as much as 4 to 5 times those required for maintenance (NRC, 2001). Because of the high nutrient demands to support milk production in early lactation, it is quite common for the dairy cow to mobilize body stores in order to meet lactation demands (Bauman and Currie, 1980; Coppock et al., 1974). During lactation, the amounts of tissue mobilized can be as much as 50-90 kg body fat and 10-15 kg body protein (Andrew et al., 1994; Komaragiri and Erdman, 1997,1998) accounting for 30 to 50% of a cow's total energy stores. In turn, utilization of body stores is reflected in lower body condition scores which have been associated with adverse affects on the health and reproductive efficiency of the animal (DeVries et al., 1999).

Rumen fermentation allows for the digestion of cellulosic plant materials, facilitating the utilization of low quality feeds and forages allowing ruminants to thrive on feedstuffs which many herbivores animals would starve on. Rumen fermentation and its concomitant production of volatile fatty acids (VFA) that are absorbed and utilized as energy sources, confer ruminants the ability to derive energy from cellulose and hemicelluloses, substrates that are virtually indigestible in herbivores that lack fermentation compartments in their digestive systems.

Not all aspects of rumen fermentation are favorable to the host animal. Rumen fermentation frequently destroys components of nutritionally dense feedstuffs such as starches, amino acids, vitamins and unsaturated fatty acids where there could actually be a nutritional advantage to direct absorption in the small intestine essentially "bypassing"

transformations from rumen fermentation. While beneficial in many regards, rumen fermentation may ultimately limit the high producing dairy cow's ability to fully exploit many nutritionally dense feedstuffs. It is during periods of peak nutritional demand, such as during early lactation where more effective utilization of high quality or nutrient dense feedstuffs might alleviate the demand placed on the cow's body stores and ultimately improve milk production as well as overall health of the dairy cow.

As a result, there is now great interest in methods and products which provide nutrient dense, nutrient limiting, and/or high cost nutrients in forms which bypass the rumen to the small intestine, the site of absorption, to improve utilization. Examples of such products include fat supplements, amino acids and vitamins where rumen bypass is allows for more favorable absorption and utilization of nutrients. This effectively allows for selective supplementation and feeding of targeted nutrients to ruminants while preventing their degradation in the rumen.

Most commercially available rumen-protected nutritional products have been developed for use in lactating dairy cow. Rumen-protected fat products using calcium soaps of fatty acids (Megalac®, Church and Dwight Inc, Piscataway New Jersey) have been sold as energy supplements for more than 25 years. Rumen-protected forms of the amino acids Met and lysine have been commercially available for more than 20 years (Smartamine-M® and Smartamine-L®, Addiseo USA Inc., Alpharetta, GA). More recently, rumen protected forms of choline and niacin (Reashure and Niashure, Balchem Corp, New Hampton, NY) have become commercially available. These are just a few examples high quality, nutrient dense, and potentially limiting nutrients and ingredients

where rumen bypass for delivery to the small intestine for absorption can be advantageous in the lactating dairy cow.

Previous work (Sharma and Erdman, 1988 and 1989) has demonstrated that dietary choline could be a limiting nutrient for milk production in the dairy cow. In addition to the use of Met as an amino acid for milk protein synthesis, Met also plays as central role in methyl group metabolism (Almquist and Grau, 1944; DuVigneaud et al., 1945). As choline is a methyl rich compound which is destroyed during rumen fermentation (Neill et al., 1978; Sharma and Erdman, 1988b), rumen-protected choline could potentially be used to spare the use of Met as a methyl donor for choline synthesis or contribute to the re-methylation of homocysteine to Met. Both Met and choline are now available in rumen-protected forms allowing for direct feeding. This thesis deals with the study of the interaction of choline and Met via the methyl sparing effect of choline in the lactating dairy cow.

Methyl Group Metabolism

One of the principal functions of the amino acid Met is as building block for proteins. However, in addition to Met's function as an amino acid, its constituent methyl group serves as readily available source of labile methyl groups required in many methylation pathways including synthesis of phosphatidylcholine, methylation of proteins, DNA, RNA and a variety of other molecules (norepinephrine, catechols and sterols). Methionine is the immediate precursor of S-adenosylmethionine (SAM), the ultimate methyl group donor that is essential in over 100 transmethylation reactions (Lobley et al., 1996). The products of transmethylation reactions can be found in every

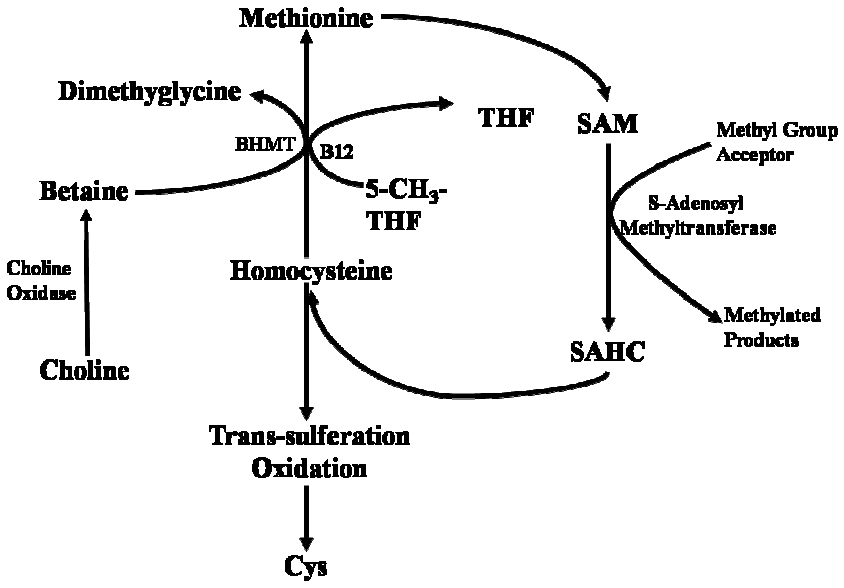
cell and have functions ranging from regulation of DNA activity to cell membrane integrity (Selhub, 1999).

Because of the limited dietary supply of Met in most common ruminant feedstuffs, coupled with the extensive utilization of methyl groups throughout the body, Met is considered either the first or the second limiting amino acid in ruminant diets (Lobley et al., 1995; Pinotti et al., 2002). Ultimately, an effective means of *de novo* methyl group synthesis is required in order to meet the competing metabolic demands for Met as an amino acid and also as a methyl group donor.

De novo Methionine Synthesis

De novo Met synthesis occurs through remethylation of homocysteine, the byproduct of SAM transmethylation reactions. After donating its methyl group, SAM is converted to S-adenosylhomocysteine (SAHC) (Figure 1). Homocysteine may then undergo either transsulfuration/oxidation to cysteine (Cys) or remethylation to reform SAM. The process of transsulfuration/oxidation is irreversible, serving both as source of the sulfur amino acid cysteine a vital component of proteins and as a route to remove excess Met and homocysteine from the body.

Figure 1.1 General overview of methyl group transfer and *de novo* methyl group synthesis pathways.



Reduced dietary supply of Met has been shown to correlate with a decrease in Cys. Reduced dietary Cys will ultimately require an increase in homocysteine transsulfuration/oxidation, further reducing the supply available for remethylation to Met. In several species it has been shown that Cys supplementation of Met deficient diets reduces the rate of transsulfuration/oxidation of homocysteine (Raguso et al., 1997). This is most likely attributed to a transsulfuration/oxidation sparing effect as cysteine cannot be utilized as a Met precursor and therefore cannot contribute to SAM or SAHC concentrations.

Re-Methylation of Homocysteine

Numerous studies have identified the negative consequences of Met deficiency and in turn methyl group deficiency which increases the level of importance of remethylation of homocysteine to Met (Selhub, 1999). Methyl group availability and therefore Met availability is required for normal metabolic function and the animal's ability to maintain adequate SAM concentrations. Synthesis of SAM is ultimately dependent on dietary intake of Met and methyl group contributors, providing a means by which SAHC can be remethylated to SAM. In humans, the SAHC to SAM remethylation cycle may occur 2 to 4 times before homocysteine undergoes final transsulfuration/oxidation. The extent of remethylation is dependent on Met intake suggesting that this process is regulated in part by methyl group availability (Benevenga, 2007). Remethylation of homocysteine may occur by two distinct pathways: Remethylation of homocysteine by *de novo* methyl group synthesis through N5-methyl-tetrahydrofolate or remethylation homocysteine by betaine derived from dietary choline.

Under normal circumstances, the tri-methylated compound choline through its oxidation product betaine, serves as the primary methyl group donor for re-methylation of homocysteine (Benevenga, 2007). However, to date only three studies in ruminants (Emmanuel & Kennelly, 1984; Lobley et al., 1996; Preynat et al, 2009) provide any quantitative information on the metabolic fates of methyl groups derived from Chol and Met.

Re-Methylation of Homocysteine by N5-Methyltetrahydrofolate

De novo synthesis of methyl group was first discovered by Du Vigneaud et al., (1945). The remethylation of homocysteine to Met can occur in all tissues with N5-Methyltetrahydrofolate (MTHF) serving as the methyl donor (Selhub, 1999). The final methyl transfer step to SAHC is Vitamin B_{12} dependent (Selhub, 1999). Therefore, this metabolic pathway is both folate and Vitamin B_{12} dependent and a deficiency of either vitamin can result in perturbation of SAM-SAHC methyl transferase system. Not surprisingly, the activity of this pathway increases during times of decreased methyl group intake (Pinotti et al., 2002). As rumen fermentation destroys feed choline, (Neill et al., 1978, 1979) it is generally accepted that this pathway is more active in adult ruminants than in monogastric animals (Pinotti et al., 2002).

Re-Methylation of Homocysteine by Betaine Derived from Choline

Betaine, the product of choline oxidation via choline oxidase in tissues, is the primary source of methyl groups for remethylation of homocysteine to Met in humans (Bennevenga, 2007). The remethylation of homocysteine by the betaine –choline pathway is dependent on the activities of two enzymes: choline oxidase and betaine-homocysteine methyltransferase. In monogastric animals, most homocysteine re-methylation occurs in the liver with limited amounts in other tissues such as the pancreas and kidneys (Selhub, 1999; Xue and Snoswell, 1986).

Xue and Snoswell (1986) compared the activity of these enzymes in adult rat and adult sheep livers. They found that choline oxidase and betaine-homocysteine methyltransferase activities were markedly lower in the adult sheep. Conversely, N5-methyl-H4folate-homocysteine methyl transferase activity was greater in sheep than rats

(Xue and Snoswell, 1986). They concluded that the low activity levels of choline oxidase and betaine-homocysteine methyltransferase was a response to rumen degradation of dietary choline, ultimately minimizing the amount of choline absorbed from the diet. Given the limited availability of choline from the diet, it is plausible that choline would be conserved and spared from catabolism as a methyl donor, thereby reducing the need for more costly *de novo* methyl group synthesis. Increased activity of the N5-methyl-H4-folate-homocysteine methyl transferase would spare the use of choline as a methyl donor and perhaps is the main route by which remethylation of homocysteine to Met can occur in the ruminant animal.

Xue and Snoswell (1986) suggested that increased activity levels of choline oxidase and betaine-homocysteine methyltransferase in both neonate and preruminant lambs were result of higher rates of dietary choline absorption in animals where an active rumen is not present. Indeed, choline deficiency has been demonstrated in the dairy calf prior to weaning and choline supplementation corrects the deficiency (Johnson et al., 1951) Likewise in pre-ruminant sheep, adequate dietary supplies of choline up-regulate choline oxidase and betaine-homocysteine methyltransferase activities (Xue and Snoswell, 1986). It has been shown that increased choline availability results in increased enzymatic activity associated with the choline-betaine remethylation pathway in monogastrics (Selhub, 1999). This same choline dependent upregulation and use of choline methyl groups for homocysteine remethylation functions in pre-ruminants (Xue and Snoswell, 1986) just as it does in on monogastrics species where it is considered to be the major source of methyl groups. (Bennevenga, 2007; Selhub, 1999).

Transsulferation and Oxidation of Homocysteine

The transsulfuration and oxidation of homocysteine (Figure 1) provides the means by which Cys is synthesized from Met. This also provides a pathway by which excess Met and homocysteine are removed, both of which are toxic at high concentrations (Selhab, 1999). In the event that methyl group donors are limited, protein sources may be mobilized for SAM, which in turn increases the production of homocysteine.

Homocysteine must be remethylated to Met or removed from the body via the transsulfuration/oxidation pathway (Selhub, 1999). The process by which homocysteine is either remethylated and or undergoes transsulfuration or oxidation, is remarkably well regulated by several complex feedback loops that respond to dietary intake of Met, the availability of methyl group donors and the animals basal need for methyl groups (Selhub, 1999). As Met availability decreases, there is a greater demand for re-methylation of homocysteine which is met in large part through the increased activity of the 5-MTHF pathway (Selhub, 1999). Synthesis of 5-MTHF is dependent on the availability of 5-MTHF reductase enzyme which is inhibited when SAM concentrations are high. However, as SAM concentrations decline the rate of 5-MTHF reductase synthesis increases and subsequently improves the rate of re-methylation (Selhub, 1999).

