ABSTRACT

Title of Document: REGULATION OF MACRONUTRIENT METABOLISM BY THE GASTROINTESTINAL TRACT OF RUMINANTS

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We set out to test the hypothesis that the gastrointestinal tract (GIT) of ruminant animals catabolizes amino acids (AAs) preferentially. We sought to determine whether this catabolism represents an obligate requirement, and whether this requirement stems from the need to generate energy or support other metabolic demands. The aim was to determine the composition of macronutrients (AAs, short chain fatty acids, and glucose) utilized by the GIT, and the influence of general and specific nutrient supplies on their routes of metabolism. Increasing protein supply to the small intestine did not alter the total amount of glucose removed by the GIT indicating, that glucose removal and therefore utilization is obligatory. In contrast, the net removal of AAs occurred at a constant proportion of arterial and luminal supplies. This translated to larger amounts of AAs removed from blood circulation, and from the lumen of the small intestine in response to increased small intestinal and blood supplies. In this respect, the net absorption of branched chain AAs was, unlike other essential AAs lower than 100%. Further, glutamate and glutamine net appearance across the whole GIT and small intestine was unaffected by protein supply. The

disproportionate utilization of BCAA, glutamate, and glutamine as compared to other AAs suggested that their metabolism occurred toward specific metabolic requirements, possibly energy production. When Krebs cycle metabolism was investigated using individual AAs, glucose, and short chain fatty acids, leucine and valine did not contribute to the flux of Krebs cycle intermediates. Conversely, α-ketoglutarate flux originated mainly from glutamate, and to a lesser extent from glutamine. Though glucose was metabolized to pyruvate and lactate, glucose did not contribute to Krebs cycle intermediates. Overall, these results indicated that glutamate plays an important role in energy metabolism, and in insuring replenishment of Krebs cycle intermediates that leave the cycle via cataplerosis. Yet, the results raised new questions that ought to be addressed in future studies. The fate of glutamine carbon, the metabolic significance of leucine and valine deamination, and the role of glucose partial catabolism to lactate need to be investigated.

REGULATION OF MACRONUTRIENT METABOLISM BY THE GASTROINTESTINAL TRACT OF RUMINANTS.

By

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Dedication

To my Loving Family

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INTRODUCTION

Ruminants are by far the most efficient farm animals in converting plant products that are inedible by humans into high quality food products such as meat and milk. However, the metabolic adaptation of ruminants to using low quality feed ingredients is coupled with a low efficiency of depositing amino acids (AAs) and energy into body tissues and milk proteins.

Gaps in our knowledge exist in relation to absorptive and post-absorptive nutrient metabolism. One area that deserves further examination is how nutrients are partitioned between catabolism and anabolism. A better understanding of these control mechanisms may provide new avenues for enhancing the efficiency of AA utilization by ruminants through formulation of diets better suited to matching the metabolic capabilities of ruminants. Furthermore such information will also be valuable in the development of predictive models of nutrient absorption and utilization in farm animals. The overarching goal is to maximize production efficiency and to decrease the impact of animal production systems and their wastes on the environment.

One aspect of the poor efficiency of nutrient utilization by ruminants relates to the high metabolic demands for AAs and energy substrates by the gastrointestinal tract (GIT) and the liver. These tissues are the most metabolically active tissues in the body, and therefore targeted investigations of these tissues may provide opportunities for decreasing these losses in nutrient utilization. The research conducted in this dissertation had the aim of determining the composition of macronutrients (AAs, short chain fatty acids and glucose) utilized by the GIT and the influence of general and specific nutrient supplies on their routes of metabolism.

The GIT has received a lot of attention not only for the role it plays in nutrient digestion and absorption, but also for its high anabolic and catabolic activity which impacts upon nutrient availability to productive tissues (e.g. muscle, mammary gland, conceptus). What is remarkable is that the small size of the GIT is coupled with a high rate of O_2 consumption and protein synthesis. In sheep for example, the GIT represents less than 10% of empty body weight (McLeod and Baldwin, 2000) yet its metabolic activity accounts for 30% of whole body oxygen consumption (Burrin et al., 1989) and 25 to 35% of whole body protein synthesis (Lobley et al., 1994). While part of the high metabolic demand for AAs by the ruminant GIT relates to the net losses of tissue and secretory proteins (Burrin et al., 1989; Neutze et al., 1997), evidence in monogastric species also indicates that AA make major contributions as substrates for energy generating pathways. In ruminants, while it would be expected that AAs make similarly large contributions to energy metabolism by the GIT, there is a paucity of information to support this role. Therefore, the extent that AA and other substrates contribute to catabolic pathways of energy generation will be a focus of this thesis.