Intercellular concentrations of SAM are regulated in part by the rate of synthesis from intercellular Met. According to Selhub (1999), the activity of two enzymes required for SAM synthesis, one having a high affinity for Met and the other a low affinity for Met, fluctuate with the intercellular concentration of Met. As Met concentration declines activity of the high affinity enzyme decline as well, reducing SAM synthesis, conserving Met and subsequently increasing the rate of 5-MTHF reductase

synthesis, the opposite effect is noted when Met is considered to be at or above normal concentrations (Selhub, 1999).

Effect of Choline and Methionine Deficiency

Methionine and choline deficiency has been shown to induce fatty liver, reduce feed efficiency, restrict growth, result in weight loss and attenuate insulin resistance and glucose intolerance (Riziki et al., 2006; Raubenheimer et al., 2006). A classic symptom of choline deficiency is fatty liver first reported by Hershey and Soskin (1931) in dogs. It has been proposed that a general deficiency of methyl groups leads to fatty liver as a result of inadequate amounts of the phospholipid phosphatidylcholine (PC) (Pinotti et al., 2002). Phosphatidylcholine is an integral component of lipoproteins that are required for export of triglycerides in the form of very low density lipoproteins (VLDL) from the liver. The attenuation of insulin resistance and glucose intolerance could be the result of an inability to oxidize free fatty acids in liver, resulting in fatty liver. Systemic weight loss seen in methyl group deficient diets may be a result of protein catabolism for methyl group supply and an inability to oxidize dietary fatty acids effectively for energy. These symptoms suggest that severely reduced methyl group availability has profound implications on normal metabolic function.

Choline Synthesis

Because of almost complete rumen degradation of dietary choline, choline must be synthesized *de novo* in the ruminant in order to meet biological demands (Dawson et al., 1981, Robinson et al., 1984; Pinotti et al., 2002). In ruminants, significant choline

synthesis occurs in the extra-hepatic tissues and to a more limited degree in the liver (Robinson et al., 1984). This contrasts with many monogastric species including humans, rats and swine that obtain a large fraction of their choline requirements from the diet, and therefore may have limited need for *de novo* choline synthesis (Robinson et al., 1984). It has been shown that pre-ruminants also receive the majority of their choline requirement via dietary sources similarly resulting in limited needs for *de novo* synthesis (Xue and Snoswell, 1986).

De novo choline synthesis in mammals is via one distinct pathway catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT). Phosphatidylethanolamine N-methyltransferase, glycine N-methyltransferase (GNMT) and guanidinoacetate methyltransferase (GAMT) all play a major role in the regulation of methyl group metabolism and in the synthesis and remethlylation of homocysteine (Williams and Schalinske, 2007). Specifically, phosphatidylethanolamine N-methyltransferase acts as a catalyst for the pathway that allows for the repeated methylation of phosphatidylethanolamine to phosphatidylmonomethylethanolamine, phosphatidyl dimethylethanolamine and finally phosphatidylcholine (Williams and Schalinske, 2007). Three methyl groups derived from SAM and ultimately Met are required for the synthesis of one molecule of choline. Ultimately this pathway consumes a far greater percentage of methyl groups then the GAMT and GNMT pathways and thus may play a larger role in the regulation of homocysteine remethylation. The byproduct of this reaction is three molecules of homocysteine that must either be degraded via transsulfuration/oxidation or remethylated to Met (Stead et al., 2006).

The sources of SAM for PEMT choline synthesis reaction may vary depending on the current nutritional status of the animal and availability of Met, folate, vitamin B_{12} (Pinotti et al., 2002). Because methyl group synthesis is metabolically costly, during Met, folate, or vitamin B_{12} deficiency it can be presumed that choline synthesis is limited, and therefore choline would be considered an essential nutrient (Pinotti et al., 2002). Deficiencies of Met, folate or vitamin B_{12} are confounded by the lack of choline absorption from the diet in ruminant animals. Therefore, the ability to supplement the diet with RPC is a possible means to spare Met from catabolism for choline synthesis and reduce the potential for methyl group deficiency.

De novo Methyl Group Synthesis

Du Vigneaud et al. (1945) was first to report *de novo* methyl group synthesis discovered by incorporation of ^{2}H from deuterium in choline. They initially theorized that the rat was capable of synthesizing methyl groups *de novo*, most likely via gut micro flora. Shortly thereafter, Sakami et al., (1950) demonstrated with C^{14}-formate that the rat was able to synthesize Met and choline methyl groups from formate in vivo and in vitro using liver tissue, confirming *de novo* synthesis of methyl groups in the mammal.

De novo methyl synthesis can occur from the demethlyation of dimethylglycine to formate. Formate is subsequently reduced to N5- Methyltetrahydrofolate (MTHF) which serves as a methyl donor for remethylation of SAHC to SAM. S-adenosylmethionine can be used in the SAM methyltransferase pathways for formation of methylated products including choline. The synthesis of Met via this route is dependent on the presence of folic acid and vitamin B_{12}.

Methyl Transfer Reactions

Methyl transfer reactions are essentially limited to two specific processes, one that allows for methyl groups to be transferred from S-adenosylmethionine to an array of methylated compounds and the second being the transfer of methyl groups from choline to homocysteine via betaine for the synthesis of SAM. Methyl groups from SAM are utilized for synthesis of methylated compounds such as creatine, norepinephrine and VLDL-lipoproteins in addition to choline via a pathway requiring sequential donation of methyl groups to form the choline precursor phosphatidylcholine.

Emmanuel and Kennelly (1984) estimated that as much as 28% of Met may be supporting choline synthesis via the SAM transmethylation pathway with 6% of choline in their studies being derived from dietary Met in lactating goats. However, there was no indication of choline supporting Met synthesis in this study based on measurements of Met enrichment following labeled choline infusion (Emmanuel and Kennelly, 1984). A later study by Lobley et al. (1996) evaluated the impact of choline and creatine infusion on Met recycling in sheep. Infusion of choline and creatine significantly decreased both Met recycling and irreversible loss rate indicating that choline spared Met from catabolism (Lobley et al., 1996).

Methionine-Choline Interaction in Poultry

Klose and Almquist (1941) found that Met deficient diets supplemented with .5% choline and .9%, homocysteine resulted in growth rates comparable to chicks fed a 1% Met supplemented diet. When choline supplementation was limited to .2% of the diet, growth was reduced dramatically, leading the conclusion that homocysteine utilization in

the Met deficient diet was dependent largely on an adequate supply of dietary choline (Klose and Almquist, 1941) This observation was confirmed by Grau and Almquist (1943) as chicks that were depleted of choline over a period of 16 to 21 days and then subsequently supplemented with homocysteine showed no improvement in growth in contrast to Met supplemented diets that significantly improved growth. Almquist and Grau (1944) went on to demonstrate that the dietary choline requirement in chicks could be partially met through supplementation with Met and betaine. They concluded that betaine could completely replace the dietary choline. However, choline showed a sparing effect on Met and betaine requirements (Almquist and Grau, 1944).

Miles et al. (1983), found that the Met sparing effects of choline were correlated positively with sulfate availability in broiler diets. Miles et al., (1987) showed that 0.11% choline or an equal molar rate of betaine would elicit an increased growth response in chicks fed basal diets marginally limited in Met, choline, and betaine. Growth response was further improved with the addition of 0.24% Met in diets containing supplemented choline and or betaine (Miles et al., 1987). However, in laying hens fed of a corn-soybean meal basal diet that was marginally limiting in choline and Met, there was no improvement in production traits with the addition of 0.044% choline while an equal molar addition of Met (0.033%) significantly improved egg production (Miles et al., 1987). They theorized that choline supplementation was capable of eliciting a positive production response only when other sulfur amino acids are at or above the absolute minimal required level. Conversely, the benefits of choline supplementation were lost when choline is not the limiting factor in the diet.

Lowry et al. (1987) determined that the minimal concentration of dietary choline for chicks to achieve maximal growth rate was 625 mg/kg. Dietary supplementation of betaine at 1000 mg/kg in a purified choline-free diet only decreased the choline dietary requirement in chicks to 467 mg/kg (Lowry et al., 1987). Based on these results, Lowry et al. (1987) theorized that betaine was not capable of meeting more than 25% of choline requirements of the chick. Additionally, Lowry et al. (1987) determined that growth responses to betaine supplementation were found only in diets with adequate dietary choline, this suggested that minimal choline requirements needed to be met first in order to induce positive growth responses to betaine.

More recent work by Dilger et al. (2007) showed that a minimum dietary concentration of 150mg/kg of preformed choline was required per day when betaine was included at 1000mg/kg diet. Furthermore, they suggested that up to 50% of the choline requirements in the growing chick could be satisfied via betaine.

Methionine-Choline Interaction in Swine

Bell et al. (1950) was one of the first to establish that Met was an essential amino acid in swine with a requirement of 0.07% to 0.27% of the growing pig diet when dietary protein concentrations were held at 10%. With Met supplementation in soybean meal based diets, they were able to demonstrate growth efficiency and biological values comparable to that of whole egg protein, the "Gold Standard" for protein at that time. Dyer et al., (1949) had previously demonstrated that choline chloride supplementation in a low protein diet could spare Met in weanling swine. They found that when 250 mg of choline chloride or 0.2% dl-Met were added to basal diets containing 20% crude protein;

growth rates were significantly improved. However, there was no significant difference between the choline supplemented diet, the Met diet and a diet supplemented with both choline and Met, suggesting that these effects were not additive. (Dyer et al., 1949). Alternatively, the amounts added individually were sufficient to meet the animal needs. Based on the results of this study Dyer et al., (1949) concluded that choline was able to effectively spare Met.

Dyer and Krider (1950) found that the Met sparing effect of choline was negated when vitamin B_{12} was supplemented. Choline supplementation to a basal diet that included vitamin B_{12} did not improve weight gain or feed efficiency (Dyer and Krider 1950). Based on these results, Dyer and Krider (1950) theorized that vitamin B_{12} could effectively spare choline.

More recently, Lovett *et* al. (1986), investigated the roles of sulfate in sparing dietary sulfur containing amino acids and specifically Met and choline. A sulfate supplemented diet with either added choline or Met improved average daily gain over the basal diet while adding both Met and choline did not further improve ADG or feed conversion ratios. According to the authors, the basal diet was not deficient in either Met or choline, both of which were included at lower levels than that of Cys. However, when Met and choline were supplemented in the diet, Cys became more limiting. Lovett *et* al. (1986) suggested that the inclusion of sulfate in the Met and Cho supplemented diets resulted in a higher growth by sparing dietary Cys.

Earlier studies in swine by Curtin et al., (1952) and Shelton et al., (1951) supported the hypothesis that dietary Cys supplementation in low protein diets can spare Met. Curtin et al., (1952) showed that supplemental Met requirements were significantly

lower when Cys was included in the diet in adequate amounts. Shelton et al., (1951) demonstrated that L-Cys could replace up to one half of the total dietary Met requirements in growing pigs. This sparing effect appears to be a direct result of the decreased demand for the transsulfuration and oxidation of homocysteine for Cys synthesis.

Rumen Degradation of Choline

Phosphatidylcholine, an immediate precursor to choline is found in relative abundance in many plants and feedstuffs and could be considered to be the primary form of choline in the ruminant diet (Neill et al., 1978). However, dietary sources of choline have been shown to have limited to no bioavailability in ruminants (Neill et al., 1978; Pinotti et al., 2002, Sharma and Erdman, 1988b). Sharma and Erdman (1989b) found nearly complete rumen degradation of choline incommonly fed feedstuffs of in situ rumen incubations. Dietary choline chloride was completely degraded using in situ incubations (Sharma and Erdman 1989b) and in dairy cows fed high amounts (> 300g) of choline chloride (Sharma and Erdman 1988b). Neill et al. (1978) found that the vast majority of feed choline in hydrolyzed and rapidly degraded to acetaldehyde and trimethlyamine. Trimethylamine was rapidly converted to methane gas in the rumen and more than 75% of choline methyl is converted into methane within 6 h in the rumen. (Neill et al., 1979)

It has been suggested by Broad and Dawson (1976) that a limited amount of dietary choline may be incorporated into rumen ciliate protozoa. However, studies by Neill et al. (1979) showed very minimal amounts of choline incorporated as PC in ciliate

protozoa and that protozoa do not appear to be even a minor contributor to absorbed choline or methyl groups in ruminant animals. Neill et al., (1979) calculated that sheep absorb less than one fiftieth the amount of dietary choline required by non-ruminants, estimating that in total they absorb no more that 20-25 mg choline per day from the diet. Further, defaunated sheep failed to show symptoms of choline deficiency, supporting the conclusion that protozoa do not provide significant sources of choline for intestinal absorption (Dawson et al., 1981). In conclusion, dietary choline whether in the common plant form of PC or as choline chloride is completely destroyed by rumen fermentation. This suggests that ruminants must *de novo* synthesize the majority of their choline needed to meet their metabolic requirements.

Effects of Choline on Milk Production

As dietary choline is completely degraded in the rumen, experiments measuring milk production responses of lactating dairy have used two approaches to deliver choline directly to the small intestine: 1) postruminal choline infusion; and 2) feeding rumen-protected forms of supplemental choline.

Postruminal Choline Infusion Experiments

Initial experiments by Sharma and Erdman (1988ab) used abomasal infusion of choline since rumen-protection technology was not available to feed choline directly at that time. In these experiments 30 to 90 g/d choline was abomasally infused over 3-4 week experimental periods. Milk production responses to postruminal choline infusion ranged from 0 to 3.8 kg/day with small increase in milk fat percentage. Generally there

was no effect of choline infusion on feed intake. Based on these experiments they concluded that postruminal dietary requirement for choline was about 30-50 g/d. While, many experiments were positive, others showed no effect, suggesting that choline might be a limiting nutrient for milk production in the dairy cow.

Subsequently, Sharma and Erdman (1988a) tested the potential for Met and choline interactions using a combination of choline or Met postruminal infusion with or without 2-amino-2methyl-1-propanol (2AMP) which is a competitive inhibitor of PEMT and theoretically would block PC synthesis using SAM. Cows receiving post-ruminal choline had greater milk fat and milk protein production than those receiving the Met even though they were administered on a molar equivalent basis (Sharma and Erdman, 1988a). Subsequent post-ruminal infusion of 2AMP with Met, resulted in a further decrease in milk protein and fat-corrected milk yield compared to cows receiving 2AMP and choline infusion (Sharma and Erdman, 1988a). These results suggested that Met was being used as a methyl donor for *de novo* choline synthesis and that choline and Met interacted in a similar manner in dairy cows as had previously been observed in monogastric animals.

Rumen-Protected Choline Supplementation Studies

Numerous studies have been conducted in order to determine the efficacy of rumen protected choline supplementation on milk production and composition (Table 1.1). Several studies have noted an increase or a tendency for higher milk yield within RPC treatment groups (Erdman and Sharma, 1991; Erdman, 1994; Hartwell et al., 2000; Pinotti et al., 2003; and Davidson *et* al., 2008). However a similar number of studies

failed to note a positive response to RPC supplementation (DiConstanzo and Spain, 1995; Hartwell et al., 2000; Janovick-Guretzky et al., 2006; Davidson *et* al., 2008; Piepenbrink and Overton, 2000). Fat yield was increased within some RPC treatments (Erdman and Sharma, 1991; Erdman, 1994; Hartwell et al., 2000; Piepenbrink and Overton, 2000; Pinotti et al., 2003; Davidson *et* al., 2008) while others showed no significant effect of RPC supplementation. (DiConstanzo and Spain, 1995; Piepenbrink and Overton, 2000; Janovick-Guretzky et al., 2006; Davidson *et* al., 2008; Hartwell et al., 2000). Protein yield in response to RPC supplementation increased within two studies (Erdman and Sharma, 1991; Erdman, 1994) and decreased in one study (Hartwell et al., 2000) which might be expected as protein levels in the study were lower than would typically be fed.

Table 1.1 Summary of dairy cow rumen-protected choline (RPC) supplementation studies with or without rumen-protected methionine (RPM) or rumen-protected betaine (RPB).

Reference	Lactation Stage[1]	Rumen-Protected Choline Fed (g/d)	DMI[2] (kg/d)	Milk Yield (kg/d)	Fat Yield[2] (g/d)	Protein Yield[2] (g/d)
Erdman and Sharma (1991)	0 to 147 d postpartum	0	21.8	36.7	1354[2]	1211[3]
		16.9	21.7	37.7	1198[2]	1187[3]
		36.1	23.2	38.9	1384[2]	1205[3]
		51.1	21.9	37.4	1387[2]	1208[3]
Erdman and Sharma (1991) 13% CP	121 to 205 d postpartum	0	21.8	31.7	1271	1088
		18.5	22.1	31.7	1243	1079
		37.2	22.2	33.4	1498	1107
		56.9	22.7	34.8	1207	1178
Erdman and Sharma (1991) 16.55 CP	121 to 205 d postpartum	0	23.7	31.6	1209	1147
		19.6	23.4	31.6	1017	1124
		40.4	23.9	33.9	1108	1226
		57.9	23.2	33.8	1160	1205
Erdman (1994)	Early lactation	0	NA	30.0	1047[3]	954[3]
		33.3		31.1	1128[3]	982[3]
DiConstanzo and Spain (1995)	28 d prepartum to 120 d postpartum	0, 15, 30, or 45g RPC, 25g RPM	No Difference From Control			
Hartwell et al. (2000) Low RUP (4.0%) (Intake is noted as pre-post partum)	28 d prepartum to 120 d postpartum	0	12.9-23.1	38.6	1530	1200
		6	12.3-22.7	38.0	1600	1160
		12	12.6-22.3	39.8	1490	1170
Hartwell et al**. (2000)** High RUP (6.2%)		0	11.7-20.9	37.2	1430	1070
		6	12.4-21.4	38.7	1500	1120
		12	12.6-20.1	34.4	1320	1020
Piepenbrink and Overton (2000) (Intake is noted as pre-post partum)	21 d prepartum to 63 d postpartum	0	12.8-18.4	39.4	1592[3]	1174[3]
		45	12.0-18.9	43.5	1836[3]	1314[3]
		60	12.9-18.3	40.2	1596[3]	1206[3]
		75	12.5-18.7	41.1	1763[3]	1262[3]
Piepenbrink and Overton (2003) (Intake is noted as pre-post partum)	21 d prepartum to 63 postpartum	0	12.6-17.8	40.0	1580	1170
		45	11.9-18.7	43.3	1780	1280
		60	12.8-18.3	39.9	1560	1180
		75	12.5-18.8	41.0	1710	1240
Pinotti et al. (2003) (Intake is noted as pre-post partum)	14 d prepartum to 30 d postpartum	0	11.3-19.4	28.5	880	868
		20	11.4-19.9	31.4	1056	966
Janovick-Guretzky *et* al. (2006)	21 d prepartum to 21 d postpartum	0	12.0-14.8	29.6	1380	1050
		15	12.1-15.7	31.6	1460	1090
Davidson et al. (2008) Premiparous	21 to 91 d postpartum	0	27.9	27.9	840	730
		(RPM) 20	20.0	28.0	770	760
		(RPB) 45	18.8	26.1	790	700
		(RPC) 40	20.2	27.5	790	740
Davidson et al**. (2008)** Multiparous	21 to 91 d postpartum	0	21.9	37.7	1030	920
		(RPM) 20	20.8	39.8	990	1040
		(RPB) 45	21.7	38.6	1110	960
		(RPC) 40	24.3	44.1	1116	1100

[1]Relative to control .[2]Calculated from milk fat percent and milk yield (kg/d). [3]Calculated from milk protein percent and milk yield (kg/d).

The variation in these results may be explained in part through differences in study design. There are few consistencies between diets, supplement quantity, length of treatments and stage of lactation. Basal diets, when comparing across experiments vary from Met limiting to adequate based on NRC recommendations. Several studies (Erdman and Sharma, 1991; Janovick-Guretzky et al., 2006; Hartwell et al. 2000) have attempted to elucidate the relationship between choline and various sources and levels of dietary protein. Erdman and Sharma (1991) were able to demonstrate a tendency for increased milk yield with RPC supplementation with the magnitude of this increase being greater in animals receiving a lower protein diet (13.0 vs. 16.5%). However, Janovick-Guretzky et al. (2006) did not observe a positive production response from RPC supplementation when feeding a diet that was in limiting in metabolizable protein flow to the small intestine but otherwise adequate based on NRC recommendations. Janovick-Guretzky et al. (2006) suggested that the amount of RPC (15g/d), half of what was used by Erdman and Sharma (1991), may not have been sufficient for a production response.

An earlier study by Hartwell et al. (2000), noted a positive production response when feeding as little as 12g/d RPC with a low RUP (4.0%). Hartwell et al. (2000), was designed in part to evaluate the effect of RUP in addition to RPC. A significant decline in DMI post parturition was seen when increased RUP (6.2%) were fed prepartum. Animals consuming a treatment diet with a lower RUP (4.0%) prepartum did not exhibit a decline in DMI and had an increase milk yield with RPC supplementation, this suggested indicating that protein level in the prepartum diet may contribute to RPC efficacy (Hartwell et al., 2000). Cumulatively, these studies suggest that the amounts

and type of protein, as well as when it is fed may be a significant factor in affecting the dairy cows response to RPC supplementation.

In addition to variation in basal diets and the quantity of choline supplementation (6 to 75 g/d) in the reported studies, there are significant differences in production status of cows used across these experiments. Studies in early to mid lactation multiparous cows receiving RPC had both positive production responses (Erdman and Sharma, 1991; Erdman, 1994; Piepenbrink and Overton, 2003; Pinotti et al., 2003; Davidson *et* al., 2008) as well as no effect of treatment (DiConstanzo and Spain, 1995; Piepenbrink and Overton, 2000; Janovick-Guretzky et al., 2006).

The time at which RPC was first administered (as early as three weeks pre partum to several months post partum), in addition to the length of time supplementation was applied (42-150 days), makes comparison between these studies difficult and further confounds the understanding of when, and under which conditions, RPC supplementation will be beneficial. Furthermore these studies are limited largely to multiparous cows with the exception of Erdman and Sharma (1991) and Davidson et al. (2008). Erdman and Sharma (1991) utilized both multiparous and primiparous cows but data was not separated by parity.

Davidson et al. (2008), reported the effects of RPC supplementation in both multiparous and primiparous cows. In multiparous Holstein cows, a significant increase in milk yield and milk protein was noted with RPC (40g/d) supplementation where cows also responded to the addition of rumen protected methionine (20g/d) (Davidson et al., 2008). Rumen-protected betaine (45g/d) had no effect on production data, suggesting

that choline may be sparing methionine via phosphatidylcholine rather than contributing to the remethylation of homocysteine (Davidson et al., 2008). However, in primiparous Holstein cows in the same study there was no effect of supplementation with RPM, RPC or RPB, suggesting that RPC responsiveness might be limited to diets which elicit a response to RPM.

Dietary Choline in Transition Cows

Transition cow production performance and physiological response to rumen protected choline supplementation has been evaluated in several studies. RPC supplementation did not affect dry matter intake pre or post partum in studies by Hartwell, *et* al., 2000, Pinotti *et* al., 2003, Piepenbrink and Overton, 2003, Janovick-Guretzky et al. 2006 or Cooke et al., 2007. However, in two studies (Oelrichs ET al.2004; Chung et al., 2005) a significant decrease in DMI was associated with RPC supplementation.

There was no effect of RPC supplementation on liver triacylglycerol (TAG) concentrations during early lactation in studies by Hartwell, et al., (2000) and Piepenbrink and Overton, (2003). In contrast, Cooke et al. (2007), observed a decrease in liver TAG concentrations during fatty liver induction when RPC was supplemented. Additionally, TAG clearance from the liver was increased with RPC supplementation following fatty liver induction (Cooke et al., 2007).

Piepenbrink and Overton, (2003), noted an increase in liver glycogen concentrations with RPC supplementation but no effect on blood ß –hydroxybutyrate (BHBA). Cooke et al., (2007) found no effect of RPC on plasma glucose or (BHBA)

during fatty liver induction. Janovick-Guretzky et al., (2006) noted a decline in plasma BHBA with RPC supplementation in Jersey but not Holstein cows while Pinotti et al. (2004) noted tendency for increased plasma glucose in response to RPC supplementation.

The effect of RPC on milk production responses in early lactation cows is far from consistent. Several studies have noted increases in milk yield, fat, or protein content (Erdman, 1994; Pinotti et al., 2000; Piepenbrink and Overton, 2003; Pinotti et al., 2004) while others have noted a decline (Hartwell et al., 2000) or no effect of RPC supplementation (Janovick-Guretzky et al., 2006).

Body condition score and weight gain/loss in response to RPC supplementation varied as well with some noting no effect of treatment (Piepenbrink and Overton, 2003; Janovick-Guretzky et al., 2006), others noting lower BCS and or increased weight loss with RPC supplementation (Hartwell et al. 2000).

Plasma nonesterified fatty acid (NEFA) concentration is frequently used as an indicator of fatty liver disease (Grummer, 1995). Studies by Hartwell et al. (2000, 2001), Piepenbrink and Overton (2002) and Janovick-Guretzky et al. (2006), reported no effect of RPC supplementation on plasma NEFA while Pinotti et al. (2003, 2004) and Cooke et al. (2007) showed decreases in plasma NEFA when RPC was supplemented.