The overall hypothesis of this thesis research is that the GIT of ruminant animals catabolizes AA preferentially. In this respect it was important to determine whether this catabolism represents an obligate requirement and whether this requirement represents the need to generate energy or support other metabolic demands.

The objectives of this project are to: 1) Determine whether changes in small intestinal protein supply alters glucose metabolism by the portal (PDV) drained viscera. 2) Determine the patterns of AA net metabolized by the mesenteric (MDV) and PDV and whether this pattern of use remains constant when intestinal protein supply is increased,

3) Determine whether the changes in small intestinal protein supply alters the extent that AA are utilized from luminal (first-pass) versus systemic (second-pass) sources. A related objective was to determine whether changes in AA use by the GIT were inversely related to GIT metabolism of glucose, and 4) Determine the contributions of individual amino acids, glucose, and short chain fatty acids (SCFA) to Krebs cycle metabolism by rumen and small intestinal cells as affected by the level of forage and concentrate in the diet.

CHAPTER 1

LITERATURE REVIEW

Amino acids

The gastrointestinal tract (GIT) of animals is considered to be a major site of AA catabolism and use for protein synthesis. Studies conducted in monogastric (pigs, humans, mice, rats) and ruminant (sheep, beef and dairy cattle) species have shown that the removal of AA by the GIT, both essential and non-essential, occurs at rates that are not proportional to its size. This high AA requirement relates to the high rates of protein synthesis and turnover (25 to 100% per day; Lobley et al., 1994) by the GIT tissues. Furthermore, energy utilization by the GIT is also high and may be related to the high rate of protein synthesis. What is unique about the GIT is that most nutrients are deliver to and extracted from the luminal aspect during absorption and from the arterial blood supply. This feature means that the GIT is capable of dictating the initial supply of nutrients to post-absorptive tissues, and that the GIT has the potential to act as a competitor with productive tissues (e.g. muscle, mammary gland) for AAs and energy substrates.

There has been some evidence in the literature that the GIT may be flexible in substrate selection for catabolism. Understanding the roles AAs serve as substrates for energy production and how their utilization is influenced by the supplies of macronutrients (i.e. protein, carbohydrates and volatile fatty acids) may provide clues to feeding strategies that reduce AA use by the GIT. This in turn would provide larger

amounts of AAs to the liver and beyond to productive tissues for tissue protein gain, and also improve the efficiency of AA utilization.

Gastrointestinal tract removal

Tissue removal of AAs can be estimated *in vivo* by measurement of the product of arterio-venous concentration difference and blood flow, which in the case of the ruminant GIT most often involves blood collected from the portal vein. When portal blood is collected, net nutrient flux values represent the removal or absorption by the whole GIT, i.e. the portal drained viscera (PDV) including the rumen, small intestines, hind gut and the pancreas. With collection of mesenteric drained visceral (MDV) blood flow, small intestinal removal or absorption can also be estimated. Based on PDV measurements, some AAs have been found to be net produced by the GIT, for example alanine and arginine. By contrast, PDV net fluxes of glutamine and glutamate are low and often negative, reflecting their net metabolism by the PDV (Burrin et al., 1991; Berthiaume et al., 2001; Rémond et al., 2003). For most essential AA, their net appearance across the MDV most often equals the amounts disappearing from the lumen of the small intestines. The latter reflecting net tissue protein balance of those tissues, i.e. the GIT is neither gaining nor losing net protein.

In fed sheep, PDV appearance of many AAs does not account for all the AAs disappearing from the small intestines (Tagari and Bergman, 1978). In that study, glutamine was found to be removed the most at two levels of protein intake, whereas portal appearance of glutamate increased and that of aspartate decreased in response to increased protein feeding. Others have measured PDV net flux of AAs across a wide