Fundamental Questions Regarding Rumen Protected Choline Supplementation

When viewed cumulatively the results of these studies suggest a possible need for choline supplementation, however two fundamental questions remain unanswered;

1) What are the dietary and physiological conditions under which choline supplementation will be beneficial to lactating cows?

2) What are the amounts of rumen protected choline that are required in dairy cow diets to affect milk production and milk composition?

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CHAPTER II

Dietary Choline Serves as Methyl Donor in Liver but not Mammary Tissue of Lactating C57BL/6 mice.

Dietary Choline Serves as Methyl Donor in Liver but not Mammary Tissue of Lactating C57BL/6 mice [1]

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FOOTNOTES:

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[2] Abbreviations used: APE, atoms percent excess; CHOL, Choline; $E_{B,}$ control sample enrichment; $E_{S,}$ treatment sample enrichment; dH_2O, deionized water; DTT, dithioerythritol; MET, methioine; SSA, sulphosalicyclic acid; tBDMS, tertiary-butyldimethylsilyl.

Abstract

The objective of this study was to determine the extent of methyl group transfer from CHOL and MET during lactation in C57BL/6 mice. Mice were pair fed a purified low protein (10%) diet supplemented with choline chloride or methionine on a methyl group equivalent basis from d 1 to d 15 of lactation. From d 11 to 15 stable isotopes of [methyl-$^2H_3$] choline and [methyl,$^2H_3$]Met replaced the unlabeled fraction. Growth data suggested that pups in both treatment groups were methyl group deficient by d 10 of the treatment period. Samples collected on d 15 indicated that free liver methionine methyl enrichment from choline was of 35% of that from MET supplementation, establishing a minimum choline contribution to remethylation in the liver. The liver was the major site of methionine remethylation from dietary choline in the mouse. Conversely, mammary tissue did not appear to be a major site of methionine remethylation from choline. The data suggested that there was an unlabeled source of methyl groups that contributes to remethylation in the mammary tissue at a rate far in excess of that from choline. There were no treatment effects on liver tissue:liver free and mammary tissue:casein enrichment ratios suggesting that both treatments resulted in similar fractional rates of methionine remethylation. The data suggested that de novo methyl group synthesis was induced for MET remethylation in the mammary gland resulted in significant MET synthesis.

Introduction

In addition to methionine's function as an amino acid, its constituent methyl group serves as readily available source of labile methyl groups required in numerous methylation pathways including synthesis of phosphatidylcholine, methylation of proteins, DNA, RNA and a variety of other molecules (norepinephrine, catechols and sterols). Methionine is the immediate precursor of S-adenosylmethionine (SAM), the

ultimate methyl group donor in over 100 transmethylation reactions (1). Methyl groups, made available via the transmethylation pathway, can be found in every cell and have functions ranging from regulation of DNA activity to cell membrane integrity (2). Numerous studies have identified the negative consequences of Met deficiency and in turn methyl group deficiency which increases the level of importance of remethylation of homocysteine to Met (2). It has been observed that Met deficient diets result in reduced rate of transsulfuration/oxidation of homocysteine and increased remethylation of homocysteine to Met (3).

Betaine, the product of choline oxidation, is the primary source of methyl groups for remethylation of homocysteine to methionine in humans, mice and rats (4,5,6). The remethylation of homocysteine by the betaine –choline pathway is dependent on the activites of two enzymes: choline oxidase and betaine-homocysteine methyltransferase. The activity of these enzymes in humans appears to be regulated in part by plasma methionine and homocysteine concentrations (7). In monogastric animals, most homocysteine remethylation occurs in the liver with limited amounts in other tissues such as the pancreas and kidneys (2,4).

To date, there is no quantitative data on the interrelationship of CHOL and MET methyl group metabolism within specific tissues of the lactating mouse. Therefore, an experiment with lactating mice was conducted with the objective of determining the extent and tissue specificity of methyl group transfer from dietary MET and CHOL during lactation.

Materials and Methods

Mice and Treatments. The experiment was reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee under Protocol R-05-57. Ten pregnant C57BL/6 mice were obtained from the University of Maryland Animal and Avian Sciences animal colony. Mice were individually housed in plastic shoebox containers under climate controlled conditions at 23°C with 12h:12h light dark cycle. Bedding was changed weekly and water bottles changed every other d with feed and water available continuously. Mice were fed a standard lab animal diet (Lab Diet 5001 Rodent Diet, PMI Nutrition International, LLC, Brentwood, MO) ad libitum throughout pregnancy until parturition.

On d 1 postpartum, the dams were switched to a pelleted low protein (10%), methyl group deficient basal diet that was otherwise nutritionally complete (8). Dam-litter pairs were blocked on litter size and d of lactation and randomly assigned within block to one of two experimental diets (Table 2.1). Dietary treatments consisted of equal molar methyl group additions of either D,L- Methionine (300 mg/100g) or Choline (9.5 mg/100g Choline-Cl).

The diets were prepared as follows: Dry ingredients were thoroughly mixed for several min with a small hand mixer, during which time a modest amount of water was added. The final mixed product resembling pliable dough was divided into spoonfuls and air dried to a constant weight prior to feeding. During d 1 to10, diets containing unlabeled Met and choline were fed. From d 11 to 15, unlabeled choline was replaced with [methyl-$^{2}H_{9}$] choline (98 atoms percent, Cambridge Isotope Laboratories, Andover, MA) while unlabeled Met was replaced with

[methyl-$^{2}H_3$]Met (methyl-$^{2}H_3$, 98 atoms percent, Cambridge Isotope Laboratories, Andover, MA).

Mice were monitored twice daily for overall health and onset of parturition. Parturition day was based on the presence of pups at the morning health check at which time, they began on their experimental diets. Feed intake and refusals were recorded daily throughout the study. Dams were weighed every 2 d beginning at on d 2 post partum, and litters every 2 d beginning at d 6.

Sample Collection. Milk samples were collected from dams on d 11, 13, and 15 postpartum using procedures similar to those previously described (9). Briefly, dams were isolated from the pups in a separate plastic shoe box container for a period of 2 h prior to milk collection. Dams had ad libitum access to feed and water during this period. At the end of the isolation period and prior to milk collection, mice were gently restrained by hand and given an inter-abdominal injection of 0.3mL containing 1IU/mL oxytocin in sterile saline. Mice were then returned to their respective containers for 5 min after which they were again gently restrained by hand and their teats were manually stimulated. Milk was collected in a silanized Pasteur pipette under vacuum from a water aspirator as using used as a source of vacuum (10). Milk was then transferred from the Pasteur pipette into hematocrit tubes via capillary action and sealed with critaseal. Hematocrit tubes were placed into individual plastic test tubes and labeled to identify mouse and date of collection. Following collection, samples were centrifuged for 20 min at maximum speed on a clinical hematocrit centrifuge (International Micro-Capillary Centrifuge Model MB, International Equipment Company, Needham Hts., MA) (10). Fat

percentage was measured using a hematocrit/creamatocrit and then stored at -20°C until further analysis (10).

Following the d 15 milk collection, dams and pups were euthanized by isoflorane asphyxiation and placed ventral side down on a marble slab that had been preciously washed with .9% saline and set on a bed of ice to rapidly reduce specimen temperature. Following a midline incision passing through the skin and peritoneum, livers and kidneys were removed and the kidneys were dissected of visible fat. Livers and kidneys were washed in ice cold .9% saline, placed in tin foil and then frozen at -20°C for later analysis. Immediately after liver removal, blood samples were from dams were collected via the hepatic portal vein in a 1 mL heparinized syringe fitted with a 21 gauge needle. Samples were gently mixed within the syringe and then separated into 100 μL aliquots and frozen immediately at -20°C.

Stomachs from the pups were removed and the milk within was expelled by gently pressing the stomach content into a 2 mL Eppendorf tube. The contents of all littermate pup stomachs were stored collectively at -20°C for later analysis. Samples of muscle from the hind limbs and mammary tissue of the dams were collected and stored at -20°C. The small intestine was removed from the stomach up to but not including the ceca. The small intestine was flushed with 20 mL of ice cold 0.9% saline in order to remove all digesta, the tissue was stored in the same manner as the previous samples. All tissue samples were lyophilized for 48 h or until reaching complete dryness, prior to chemical analysis.

Sample Preparation and Analytical Procedures. Milk fat was separated from the protein and water fraction by scoring the glass hematocrit tube and gently breaking the

tube at the junction of the fat and non-fat fraction. The non-fat fraction was placed in a 1.5mL micro-centrifuge tube, centrifuged at 250 × *g* for 1 min or until entirely expelled. One mL deionized H_2O (dH_2O) was added, the sample vortexed, and 1M HCl added to adjust the pH to < 4. Samples were vortexed vigorously and then centrifuged at 12000 × *g* for 10 min to precipitate the casein pellet. The supernatant was removed, and 1 mL dH_2O was added along with 1 drop of 6M NaOH to neutralize the HCl. Samples were re-suspended followed by a second precipitation with 1M HCl and centrifugation. The supernatant was removed, the casein pellet was washed twice with 0.5 mL of dH_2O and the final pellet frozen and lyophilized as described above.

Dried casein samples were hydrolyzed with 0.5 mL of 2N HCl containing 0.05% dithioerythritol (DTT) at 100°C for 24 hours. Cooled hydrolysates were transferred to 2.5 mL micro-centrifuge tubes and centrifuged briefly, reaching a final speed of 10,000 rpm (Eppendorf MiniSpin® rotor 5452000018, Hamburg, Germany) to precipitate debris. The supernatant (0.25mL) was applied by pipette to mini-columns with 1g cation exchange resin (AG 50W-X8, 100-200 mesh, H+ form, Bio-Rad, Hercules, CA) previously prepared by washing with 12 mL dH_20. Following sample application, columns were monitored for elution cessation at which point a wash of 8 mL dH_20 was applied. Waste elution containing acids, salts and neutral compounds was discarded. Amino acids bound to the resin were eluted with 4 mL of 2M NH_4OH followed by 2 mL dH_20. The NH_4^+ and dH_20 elutions were collected in 8 mL glass pots and then covered with punctured caps. The samples were frozen overnight at -20°C and then lyophilized until completely dry.

Dried samples were transferred to a 1 mL V-vials with 150 µL of dH_20 and then dried completely under a stream of N_2 gas at 40°C prior to preparing the tertiary-butyldimethylsilyl (tBDMS) derivatives. To the dried sample, 50 µl of N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane and 50µl of dimethylformamide was added. Samples were capped, vortexed and heated at 90°C for 20 min. then transferred to auto sampler vials for GC-MS analysis on an Agilent 6890 Series GC system with a 5973 Network Mass Selective Detector with an Aletch EC-5 30-m column (ID 0.250 µm, 0.25 µm film thickness). The initial column temperature was set at 100°C with a 1 min hold followed by a single ramp of 10°C/min to a final temperature of 300°C. Sample injection volumes were 1µl with the split adjusted as required to obtain satisfactory peak areas. Ions were monitored with mass-to-charge for Methionine 292 and 295, which elutes between 7.65 and 11.96 min.

Thawed blood samples (100 µl) were deproteinized by the addition of 1 mL of 10% sulphosalicyclic acid (SSA). Samples were vortexed vigorously and then placed on ice for 10 min in order to improve protein precipitation followed by centrifugation at12,000 × g for 10 min. Supernatants were prepared on resin mini-columns as above for casein hydrolysates with the exception that 0.5 g of resin was prepared and accordingly elution solutions were reduced in volume by half. Samples were lyophilized, transferred to 1 mL vials with 0.35 mL of 0.1M HCL and then dried completely under a stream of N_2 gas at 90°C The tBDMS derivatives were prepared as for casein hydrolysates except that 30 µL N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane and 30µl of dimethylformamide was added. Samples were capped, vortexed thoroughly, and heated at 90°C for 30 min and then transferred to auto

sampler vials for GC-MS analysis of methionine and glycine. Column, temperature, and flow settings along with the retention times and ions monitored were identical to those used for casein analysis. Injection volume was increased to 2 µl and the split was 50% of that for casein hydrolysate analysis in order to obtain a satisfactory area.

Dried liver and mammary tissues were pulverized to a powder in a cryogenic impact mill (Spex Certi Prep Freezer Mill 6850, Metuchen NJ). Subsamples of 100g were homogenized with 15% ice cold sulphosalicyclic acid in 2.5 mL Eppendorf tubes. In order to achieve complete deproteinization of free Methionine, samples were further homogenized using a VWR AHS-200 Power Max Homogenizer (VWR International, West Chester, PA) with a 5 mm diameter generator. Samples were place on ice for 15 min following homogenization and then processed in a manner identical to the blood samples. Sample pellets following deproteinization were stored at -40°C for further analysis.

Liver and mammary free methionine enrichment was analyzed by GC-MS (HP 6890; Agilent) fitted with a HP-5 column (0.250 µm ID, 0.25 µm film thickness, 51 m) and mass spectrometer detector (HP 5973N Mass Selective Detector, Agilent). The initial column temperature was set at 100°C with a one min hold followed by an initial ramp of 40°C/min to a temperature of 220°C and a final ramp of 10°C/min with a final temperature of 300°C. Sample injection volumes and split were adjusted as required to obtain a satisfactory peak areas. Ions with mass-to-charge were monitored for methionine 292-295 at a retention time of 11.1 min.

For liver tissue, following initial deproteinization, samples were re-suspended in 1 mL ice cold methanol, vortexed for 15 s and then placed on ice for 5 min. Samples were

centrifuged at 1200 × *g* for 10 min, the supernatant containing fat was discarded and the process was repeated. Samples were dried completely in a convection oven at 50°C. Dry liver samples (10 mg) were transferred to hydrolysis tubes, containing 2 mL of 2M HCL with 0.05% DTT. Samples were heated for 18 h at 110°C to facilitate complete deproteinization of the tissue. Once cool, a 1 mL fraction of the sample was processed in a manner identical to the blood samples with the exception of rinsing the resin bed with 16 mL of dH_2O following sample application to ensure the elute was at a neutral pH.

Approximately 100 mg of the labeled methionine diet was ground by mortal and pestle and hydrolyzed with 4 mL of 2M HCL with 0.05% DTT for 18 h. The sample was then filtered (#4 Whatman) to remove particulate matter. Half of the filtrate was then applied to a mini-column containing 2 g 50W-X8 100-200 mesh hydrogen form resin beds (Bio-Rad, Hercules, CA) that was prepared with 24 mL d H_20. Following sample application, the resin bed was washed with dH_20 until the elution reached a neutral pH. The sample was then eluted with 8 mL 2N NH_4OH and 6 mL dH_20 into a 40 mL plastic vial with a snap top closure. The sample was lyophilized as described above. Once dry the sample was taken up in 2 mL dH_20, 400 μL was transferred to a 2 mL vvial and blown down under a stream of N_2 gas at 40°C. To the dry sample 50 μL N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane and 50 μLof acetonitrile was added. The sample was heated at 90°C for 20 min and analyzed in the same manner as the liver free methionine.

Calculations and Statistical Analysis. Methionine isotopic enrichment was expressed as atoms percent excess (APE):

$$APE = 100 \times (R_i - R_o)/(1 + R_i - R_o)$$

Where R_o and R_i are the ratios of m/2 295/292 for a sample at natural abundance and an enriched sample respectively. This corrects the observed sample enrichment (R_i) for the natural isotopic abundance based on the enrichment of control samples (R_o) and establishes the isotopic enrichment resulting from tracer feeding (APE) (1).

Statistical analysis of pup growth, dam weight and milk isotopic data collected over time were analyzed as repeated measures using ANOVA. Statistical analysis was completed using the MIXED procedure of SAS 9.3 (SAS/STAT Inst. Inc., Cary, NC) with the repeated option and autoregressive covariance structures. The statistical model included: Block, Treatment, Day and Day by Treatment interaction with dam within treatment as a random effect used as the error term for testing treatment effects. Day and day X treatment interactions were tested against residual error. Data are presented as least squares means with pooled SEM with differences between treatment means considered significant when $P \leq 0.05$.

Statistical analysis of the effect of dietary supplement on methionine isotopic enrichment of mouse blood, casein and tissue on d 5 postpartum were analyzed using the MIXED option of SAS 9.3. Isotopic enrichment and ratios were treated as a continuous response variable, treatment was treated as the fixed effect and block was the random variable. The statistical model included: block, treatment and block by treatment interaction. Block and block by treatment interactions were tested against residual error. Data are presented as least squares means with pooled SEM with differences between treatment means considered significant when $P \leq 0.05$.

Results

Dam and pup weight changes. Dam food intake did not differ between diets (Table 2.2). Dam weights measured every other d were measured from d 2 to d 12 postpartum, a significant effect of diet ($P = 0.002$) was noted for the average dam weight across the entire period with methionine fed mice weighing slightly more than there choline counterparts. Analysis of dam weights by d indicated that the effect of diet was only significant at d 4 ($P = 0.04$), but no statistical significance existed for the remaining days. Dam d 2 to 12 total weight gain/loss was not affected by treatment. Additionally gain/loss by d from d 2 to d 12 post parturition was not significantly different between the treatment groups (Tables 2.3 and 2.4).

Table 2.5 summarizes pup litter weights and average weights. There was no significant effect of diet on average litter weights from d 6 to 12. The average pup weight was significantly different ($P = 0.04$) from d 6 to 12 with regards to treatment, methionine pups weighing slightly more on average from d 6 to d 12 than their choline counterparts. The average pup weight was highly significant ($P = 0.03$) at d 6 but was not considered significant at d 8, 10 or 12 as reported in table 2.8 and figure 2.3. Pup average daily gain is summarized in Table 2.6, there was no significant effect of treatment on the average of daily gain over d 6-12. There was a trend for higher gain among the choline treatment ($P = 0.07$) for d 6-8, 8-10 and 10-12 (Figure 2.2). Total weight gain from d 6 to 12 post parturition for the litter and average pup was not significantly different between the two diets as summarized in Table 2.6.

Diet, Tissue, blood and milk enrichment.

Analysis of the methionine supplemented diet indicated methionine methyl group enrichment of 54.89%. Methionine enrichments for liver, blood, and mammary tissue collected on d 15 postpartum are shown in Table 2.7. As expected, methionine enrichment was 3 to 10 fold greater in dams fed MET vs. those fed CHOL. For dams fed MET, liver tissue and free methionine along with whole blood and mammary tissue methionine enrichments were similar. In dams fed CHOL, methionine enrichments were much greater in liver, than in blood, mammary tissue or casein.

Dams receiving CHOL blood enrichment values of 4.08, roughly half the enrichment values of the liver free and liver tissue methionine (9.26 and 8.27). In contrast to dams fed MET, mammary tissue and casein enrichments were lower than in whole blood and liver.

Relative enrichment ratios are summarized in Table 2.7. As expected, enrichment ratios between liver tissue and liver free methionine were not different in both treatments. However, enrichment ratios of casein:whole blood were lower and liver tissue: free methionine were greater ($P < 0.01$) in animals fed CHOL vs. MET suggesting substantial differences in methyl transfer from choline in mammary vs. liver tissue.

Discussion

Production Measures. In an earlier study in C57BL/6 mice fed a protein adequate diet had pups that weighed approximately .5 g more on d 10 of lactation than was observed in this study on d 12 of lactation (11). This data indicates that pups in both treatment groups were methyl group deficient prior to d 10 of the treatment period. MET treatment pups initially performed better based on calculations of growth rates at d 6 ($P =$

0.03). By the conclusion of the study there was no significant difference in the average pup weights between the diets. Cumulatively this data suggests that mice receiving the MET supplemented diet were better able to synthesize milk than their CHOL supplemented counterparts during the early stages of lactation, but experienced a more rapid decline in production once body stores of MET became limiting. Both treatments groups appear to have reached a peak rate of average daily gain on or before d 6 with a decline in pup average daily gain across d 6-12 in contrast to pups on protein adequate diets (11).

Tissue, blood and milk enrichment. Both treatment diets contained casein as the protein component and therefore also contained MET, this may have reduced the rate at which body stores of protein would have been catabolized to meet lactation demands. However, there were no treatment effects on liver tissue:liver free and mammary tissue:casein enrichment ratios suggesting that both treatments resulted in similar fractional rates of MET remethylation.

Mice receiving the CHOL supplemented diet had a MET liver tissue enrichment of 9.26 APE, establishing a minimum choline contribution to homocysteine remethylation in the liver. These results are consistent with earlier studies that identified the liver as a major site of homocysteine remethylation in other mammals (4, 12, 13)

In contrast, mammary tissue does not appear to be a major site of MET remethylation from CHOL in the lactating mouse, as MET enrichment in CHOL fed mice was half that of whole blood enrichment. This would indicate that there is an unlabeled source of methyl groups that contributes to remethylation in the mammary tissue at a rate

far in excess of that from CHOL. This data is consistent with earlier studies in lactating Sprague-Dawley rats which demonstrated that free CHOL taken up by the mammary tissue remains predominately as CHOL or CHOL metabolites (14).

Casein MET enrichment in both treatment groups was roughly 30% greater than that of the mammary tissue. This would indicate a more highly labeled source of MET than that found in the mammary tissue is serving as the pre-cursor pool for casein synthesis. An earlier study in lactating goats (15) in which carbon labeled CHOL and MET were infused via a jugular catheters demonstrated that the protein and whey components of milk were derived largely from the free MET pool. Our data suggest free MET from the diet as well as from CHOL remethylation was a major contributor to casein synthesis based on casein and whole blood enrichments.

The present study indicates whole blood MET enrichment is significantly lower than casein enrichment in animals receiving the MET supplemented diet. This may be explained in part by the use of whole blood in lieu of plasma. Accurately identifying precursor pool MET enrichment through whole blood is limited due to the unlabelled fraction of red blood cells that undoubtedly dilute the enriched plasma fraction. Female C57BL/6 mice at sixteen weeks of age have a packed cell volume of 51.2% that is comprised almost entirely of red blood cells (17). Red blood cells may obtain up to 63% of their amino acids primarily from tissue rather than the free plasma pool which would be more highly labeled (16). In addition relative labeling of the MET diet is roughly half that of CHOL due to the unlabeled fraction of MET from casein.

In all likelihood, the dietary MET enrichment of 54.89% for the MET treatment is probably a closer estimate to the plasma free MET pool than the actual value of the whole blood sample, the same is likely true for the CHOL diet.

TABLE 2.1 Ingredient composition of experimental diets[1]

Ingredient	MET	CHOL
	g/100 g diet	
Dextrose[2]	70.00	70.00
Vitamin free casein[2]	10.00	10.00
Solka-floc[3]	5.00	5.00
Soybean oil[2]	9.00	9.00
AIN 93 Mineral Mix[4]	4.00	4.00
AIN 76 Vitamin Mix[5]	1.50	1.50
Choline choride[6]	—	0.095
D,L Methione[6]	0.30	—

[1] Treatments consisted of 300 mg/100g diet added methionine (MET) or 95 mg/100g added choline (CHOL)
[2]Source: Dyets, Inc.
[3]Source: International Fiber Corporation
[4]Source: MPBio
[5]Source: ICN Biomedicals, Inc.
[6]Source: Sigma Chemical Company

TABLE 2.2 Least squares means for feed and methyl group intake by treatment.

	------- Treatment -------			
Item	MET	CHOL	SEM[1]	P-value[2]
Feed, g/d	6.57	6.01	0.37	0.27
Methyl intake, mol/d				
Casein	2.84	2.62	0.16	0.33
[methyl,$^{2}H_3$] Met	3.17	---	0.19	0.97
[methyl-$^{2}H_9$] Choline[3]	---	3.18	0.18	0.97
Total methyl, mol/d	6.00	5.81	0.35	0.64

[1] Standard error of the mean.
[2] Probability that treatment means are not different, when the null hypothesis is rejected.
[3] Estimated from dietary inclusion and isotopic purity.

TABLE 2.3 Dam and pup weights (g) by treatment for d 2 to d12 post parturition.

	---------- Treatment ----------			
Days Postpartum	MET	CHOL	SEM[1]	P-value[2]
Average Dam, g				
2-12	25.50	23.18	0.51	0.002
2	25.46	24.20	1.31	0.47
4	27.93	23.65	1.60	0.04
6	25.92	23.73	1.31	0.21
8	24.82	23.20	1.13	0.31
10	24.97	22.32	1.13	0.10
12	23.92	22.02	1.13	0.24
ADG, g, 2-12	-0.35	-0.27	0.11	0.63
Pup Data				
Litter Weight, g, d 12	14.88	13.96	0.45	0.16
Pup Weight Average, g d 6- d 12	3.71	3.42	0.09	0.04

[1] Standard error of the mean.
[2] Probability that treatment means are not different, when the null hypothesis is rejected.

FIGURE 2.1 Average pup weight d 6 to d 12 in grams Error bars indicated the standard error of the mean. Significance (P =0.03) on d 6 is indicated by *.