range of feeding conditions. In some reports, intestinal supplies of glutamate and glutamine were completely removed by the PDV in sheep and cattle, and the amounts removed were not affected by level of feed intake (Huntington and Prior, 1985; Nozière et a., 2000), protein supply (Bruckental et al., 1997) or by fasting (Heitmann and Bergman, 1980). However, in other studies (Burrin et al., 1991; Berthiaume et al., 2001; Rémond et al., 2003), only glutamine was completely removed by the PDV, whereas glutamate was net absorbed by the PDV. Glutamate absorption is also responsive to level of nutrition, with net absorption greater in lambs fed ad libitum compared to those fed at maintenance (Burrin et al., 1991). Most of these data in ruminants represent fluxes across the PDV. In studies where MDV net flux has been measured in ruminants, glutamine and glutamate were also found to be net absorbed from small intestine (Berthiaume et al., 2001; Rémond et al., 2003). Therefore, the removal of glutamate and glutamine by the PDV can be attributed to arterial use by the rumen and hind gut tissues, in addition to that removed from the small intestinal lumen

All EAA are net absorbed by PDV of well fed ruminants (Tagari and Bergman, 1978; Burrin et al., 1991; Nozière et al., 2000; Berthiaume et al., 2001), but to varying extents. Net removal of EAA by the PDV was negative only when ruminants were fed to 0.5 times maintenance energy and protein requirements (Nozière et al., 2000), indicating that the PDV net removed EAA from the systemic circulation and from the lumen of the small intestines. In sheep fed to maintenance (Burrin et al., 1991) or subjected to short term fasting (Heitmann and Bergman, 1980) EAA are net absorbed into the portal vein.

Despite the appearance of AA in the portal drainage, PDV removal occurs at rates that are higher than for the MDV (MacRae et al., 1997; Lobley et al., 2003). The

difference in EAA net absorption is due to the removal of those AA from the circulation by the forestomach and hind gut (Rémond et al., 2000; Rémond et al., 2003). The GIT of sheep fed a forage diet removes 0.17 to 0.39 of EAA infused into the small intestines while systemic removal was 0.06 to 0.13 of arterial supply (MacRae et al., 1997). In that study, the arterial supply of EAA, except for phenylalanine and histidine, provided 0.75 to 0.82 of the EAA sequestered by the GIT. In this connection, systemic AA removal by the GIT correlates well with fluctuations in arterial AA concentration (Lobley et al., 2001). These findings highlight the importance of the blood circulation in the provision of AAs to the GIT.

Protein synthesis

In sheep, protein synthesis in MDV and PDV account for 0.20 and 0.35 of whole body protein synthesis rate (Neutze et al., 1997; Lobley et al., 1994). Furthermore, protein turnover in the small intestines occurs at a greater rate in the mucosa than in the serosa (0.64 and 0.42/d; Lobley et al., 1994).

At a minimum, AA utilization by the GIT should reflect their requirements for synthesis of mucins, enzyme secretions and GIT tissue proteins. In this respect, the proportional removal of EAA from luminal and arterial supplies by the GIT of ruminants correlates well with their profile in whole GIT tissue proteins (MacRae et al., 1997).

Despite the high rates of protein synthesis by GIT tissues, protein retention does not exceed 0.03 of whole body protein gain (MacRae et al., 1993). This is due in part to the losses attributed to the incomplete reabsorption of intestinal secretion. For example, the reabsorption of small intestinal mucin, which is rich in threonine and valine (Mukkur et al., 1985), does not exceed 0.75 at the ileum (van Bruchem et al., 1997).

Protein synthesis is also energetically demanding. In cattle, up to 0.30 of heat production has been attributed to protein synthesis (Lobley et al., 1980). Consequently, protein synthesis places a high demand on energy substrates including AAs.

Catabolism

The high rate of AA utilization by the GIT, in particular the NEAA, occurs at rates that exceed their requirements for protein synthesis alone (Reeds et al., 2000). The mismatch between tissue composition and AA utilization by the GIT reflects the metabolism of some AA in pathways other than protein synthesis, for example NEAA synthesis (Wu, 1998), or in catabolic routes for energy production (Reeds et al., 2000). The metabolic pathways of all twenty amino acids converge to five end-products, all of which may enter the Krebs cycle (**Figure 1.1**). As a result, the carbon skeleton of AA can be diverted towards gluconeogenic (alanine, lactate) or ketogenic end-products (acetoacetate), completely oxidized to CO_2, or their carbon skeletons can be used for NEAA and lipid biosynthesis.

Non-essential amino acids. Glutamine has been singled out as the main respiratory substrate for the rat small intestines (Windmuller and Spaeth, 1974, 1978, 1980). In those studies more than half of glutamine carbon appeared as CO_2, which accounted for one-third of total CO_2 production by the small intestines. However, uncertainty has since emerged with respect to the fates of glutamine carbon and nitrogen and whether glutamine oxidation in the GIT represents a requirement or occurs as a passive response to supply and concentration.