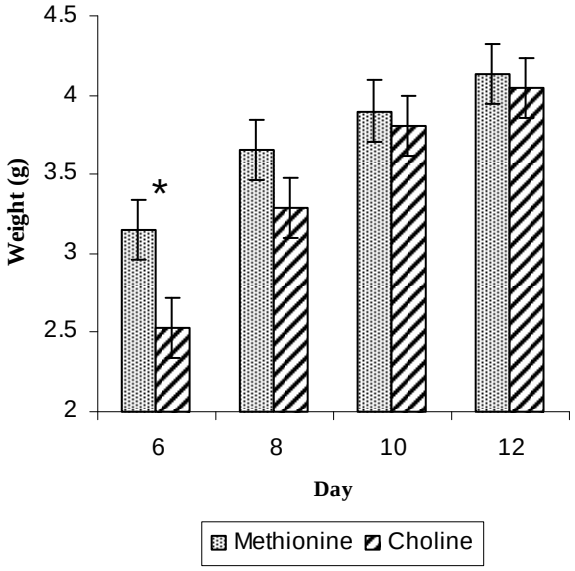

TABLE 2.4 Methionine enrichment atoms percent excess and ratios of mouse blood, casein and tissue on d 5 of supplementation with [methyl-$^2H_3$] Methionine[2] or [methyl-$^2H_3$] Choline[3].

--------- Treatment --------				
Source	MET	CHOL	SEM[1]	P-value[2]
Whole Blood	24.37	4.08	2.07	0.0001
Casein	32.04	2.99	0.48	0.0001
Mammary Tissue	19.51	1.58	1.31	0.0001
Liver Free	26.61	9.26	1.21	0.0001
Liver Tissue	22.75	8.02	1.29	0.0001
Liver tissue:Liver free	0.85	0.89	0.02	0.89
Liver free:Whole blood	1.15	2.34	0.18	0.01
Casein:Whole blood	1.37	0.72	0.11	0.01
Mammary Tissue:Casein	0.61	0.65	0.05	0.67

[1] Pooled standard error of the mean.
[2] Probability that treatment means are not different, when the null hypothesis is rejected.

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CHAPTER III

Choline Methionine Methyl Metabolism in Lactating Cows

RUNNING HEAD: CHOLINE:METHIONINE METHYL METABOLISM IN LACTATING COWS

Rumen Protected Choline Effects on Methyl Group Metabolism in Lactating Dairy Cows

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ABSTRACT

The objectives of this study were to determine the metabolic fates of methyl groups derived from dietary choline and methionine (**Met**) and the bio-availability of rumen-protected choline in lactating dairy cows. Four multiparous Holstein cows in mid lactation were fed a nutritionally complete basal diet except for methionine that was limited to 1.49 % of metabolizable protein. Treatments included the basal diet or the basal diet plus 15g/d rumen-protected choline chloride (**RPC**) in single reversal design with 2 wk experimental periods. To measure metabolic fates of Met and choline, cows received a continuous jugular vein infusion of [1-^{13}C] Met, [methyl-^{13}C] Met, and [methyl-$^{2}H_9$] choline for 12 h on the final day of each experimental period. Milk was collected at 3 h intervals and blood samples taken over the last 6 h. Supplementation with RPC did not affect total milk yield or milk fat and protein yields which averaged 39 kg/d, 1634 g/d and 1110 g/d respectively. Total Met flux, Met irreversible loss, and remethylation were not affected by treatment and averaged 15.2, 11.5, and 4.2 mmoles/h suggesting that 24% of Met was remethylated in the liver. Based on plasma casein tracer:tracee ratios for both ^{13}C-Met and D_3-Met, a minimum of 27% of casein Met methyl group results from *de novo* methyl group synthesis occurs in the mammary gland. Further, upwards of 40% Met present in casein is the result of transmethylation with choline serving as the ultimate methyl donor. Based on differences in Met methyl flux rates, bio-availability of RPC was estimated to be 72%. These results illustrate the central role of Met and choline in methyl metabolism and the importance of methyl group transactions in the high producing dairy cow.

Key Words: choline, methionine, methyl metabolism, lactating dairy cows

INTRODUCTION

Methionine for lactating cows along with lysine is considered to be the first or second limiting amino acid in most monogastric animal and ruminant diets (Schwab et al., 1976). In addition to its role as a building block in protein synthesis, methionine also serves as the precursor for S-adenosylmethionine, which is involved in more than 100 transmethylation biosynthetic end products (Lobley et al., 1996). After donating its methyl group, S-adenosylmethionine is converted to S-adenosylhomocysteine, which in turn can be re-methylated to re-form methionine.

In monogastrics, the tri-methylated compound choline is derived mainly from dietary sources and is the primary methyl donor for re-methylation of homocysteine to form methionine (Benevenga, 2007). Furthermore, choline itself is considered an essential nutrient due to its role as a precursor of phosphatidylcholine. Phosphatidylcholine is an essential component of cell membranes and it is an integral component of lipoproteins that are essential for the removal of triacylglycerol from the liver. A classic symptom of choline deficiency is fatty liver and it has been proposed that a general deficiency of methyl groups leads to fatty liver (Pinotti et al., 2000). Models of experimentally induced fatty liver in dairy cows have shown that rumen-protected choline (RPC) is effective in preventing and alleviating fatty liver (Cooke et al. 2007).

In ruminant animals, dietary choline is extensively degraded in the rumen. Therefore choline must be synthesized endogenously or provided in a rumen-protected form to meet the animals needs (Pinotti et al., 2000). The *de novo* synthesis of choline

requires three moles of methyl from S-adenosylmethionine, this further limits the net supply of methionine for protein synthesis.

While some studies have demonstrated a milk production response to dietary choline, others have not (Pinotti et al., 2000). To date, there have been only three experiments (Emmanuel & Kennelly, 1984; Lobley et al., 1996; Preynat et al, 2009) that provide quantitative information on the metabolic fates of methyl groups derived from choline and methionine in ruminants. The lack of quantitative knowledge of methyl group transactions has limited our ability to predict when, and under what conditions, RPC supplementation will be effective. Because of these limitations, an experiment was conducted with the objectives of determining the metabolic fates of methyl groups derived from dietary choline and methionine and estimating the bio-availability of RPC in lactating dairy cows.

MATERIALS AND METHODS

Cows and Treatments

The methods and materials used in this experiment were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee (Protocol #R-08-06). Four multiparous Holstein cows (672 ± 32 kg) in mid-lactation were used in the experiment. Cows were housed in tie stalls bedded with wood shavings and individually fed a total mixed ration (TMR) that was formulated to meet the nutrient requirements of a 650 kg Holstein dairy cow, 40 mo age and 90 days in milk producing 39 kg/d milk containing 3.75% fat and 3.10% protein with an estimated feed consumption of 23.6 kg/d (NRC, 2001). In order to challenge the cow's methyl group

needs, the basal diet Met concentration was restricted to 1.49 % MP. Feed intake and feed refusals were recorded once daily and water was available ad libitum. Ingredient and chemical composition of the basal diet is presented in Table 3.1

Dietary treatments consisted of either the basal diet alone or the basal plus 15 g rumen-protected choline chloride (**RPC**) (Reashure, Balchem Corporation, New Hampton, and New York). In the RPC treatment, RPC was mixed with 394g ground corn and top dressed on the basal TMR once daily when cows were offered fresh TMR. Similary, the Control group received 454 g ground corn as a daily top-dress. Treatments were applied in a single reversal design with 3 week (**wk**) experimental periods. Cows were fed the basal (Control) diet during the first wk of each experimental period to allow for isotope washout and minimize treatment carryover followed by treatment diets during the last 2 wk of each period.

Isotope Infusion Procedures

Methionine and choline methyl transfer and irreversible loss were measured on d 21 of each period using stable isotopes of Met and choline. Infusion and blood sampling catheters were placed during the morning of d 20 of each period. Infusion catheters (75 cm) were constructed from Tygon Microbore® Tubing (ID 1.02 mm, OD 1.78mm; (Saint-Gobain Performance Plastics, Paris, France) and sterilized with 70% ethanol. Prior to insertion catheters were flushed with .9% sterile saline solution containing 100 IU/mL heparin. Cows were haltered and restrained in a squeeze chute during catheter insertion. A 10 by 10 cm area of the jugular groove located at the midpoint between the withers and the point of shoulder was clipped with a surgical clipper and then washed with 70%

ethanol solution. For each catheter, a sterile 7 cm 12-guage stainless steel trocar was inserted into the jugular vein through which the sterile catheter was inserted 20 inches. The catheter was flushed with copious amounts of a 0.9% sterile saline solution followed by 500 IU/mL heparin saline solution in order to prevent clotting in the catheter overnight. The catheter was plugged with a 17-gauge blunt that was secured with a small strip of Elastoplast (Beirsdorf Ag, Berlin, Germany) bandage. Immediately cranial to the catheter insertion site, a 2"x3" butterfly strip of Elastoplast bandage was applied with Kamar adhesive (Kamar, Inc., Steamboat Springs, Colorado) to secure the infusion line and prevent catheter movement at the site of insertion. Finally, an elastic neck girdle was placed on the animals to protect the catheter insertion sites overnight and throughout the infusion period.

A single infusion solution consisting of 3.4 g [1-^{13}C]Met, 3.4 g [methyl-^{13}C] Met, and 6.4 g [methyl-$^{2}H_3$] choline (Cambridge Isotope Laboratories, Andover, MA), were dissolved in 1475 g in 0.9% sterile saline. The rate of infusion (30 mL/h) was calculated to allow for a 4% Met enrichment of blood plasma Met based on estimates of Met turnover in the lactating dairy cow (Bequette et al., 1997; NRC, 2001). The tracer solution concentrations were calculated to limit any potential excess of Met and or choline that might have unduly influenced methyl group metabolism during the infusion period.

Cows were milked in their tie stalls at 0600 h using a portable milker after which isotope infusion commenced using Minipulse-3® peristaltic infusion pumps (Gilson, Inc., Middleton, WI) calibrated to deliver a constant rate of infusion (30 mL/h) over the next 12 h. Actual infusion rates were calculated using the change in weight of the infusion

solution as measured throughout the infusion period and the individual isotope concentration. Cows were milked every 3 h throughout the infusion period, with milk weighed, sub sampled and stored at 4°C for analysis. Oxytocin (20 IU) was infused via the blood sampling catheter in instances where milk letdown did not occur after normal milking preparation. Milk fat, protein, and SCC were determined by infrared analysis (Foss Milk-O-Scan, Foss Food Technology Corp., Eden Prairie, MN) on fresh samples from individual milkings collected over the last 2 d prior to isotope infusion.

Blood samples were collected via the second non-infusion catheter at 0600 h and hourly from 1400 to 1800 h. Blood was collected in a syringe containing 250 IU heparin solution (10 IU/mL blood collected). Following collection syringes were gently mixed and placed on ice prior processing. Catheters were then flushed with 10 mL of 100 IU/mL heparin solution in 0.9% sterile saline, the block replaced and the catheter secured with a small strip of Elastoplast®. Blood samples were centrifuged in 1000 × *g* for 20 min at 4°C. The plasma fraction was pipetted into six 1 mL fractions and one 5 mL fraction with any remaining plasma stored as one fraction. Samples were immediately frozen at -20°C.

Casein Isolation and Analysis

Immediately following the completion of each milking, milk was thoroughly mixed and two 12 to 14 mL subsamples of milk were collected and placed on ice until processed within 20 min post collection. Samples were centrifuged in at 1000 × *g* for 20 min at 4°C.

The remaining casein, sugar and water fraction was vortexed to break up the casein pellet. The two subsamples were transferred to a single 50 mL polycarbonate centrifuge tube (3118-0050, Nalgene, Rochester NY). A volume of distilled H_20 (**dH_20**) equal to that of the sample was added leaving a final volume of 30 to 40 mL to which 1M HCL was added until casein precipitation occurred at a pH of 4.5 to 4.7. The casein samples were refrigerated overnight to aid precipitation. Following refrigeration, the supernatant was gently poured off and samples were re-suspended in 30ml of dH_20. Samples were briefly vortexed, centrifuged at 1000 × g for 15 min at 4° C, the supernatant removed and the sample re-suspended in dH_20, followed by a another centrifugation 1000 × g for 15 min at 4° C and subsequent removal of supernatant. Finally 30 mL of dH_20 was added to the samples, they were vortexed and transferred to a 45 ml polypropylene sample vial with punctured caps (03-341-75A, Fisher Scientific) and stored at -20°C. Frozen samples were lyophilized for 48 h or until reaching complete dryness with dried samples stored at -20°C.

A 20 mg sample of dry casein was placed into a 15 mL hydrolysis tube to which 2 mL of 2 M HCL containing 0.05% dithioerythritol was added. The capped sample tube was vortexed vigorously for several seconds and then placed on a heating block at 110°C for 18 h. Samples were cooled and transferred to 2.5 mL micro-centrifuge tube**;** samples were centrifuged briefly at 1300 rpm to collect any undigested debris.