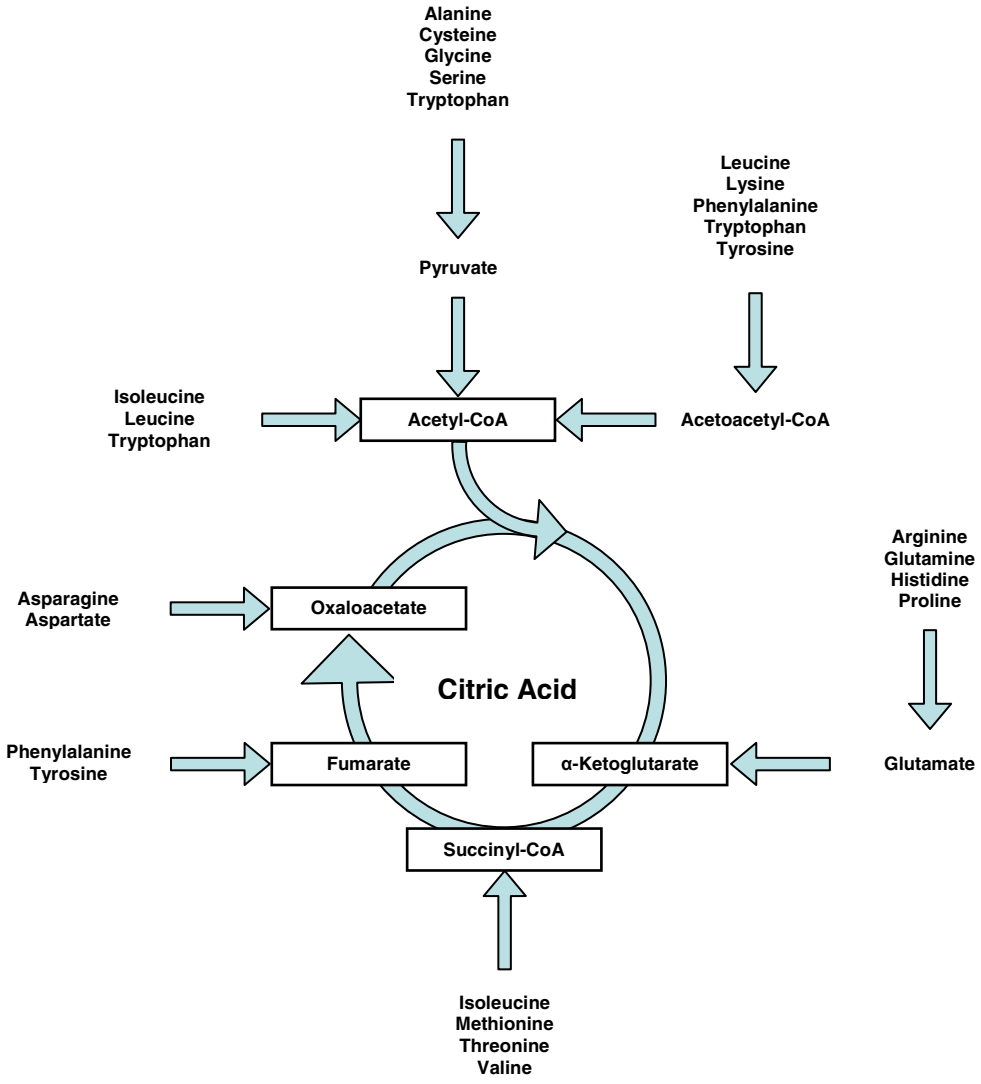

Figure 1.1. Points of entry of amino acids to the citric acid cycle. Some amino acids have more than one point of entry because their metabolism yields two end products.

Firstly, glutamine oxidation is believed to occur through a pathway that involves the intermediary catabolism to glutamate and thence to α-ketoglutarate (Windmuller and Spaeth, 1974). However, studies conducted using [1-^{14}C], [5-^{14}C] or [U-^{14}C]glutamine tracers in rats have shown that glutamine oxidation is incomplete and that this catabolism could involve pathways other than the Krebs cycle (Watford, 1994). It was suggested that the appearance of labeled CO_2 from glutamine provided unequivocal evidence of glutamine decarboxylation, which is similar to previous observations (Windmuller and Spaeth, 1974). However, the lack of labeling in α-ketoglutarate, malate, citrate, and fumarate ruled out the Krebs cycle as a possible pathway for glutamine oxidation (Watford, 1994).

Glutamine-N is important in purine and pyrimidine synthesis (Gate et al., 1999). In rapidly dividing cells of the small intestine, which are replaced every 1-4 d in ruminants (Attaix and Meslin, 1991), nucleotide biosynthesis may be the most important process driving glutamine utilization, not energy production as previously thought (Newsholme et al., 1985). The utilization of glutamine nitrogen for purine and pyrimidine biosynthesis does not reflect a large quantitative requirement for glutamine because only 5% of glutamine amido-N is recovered in RNA and DNA extracted from GIT tissues (Gate et al., 1999). However, it has been suggested that the high rate of glutaminolysis in many tissues is required in order to maintain a small, yet metabolically flexible, nucleic acid precursor pool for immediate repair responses to damage (Newsholme et al., 1985).

Second, studies have shown that in pig small intestine glutamate and glucose contribute more to oxidative energy generation than does glutamine (Stoll et al., 1999). Therefore glutamine catabolism through deamidation may reflect a requirement for glutamate, and not for glutamine *per se* (Reeds and Burrin, 2001). In fact, glutamate addition to isolated sheep enterocytes decreased glutamine oxidation (Oba et al., 2004). Equally important is the fact that other NEAAs, namely glutamate and proline, could substitute for glutamine in non-protein synthetic metabolic pathways involving arginine and citrulline synthesis (Wu, 1996; Brunton et al., 1999).

TABLE 1-1.

Pathways supported by glutamine metabolism in small intestinal mucosa[1]

Amido-N end product	Intermediary metabolic products
Purine	Ornithine
Pyrimidine	Arginine
Amino sugars	Proline
	Polyamines
	Ammonia
	Alanine

[1] Reeds and Burrin 1991.

Lastly, glutamine removal by the GIT of ruminants exceeds that of other AA, yet oxidation occurs at rates lower than those observed in the simple stomached animals. Only 9 to 25% of glutamine carbon appeared as CO_2 with dairy cow enterocytes (Okine et al., 1995) as compared to 55-70% with rat and piglet enterocytes (Windmuller and Spaeth, 1974; Stoll et al., 1999). In addition, glucose provision to sheep enterocytes

reduced glutamine oxidation but had no effect on CO_2 production from glutamate (Oba et al., 2004). It is important to note that the results in ruminants are from animals at different physiological conditions (lactating cows vs growing sheep) and substrate concentrations in the incubation media (1.0-6 mmol/L).

Enteral glutamate has been shown to be extensively removed on first pass in pigs, and glutamate catabolism accounted for 0.10-0.36 of total CO_2 production by the PDV (Stoll et al., 1999; van der Schoor et al., 2001) compared to 0.19 for glutamine (Stoll et al., 1999). These observations support the suggestion that glutamate is a more important substrate for catabolism than is glutamine (Reeds and Burrin, 2001). A further observation that supports this view is that in sheep enterocytes glucose addition did not reduce glutamate oxidation to CO_2 (Oba et al., 2004), and therefore glutamate catabolism may represent a requirement.

In rats, small intestinal catabolism to CO_2 of aspartate (0.51) and arginine (0.14) is also significant (Windmueller and Spaeth, 1975; 1976). Also, the synthesis of other metabolic end-products (lactate) and AAs (alanine, proline, citrulline and ornithine) from glutamine, glutamate, aspartate and arginine is significant. In fact, the portal appearance of alanine, in all species studied, far exceeds intake or intestinal disappearance (Stoll et al., 1998; Stoll et al., 1999).

Essential amino acids. Many of the studies carried out that have examined EAA oxidation by the ruminant GIT have used leucine tracers (Pell et al., 1986; MacRae et al., 1997; Capelli et al., 1997; Yu et al., 2000). In sheep fed lucerne pellets, leucine oxidation accounted for 0.17 of arterial leucine sequestered by the GIT (MacRae et al., 1997). Changes in leucine catabolism have been shown to occur as a result of infection with

intestinal parasites. Here, leucine oxidation increased from 0.12 to 0.21 of leucine sequestered by the GIT of sheep challenged with parasitic nematodes (Yu et al., 2000). In this study, the increase in leucine oxidation correlated with an increase in GIT protein turnover with no change in the fractional rate of leucine oxidation. Diet alterations have also been shown to affect EAA oxidation by the dairy cow GIT. Increasing the supply of metabolizable protein to dairy cows increased leucine oxidation from 0.16 to 0.22 of small intestinal utilization (Lapierre et al., 2002).