Amino acids were separated from acids, salts and other neutral compounds using a 2 g AG 50W-X8 100-200 mesh hydrogen form resin (Bio-Rad, Hercules, CA) in 12 mL mini-prep columns were the resin bed had been previously prepared by washing with 24 mL dH_20. One mL supernatant was added to the column and the resin bed was

monitored for elution cessation, at which point, 16 mL dH_20 was applied to the column. Column retained amino acids were eluted with 8 mL of 2M NH_4OH followed by 4 mL dH_20. The ammonia and water elutions were collected in 45 mL polypropylene sample vial with punctured caps (03-341-75A, Fisher Scientific) and frozen overnight at -20°C and then lyophilized until completely dry.

Once dry, 0.5 mL of 0.1 M HCL was added, carefully pippeting around the bottom and lower edges of the vial to ensure that the sample was completely solubilized. A 100 µL subsample was transferred to a 1 mL V-Vial and dried under N_2 gas. To the dried sample, 50 µL of N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (**MTBSTFA**) and 50 µL of dimethylformamide (**DMF**) were added. Samples were capped, vortexed and heated at 90°C for 20 min. Samples were transferred to auto sampler vials and analyzed by GC-MS analysis on an Agilent 6890 Series GC system with a 5973 Network Mass Selective Detector using a HP-5 60 meter column (ID 250.0 µm, 0.25 µm film thickness). The initial temperature was set at 160°C followed with a ramp of 10°C/minute to 300°C. Injection volumes were 1 µL, split was adjusted in order to obtain a satisfactory area. Ions with mass-to-charge of 246 to 249 were monitored for Gly at a retention time of 12.02 min while Met was monitored with mass-to-charge ratios of 292 to 295 and 320 to 323 at a retention time of 16.43 min.

Plasma Amino Acid Analysis

Thawed plasma samples (0.5 mL) were deproteinized with 0.5 mL of 15% sulphosalicyclic acid (**SSA**). Following the addition of SSA, samples were vortexed vigorously then placed on ice for 10 min in order to improve protein precipitation.

Samples were centrifuged for10 min at 1300 rpm. The supernatant was applied by pipette to 0.5 g AG 50W-X8 100-200 mesh Hydrogen form resin (Bio-Rad, Hercules, CA) in 12 mL mini-prep columns previously by washing with 6 mL dH_20. Following initial elution, an additional wash of 8 mL dH_20 was applied. Amino acids were eluted with 2 mL of 2M NH_4OH followed by 1 mL dH_20 and collected in 4 mL Wheaton vials. Vials were capped with punctured tops and frozen overnight at -20°C and then lyophilized until completely dry.

Dried samples were transferred to 1 mL V-Vials with 0.35 mL of 0.1M HCL and then dried under N_2 gas. To each sample 30 µL MTBSTFA with 1% tert-Butyldimethylchlorosilane and 30 µL acetonitrile was added. Samples were capped, vortexed thoroughly and heated at 90°C for 20 minutes. Once cooled, samples were transferred to auto sampler vials for analysis via GCMS analysis was conducted on an Agilent 6890 Series GC system with a 5973 Network Mass Selective Detector using ah a HP-5 51 m column (ID 250 mm, 0.25 µm film thickness). The initial temperature was set at 100°C with a one minute hold followed by two ramps one of 40°C/minute to 220°C and a final ramp of 10°C/minute to 300°C. Injection volumes were 1 µL, split was adjusted in order to obtain a satisfactory area. Glycine and Met ions monitored were as for casein but at retention times of 7.09 and 11.09 min, respectively.

Plasma samples for amino acid (**AA**) analysis followed the same protocol as that for Met and Gly with the exception of the addition 0.125 g of an amino acid standard containing 3 mg [U-^{13}C] algae (Table 3.2), 13 nmole S-methyl-D_3-methionine, 100 nmole L-glutamine-amide-^{15}N, 10 nmole L-tryptophan-[indole- D_5] were added to 0.5 g plasma prior to analysis by GCMS. Analysis was conducted on an Agilent 6890 Series

GC system with a 5973 Network Mass Selective Detector with a HP-50+ 30 m column (ID 250 µm, 0.25 µm film thickness. The initial column temperature was set at 100°C with a one min hold followed by two ramps one of 10°C/min to 280°C and a final ramp of 50°C/min to 300°C. Injection volumes were 0.1 µL, split was adjusted in order to obtain a satisfactory area. Ions monitored are summarized in Table 3.3.

Plasma urea preparation was identical to the plasma amino acid with the exception of 3.7 nmole of labeled urea [$^{15}N_2$] standard utilized in lieu of the amino acid/algae tracer. Following elution 30 µL of the sample was transferred to a 1 mL V-Vial and dried under N_2 gas. Samples were derivatized in 50 µL of DMF and 50 µL of MTBSTFA with 1% BDMCS at 110° for 30 min. Samples were analyzed by GCMS as per AA except the initial temperature was set at 160°C followed by two ramps: one of 10°C/min to 230°C and a final ramp of 50°C/min to 300°C. Injection volumes were 1 µL, split was adjusted in order to obtain a satisfactory area. Urea ions were monitored as a mass:charge ratios of 231 and 233 at a column retention time of 4.38 min and dwell time of 50.

Isotopic purity of infusion isotopes [1-^{13}C] Methionine and [$^{13}CH_3$] Methionine were completed in a manner identical to that for plasma analysis with the following exceptions. Five mg of the labeled isotopes were taken up in 1 mL of 0.1N HCL and vortexed thoroughly. Ten µL of each solution were dried under N_2 gas and samples were derivatized with 50 µL of MTBSTFA with 1% BDMCS and 50 µL acetonitrile. The isotopic purity of the [1-^{13}C]Met tracer was calculated to be 99.78 APE (M+1) and [$^{13}CH_3$] Met tracer isotopic purity was 98.85 APE (M+1) based on GCMS analysis.

Methionine standard solutions of unlabeled Met, [1-^{13}C] Met, [$^{13}CH_3$] Met, and [D_3 Methyl] Met were used to create four standard curves. One standard curve was of unlabeled Met, and three standard curves were a solution of unlabeled Met and one of the labeled Met standards. The curves were calculated to have an APE of 1 to 5. The standards were processed and analyzed in the same manner as for plasma Met samples. Plasma samples for urea concentration analysis were processed in the same manner as plasma Met. Plasma urea concentrations were corrected using a standard curve of unlabelled urea and [$^{15}N_2$] urea. The standards were processed and analyzed in the same manner as the plasma urea samples.

Calculations

Data from standard curves for Met were used in calculations of the tracer:tracee (**TTR**) ratios. Tracer:tracee ratio is defined as the moles of tracer (labeled ion) per 100 mol of the tracee (unlabeled ion) and calculated as:

$$TTR = ((E_X / E_Y) - \text{intercept}) / \text{slope} \quad (1)$$

The above equation corrects the tracer ($\mathbf{E_X}$):tracee ($\mathbf{E_Y}$) ratio using the slope and intercept derived from the respective standard curve.

As 1-^{13}C-Met, in contrast to $^{13}CH_3$-Met, does not lose its labeled atom during transmethylation, 1-^{13}C-Met flux can be used to calculate total Met carboxyl flux using the equation:

$$\text{Met carboxyl flux} = (E_i / E_{Mi}) \times R \quad (2)$$

This equation calculates Met carboxyl flux using M+1 TTR 1-^{13}C-Met (E_{Mi}) and corrects for isotopic purity of the tracer (E_i), tracer infusion rate (R, mmol/hr) and expresses the total Met flux in the context of 1 h.

Total Met methyl flux was calculated as:

$$\text{Met methyl flux} = (E_i / E_{Mi}) \times R \quad (3)$$

This equation calculates Met methyl flux using M+1 TTR $^{13}CH_3$-Met and corrects for isotopic purity of the tracer (E_i), infusion rate R and expresses methyl flux in the context of 1 h.

Methionine remethylation (mmol/h) was calculated based on the difference between the 1-^{13}C-Met flux and the $^{13}CH_3$-Met methyl flux:

$$\text{Met remethylation} = \text{Met methyl flux} - \text{Met carboxyl flux} \quad (4)$$

Where Met methyl flux and Met carboxyl flux are expressed as mmol/hr.

Fractional remethylation is ratio of the rates of Met remethylation and $^{13}CH_3$ Met methyl flux:

$$\text{Fractional remethylation} = \text{Met remethylation} / \text{Met methyl flux} \quad (5)$$

Relative transmethylation activity can be established using plasma concentrations of 1-^{13}C-Met and $^{13}CH_3$-Met given that the concentrations in the infusion solution and the infusion rate are known. In this study, relative transmethylation is defined as the ratio of plasma $^{13}CH_3$-Met:1-^{13}C Met. A relative transmethylation ratio of 0.9 would indicate that roughly 10% of Met is undergoing transmethylation whereas a ratio of 0.1 would

indicate that 90% of Met is undergoing transmethylation. Relative transmethylation activity (**RTMR**) was estimated as:

$$RTMR = [M+1\ TTR\ ^{13}CH_3\text{-Met}] / [M+1\ TTR\ 1\text{-}^{13}C\text{-Met}] \quad (6)$$

Where [M+1 TTR 1-^{13}C Met] was calculated as the difference between the 321:320 TTR (1-^{13}C-Met and $^{13}CH_3$-Met) and the 292:293 TTR ($^{13}CH_3$-Met).

Statistical Analysis

Daily feed intake and milk production were averaged over the 7 d of each experimental period. Milk composition data, were averaged by cow over the last 2 d of each experimental period. Milk yield and composition were used to calculate fat, protein and lactose yields. Fat-corrected milk (3.5%) was estimated using the equation of Tyrell and Reid (1965). All production data was analyzed using the GLM procedure of SAS 9.22 (SAS/STAT Inst. Inc., 293 Cary, NC). The model included fixed effects of treatment, cow and period. Statistical analysis of plasma amino acids and urea concentration, casein isotopic enrichments and flux rates were analyzed as repeated measures ANOVA using the MIXED procedure of SAS 9.2 (SAS/STAT Inst. Inc., 293 Cary, NC) with the repeated option and autoregressive covariance structures. The statistical model included the fixed effects of treatment, cow, and period. Finally, differences between means of enrichment ratios within treatment were analyzed using the TTEST procedure of SAS 9.2. All data are presented as least squares means with a pooled SEM where differences between treatment means considered significant when $P \leq 0.05$.

RESULTS

Feed intake was not affected by treatment and averaged 24.0 kg/d across treatments (Table 3.4). While numerically greater for RPC there was no treatment effect on milk, milk fat, or milk protein yields which averaged 39 kg/d, 1634 g/d and 1110 g/d, respectively (Table 3.4) across treatments.

Plasma amino acid and urea concentrations are summarized in Table 3.5. There was no indication of a treatment effect on plasma amino acid concentrations. However, there was a trend ($P < 0.09$) for higher urea concentrations with the RPC treatment.

Isotopic enrichment plateaus were achieved during the 12 h infusion. As example, Figure 3.1 shows the tracer:tracee ratio plasma and casein D3-Methyl methionine during by infusion [methyl-$^{2}H_3$] choline infusion. Somewhat surprisingly, casein Met-Methyl enrichment plateaued earlier and was less variable (SEM = 0. 01) than plasma enrichment. However, differences due to time of sampling were not significant and mean concentrations for all sampling periods were used in the flux measurements.

Whole body Met flux and transmethylation relative activity as measured in plasma and casein are in Table 3.6. Rumen-protected choline had no effect on methionine methyl or carboxyl flux, or fractional remethylation although in each instance, they were numerically lower than the Control. Methionine carboxyl flux averaged 11.45 mmole/h which would correspond to a daily equivalent of 41 g/d. Due to Met remethylation, methyl flux was greater than carboxyl flux. Fractional Met remethylation averaged 0.242

across treatments suggesting that at a minimum, 24% of Met is re-methylated using the difference between Met methyl and Met carboxyl flux rates.

Transmethylation as measured in plasma was not affected by treatment. There was a tendency for greater transmethylation (P= 0.09) in the Control vs. RPC as measured in casein (Table 3.6). However the ratio of plasma:casein relative transmethylation was not different between RPC and Control. For all tracers measured, there was no difference in plasma or casein enrichment between Control and RPC.

Treatment had no effect on plasma:casein enrichment as measured by $^{13}CH_3$ Met and 1-^{13}C-Met. Moreover, the ratio of $^{13}CH_3$-Met plasma:$^{13}CH_3$-Met casein and 1-^{13}C-Met plasma:1-^{13}C-Met casein were not significantly different within the Control or RPC treatments. However, within treatment ratio of ($^{13}CH_3$-Met plasma:$^{13}CH_3$-Met casein): (D_3-Met plasma: D_3-Met casein) enrichment was significantly different (-2.78, P = 0.04) for Control and tended be different (-2.74, P = 0.06) for the RPC treatment.

DISCUSSION

While supplementation with RPC did not elicit a significant production response, this experiment had insufficient numbers of cows to measure production responses. Rather our objectives were designed to identify the effect of RPC on several biological measures.