Very low rates of luminal leucine oxidation have been reported for sheep (0 to 0.05) as compared to the extent of arterial leucine oxidation (Capelli et al., 1997; MacRae et al., 1997b; Yu et al., 2000). These observations are in agreement with the observation that 0.80 EAA sequestered by the GIT, including leucine, occurs from the systemic supply (MacRae et al., 1997b).

In the most comprehensive study to date in ruminants, ^{13}C-tracers of leucine, methionine, lysine and phenylalanine were infused via the jugular vein into sheep, and $^{13}CO_2$ release by the PDV monitored (Lobley et al., 2003). Here, there was detectable oxidation of only leucine and methionine, amounting to 8% and 3%, respectively, of their sequestration from the arterial blood supply to the PDV (Lobley et al., 2003). In that study, no significant oxidation of lysine and phenylalanine was detected. A proviso in that study was that the tracers were administered into the blood circulation only, and so oxidation of the AAs represented that by the arterial facing aspect of the GIT tissues, not the intestinal lumen aspect. In a series of studies in pigs, however, where the ^{13}C tracers were given via the feed and infused into the blood supply, oxidation of many of these AA was observed (Stoll et al., 1999; van der Schoor et al., 2001; van Goudoever et al., 2001).

In one of these studies, leucine oxidation by the PDV of piglets fed a diet adequate in protein accounted for 0.18 of total CO_2 production by PDV with duodenal leucine (0.12) contributing more to total oxidation than leucine from the systemic circulation (0.06, van der Schoor et al., 2001). In a companion study by this group, lysine was also demonstrated to be oxidized by the GIT of pigs, with lysine oxidation by the PDV accounted for 0.31 of whole body lysine oxidation (van Goudoever et al., 2001). However, unlike leucine, which was oxidized from both the arterial and luminal supplies to the GIT, only dietary (luminal) lysine was oxidized.

Threonine is also catabolized by the PDV of pigs, however, most of the enzyme activity for threonine catabolism (threonine dehydrogenase) is localized to the liver and pancreas (Le Floc'h et al., 1997). Therefore, glycine production from threonine in the pancreas is the major contributor to threonine catabolism by the PDV (Le Floc'h and Sève, 2005). Others have reported no catabolism of threonine to glycine by the GIT as indicated by the lack of labeled 13C incorporated into glycine released by the GIT (Schaart et al., 2005). These authors did report $^{13}CO_2$ production by the PDV, but only when labeled threonine was derived from the arterial blood. Consequently, threonine oxidation by the PDV accounted for 0.13 to 0.50 of whole body threonine oxidation.

Metabolic plasticity and AA catabolism

Cells lining the GIT of rodents (Fleming et al., 1997) and ruminants (cattle: Harmon, 1986; Okine et al., 1995; sheep: Oba et al., 2005) exhibit metabolic plasticity in substrate selection for catabolism. There are, however, notable differences between species with respect to the major substrates metabolized.

In rat small intestinal mucosa, glucose addition to the incubation media did not alter catabolism of glutamine to CO_2 (Watford, 1994; Fleming et al., 1997), whereas glutamine addition reduced CO_2 production from glucose (Fleming et al., 1997). By contrast, in ruminants, glutamine oxidation was reduced when the concentration of other substrates was increased. Here, glutamine oxidation by isolated rumen epithelial cells from steers was decreased in the presence of glucose and butyrate (Harmon et al., 1986). In similar studies conducted with sheep and dairy cow enterocytes, glucose addition decreased glutamine oxidation (Oba et al., 2004; Okine et al., 1995). Despite the decrease in glutamine oxidation in response to glucose addition, glutamate oxidation was not affected (Oba et al., 2004).

In rat enterocytes, glutamine carbon does not appear in the Krebs cycle intermediates despite the production of labeled CO_2 from the glutamine radio-tracer (Watford, 1994). The authors suggested that glutamine undergoes partial catabolism to lactate and alanine. In the sheep GIT tissues, glutamine is used primarily for protein and nucleotide synthesis (Gate et al., 1999). Because purine and pyrimidine synthesis occurs in the cytosolic compartment, partial catabolism of glutamine may also generate lactate and alanine.