Plasma free amino acids concentrations were not affected by RPC. Most notably, Met concentrations as measured in plasma were not significantly different between the treatment and control. If RPC supplementation was sparing Met then we would have

anticipated an increase in free Met and subsequently a decrease in plasma glycine concentrations but this did not occur (Selhub, 1999).

Previously, Lobley et al. (1996) demonstrated a reduced need for homocysteine remethylation in sheep fed a Met limiting diet with supplemental choline infusion. The results of that study suggested that choline may be sparing Met through reduced *de novo* choline synthesis, rather than increased the rate of homocysteine remethylation (Lobley et al.,1996). More recently, Preynat et al., (2009) demonstrated an increased rate of Met remethylation in Met supplemented diets; counter to the theory that adequate dietary methionine would reduce the need for remethylation of homocysteine. In the current study there was no effect of RPC supplementation on the rate of homocysteine remethylation. However, our average remethylation rates (4.2 mmol/h) across treatments, were much greater than reported by Preynat et al. (2009), (2.6 mmol/h). Conversely, average Met methyl flux was lower (14.1 mmoles/h) in this experiment than reported by Preynat et al. (2009) (22.2 mmoles/h). Perhaps, the greater rates of Met remethylation in the present study were reflected in reduced rate of total Met flux when compared to those reported by Preynat et al. (2009).

This experiment did not show a difference between RPC and Controls in fractional rates remethylation (Table 3.6). However, relative transmethylation rate as measured in casein TTR tended to be higher in the Control than in the RPC treatment (P=0.09). The ratio of $^{13}CH_3$-Met plasma:$^{13}CH_3$-Met casein and 1-^{13}C-Met plasma:1-^{13}C-Met casein were not different between Control and RPC indicating that Met methyl loss was minimal within the mammary tissue.

In both the Control and RPC groups comparison of D_3-Methyl Met enrichment in plasma and casein with that of 1-C^{13}-Met enrichment in plasma and casein suggested that upwards of 40% Met present in casein had undergone transmethylation with choline serving as the ultimate methyl donor. Furthermore this data suggests that choline is serving as a major contributor of methyl groups for methionine remethylation within the mammary tissue.

In an earlier study in lactating goats (Emmanuel and Kennelly, 1984) where uniformly labeled ^{14}C choline was infused for 5 h, ^{14}C was not recovered in either plasma or casein Met. This contrasts with this study where choline methyl was recovered in both fractions.

Conceivably, the longer infusion period in this study (12 h) may have allowed for choline-methionine transmethylation to occur in the liver providing a plasma free pool of labeled methionine from which casein could be synthesized. Additionally, the longer infusion period appears to have allowed for [methyl-$^2H_3$] choline to be taken up by the mammary tissue and subsequently act as a methyl donor for casein Met synthesis. This study is the first to demonstrate the mammary utilization of choline methyl for homocysteine remethylation.

Finally, choline biovailability can be indirectly estimated from methyl flux rates between treatments. The RPC treatment provided and additional 0.107 moles (15g) of choline chloride compared with the control diet. Conceivably this would make an additional 4.47 mmoles/h of choline methyl available for Met synthesis assuming one methyl group per mole choline (Selhub,1991). Based on the difference in Met methyl flux between the RPC and Control (3.25mmoles/h), this would suggest that the

bioavailability of RPC is approximately 72% if the difference noted between the control and treatment groups are due to solely to a treatment effect.

Table 3.1 Ingredient and chemical composition of basal diet (DM basis)

Ingredients	% of DM
Corn silage	47.88
Alfalfa hay	7.42
Corn grain, ground	11.75
Citrus pulp	3.71
Soybean meal	14.85
Wet brewers grains	7.79
Calcium Carbonate	0.65
Salt	0.34
Magnesium oxide	0.15
Sodium bicarbonate	0.51
Potassium magnesium sulfate	0.10
Calcium phosphate monobasic	0.38
Trace minerals and vitamins[1]	0.10
Chemical composition (% of DM)	
DM	53.89
CP	17.69
RUP[2]	6.73
RDP[2]	10.97
MP	8.00
Lysine, % of MP	5.49
Methionine, % of MP	1.49
NDF	30.00
$NE_{L,}$ Mcal/kg[2]	1.59
Ca	0.84
P	0.42
Na	0.31
Mg	0.35
S	0.35
K	1.42
Cl	0.44

[1]Trace mineral and vitamin mix provided (per kg DM) 15 mg Mn, 50 mg Zn, 10 mg Cu, 0.60 mg I, 0.20 mg Co, 0.30 mg Se, 3,000 IU Vitamin A, 1600 IU IU Vitamin D_3, and 50 IU Vitamin E.
[2]Estimated from NRC (2001).

Table 3.2 Amino acid composition of [U-^{13}C] algae

Amino Acid	µM/g Algae Solution
Alanine	0.297
Arginine	0.129
Aspartic Acid	0.247
Glutamic Acid	0.266
Glycine	0.307
Histidine	0.057
Isoleucine	0.085
Leucine	0.232
Lysine	0.310
Methionine, D_3	0.037
Phenyalanine	0.101
Proline	0.174
Serine	0.190
Threonine	0.156
Tyrosine	0.083
Valine	0.158

Table 3.3 Plasma amino acid ions monitored.

Amino Acid	Ions Monitored (m/z)	Retention Time, min	Dwell Time[1]
Ala	262, 263	7.02	100
Gly	246, 248	7.51	100
Val	288, 293	8.25	100
Leu	302, 308	8.59	100
Ile	302, 308	9.02	100
Pro	286, 291	10.00	100
Ser	390, 393	11.46	100
Thr	404, 408	11.67	100
Met, D_3	320, 322	12.24	100
Asp/Phe	232, 242, 418, 422	13.48	40
		13.52	40
Orn	286, 291	14.10	100
Glu	432, 437	14.47	100
Lys/Arg	300, 306, 417	14.98	60
		15.15	60
Gln-1	431, 436	16.17	100
Arg	442, 448	16.44	100
Tyr	302, 304	17.62	100
His	440, 446	18.03	100
Trp, D_5	244, 249, 375, 380	19.56	40

[1]1/100 s.

Table 3.4 Effect of rumen-protected choline (RPC)[1] supplementation on dry matter intake (DMI) and milk production

	------ Treatment -------			
Item	Control	RPC	SEM[2]	P-value[3]
DMI, kg/d	23.3	24.8	1.14	0.42
Milk, kg/d	37.3	40.8	2.10	0.36
Fat, %	4.19	4.15	0.26	0.64
Protein, %	2.90	2.82	0.06	0.39
3.5% FCM[3]	41.8	44.8	3.8	0.60
Fat, g/d	1587	1680	181	0.75
Protein, g/d	1079	1142	36	0.34
SCC[4]	689	19	472	0.42

[1] Cows were supplemented with 15 g/d rumen-protected choline chloride.
[2] Standard error of the mean.
[3] Probability that treatment means are not different, when the null hypothesis is rejected.
[4] Somatic Cell Count; One cow during the control period had SCC about 2 million but did not show signs of clinical mastitis or illness causing and elevated mean SCC for the Control treatment.

Table 3.5 Effect of rumen-protected choline (RPC)[1] supplementation on plasma essential and nonessential amino acid (AA) and urea concentrations.

	---- Treatment ----			
Item	Control	RPC	SEM[2]	P-value[3]
Essential AA (µM/L)				
Ile	124.0	123.8	14.7	0.98
Leu	185.6	182.6	21.6	0.86
Lys	59.8	59.1	6.4	0.93
Met	39.0	37.3	1.5	0.31
Phe	55.0	61.4	3.8	0.21
Thr	66.1	63.1	7.4	0.79
Val	301.5	303.4	42.7	0.94
Nonessential AA (µM/L)				
Ala	179.4	191.0	18.0	0.34
Asp	6.7	6.6	0.4	0.90
Gln	167.6	157.9	17.7	0.64
Glu	26.5	26.5	3.4	0.99
Gly	174.0	140.3	14.9	0.16
Ser	70.9	61.6	6.2	0.33
Urea (mmol/L)	6.2	7.6	0.3	0.09

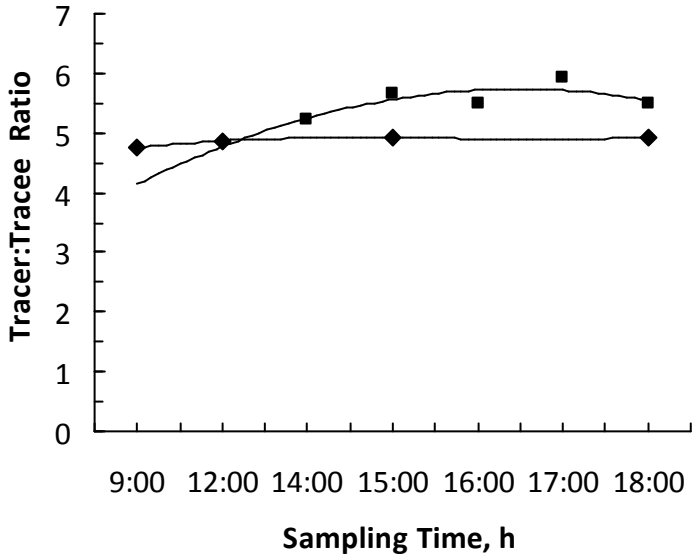
[1] Cows were supplemented with 15 g/d rumen-protected choline chloride.
[2] Standard error of the mean.
[3] Probability that treatment means are not different, when the null hypothesis is rejected.

Table 3.6 Effect of rumen protected choline (RPC)[1] supplementation on methionine flux, re-methylation, whole body and mammary transmethylation relative activity, 1-^{13}C, $^{13}CH_3$, and D_3, methionine tracer:tracee ratio[1].

	---- Treatment----			
Item	Control	RPC	SEM[2]	P-value[3]
Methionine Methyl Flux (mmol/hr)[4]	16.81	13.56	5.60	0.69
Methionine Carboxyl Flux (mmol/hr)[5]	12.56	10.34	3.97	0.71
Re-methylation (mmol/hr)[6]	4.57	3.82	2.04	0.80
Fractional Re-methylation[7]	0.258	0.227	0.03	0.52
Transmethylation relative activity				
Plasma[8]	0.751	0.790	0.03	0.39
Casein[8]	0.850	0.877	0.009	0.09
Plasma:Casein Ratio	0.901	0.884	0.03	0.73
Methionine TTR (mole:100 mole)				
Plasma D_3–Met	6.27	6.13	0.46	0.85
Casein D_3–Met	4.93	4.92	0.02	0.44
Plasma:Casein D_3–Met	1.27	1.25	0.08	0.92
Plasma 1-^{13}C-Met	5.47	6.36	0.95	0.58
Casein 1-^{13}C-Met	1.55	1.55	0.98	0.97
Plasma:Casein 1-^{13}C-Met	3.55	4.15	0.62	0.56
Plasma $^{13}CH_3$–Met	5.10	5.57	2.13	0.47
Casein $^{13}CH_3$–Met	1.22	1.35	0.10	0.86
Plasma:Casein $^{13}CH_3$–Met	4.05	4.00	1.39	0.98
Plasma:Casein $^{13}CH_3$–Met :	-.50	----	0.65	0.21
Plasma:Casein 1-^{13}C-Met	----	0.14	2.02	0.89
Plasma:Casein $^{13}CH_3$–Met :	-2.78	----	1.71	0.04
Plasma:Casein D_3–Met	----	-2.74	1.91	0.06

[1] Cows were supplemented with 15 g/d rumen-protected choline chloride.
[2] Standard error of the mean.
[3] Probability that treatment means are not different, when the null hypothesis is rejected.
[4] Met Carboxyl Flux = (E_i / E_{Mi})*R (M+1 TTR 1-^{13}C Methionine (E_{Mi}), isotope purity of tracer (E_i), tracer infusion rate (R).
[5] Met Carboxyl Flux = (E_i / E_{Mi})*R M+1 TTR 1-^{13}C Methionine (E_{Mi}) and isotope purity of tracer (E_i), tracer infusion rate (R).
[6] Re-methylation = Met Methyl Flux - Met Carboxyl Flux
[7] Fractional Re-methylation = Met Re-methylation / Met Methyl Flux
[8] RTMR = [M+1 TTR $^{13}CH_3$-Met / [M+1 TTR 1-^{13}C -Methionine] [M+1 TTR 1-^{13}C-Met] was calculated as the difference between the 321:320 TTR (1-^{13}C-Met and $^{13}CH_3$-Met) and the 293:293 TTR ($^{13}CH_3$-Met).

Figure 3.1 Mean plasma (◆) and milk casein (■) [D_3-Methyl] methionine enrichments across treatment and sampling time as measured by tracer:tracee ratio during isotope infusion on d 14 of each experimental period. Choline [D_3-Methyl] infusion began at 0900 h and continued through 1800 h.



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