Postabsorptive amino acid utilization

The ability to decrease AA catabolism by the GIT should, in turn, lead to an increase in portal AA absorption and consequently provide greater supplies of AAs to the liver and beyond to productive tissues for milk casein and muscle protein production.

The selective removal of AAs by the GIT tissues has the potential to create an imbalance in the pattern and quantity of AAs available to productive tissues (e.g. muscle,

mammary gland, and uterus) for anabolic use. One consequence of reducing GIT tract utilization of AA is their increased supply to the liver. In a study employing pregnant, non-lactating dairy cows, AA removal by the liver exceeded the requirements for urea synthesis, and therefore it was suggested that AA utilization supports primarily hepatic protein synthesis (Wray-Cahen et al., 1997). The pattern of free AAs available to non-splanchnic tissues is altered relative to that which is absorbed from the GIT as a result of hepatic removal of AAs from the portal circulation (Wray-Cahen et al., 1997; Lobley et al., 2001; Raggio et al., 2004). Despite the hepatic removal of AAs, when mesenteric supply of AA is increased by infusion the arterial blood concentration for most AAs is increased.

Due to the low extraction of BCAA and lysine by the liver of ruminants (Lobley et al., 2001; Raggio et al., 2004), larger amounts of these AAs appeared in the peripheral circulation in response to increments of metabolizable protein (Raggio et al., 2004). This resulted in an increased removal by the mammary gland and translated into a significant increase in milk output and milk protein yield. Feed intake has also been shown to increase arterial AA concentration. The arterial AA concentrations of sheep fed increasing levels of a dried grass diet (0.5 to 2.5 × maintenance energy) were elevate with each increment, and, consequently, this lead to an increase net removal of several AA by hind leg tissues (Hoskin et al., 2001; Hoskin et al., 2003; Savary-Auzeloux et al., 2003). This response likely resulted in a net muscle protein gain, as several of these AA (phenylalanine, tyrosine, and serine) cannot be catabolized by the leg tissues.

To date there are several questions about AA metabolism by the GIT of ruminants that remain unanswered. These questions include:

1. Are AA catabolized by the GIT as part of a fixed metabolic requirement?

2. If AA catabolism is not fixed how would changing dietary conditions, such as feeding carbohydrates or forages affect it?

3. Does AA oxidation represent a complete or partial catabolism? To what metabolic intermediates AA contribute to?

Glucose

Ruminants have evolved to survive on and utilize plant materials that are high in carbohydrates, mainly cellulose, hemicellulose, pectins, fructans and starches. In forage-based diets, very little starch escapes rumen microbial fermentation and so the small intestines 'sees' negligible carbohydrate monomer for digestion and absorption (Janes et al., 1985). In addition to rumen escape starch, some glucose is absorbed from the small intestine that arises from digestion of microbial polysaccharides (MacRae and Armstrong 1966; McAllan and Smith, 1974) and from intestinal muco-polysaccharides (mucins; Mukkur et al., 1985). Despite the low dietary supplies, glucose turnover rate in ruminants is only slightly lower than in non-ruminants on a body weight basis (Annison and White, 1961; Leng et al., 1967). The latter reflects ruminants' reliance on gluconeogenesis to meet their requirements for glucose in the starved and fed states.

In modern intensive animal production cereal grains that are rich in starch 58-77%; Huntington, 1997) have been widely used as a means to increase production performance in beef finishing and dairy operations. The additional benefit of supplementing forage based diets with grains to ruminants relates to changes in the pattern of short chain fatty acid production in the rumen and the "leakage" of starch to the small intestine.

Grain supplementation increases the proportion of propionate produced in the rumen as compared to other short chain fatty acids (Bergman, 1990). The increase in propionate production increases the availability of this short chain fatty acid for hepatic gluconeogenesis (Leng et al., 1967). Another reason for the improved performance is that dietary starch reaching the small intestines undergoes digestion by pancreatic and

intestinal enzymes, resulting in glucose available for absorption (Janes et al., 1985). In either case, the overall result is an increased glucose supply to the gastrointestinal tract and beyond to other tissues.

The gastrointestinal tract expresses the full compliment of glycolytic enzymes. What remains less certain is the metabolic need for glucose metabolism by the GIT. That is, does glucose metabolism occur to provide energy via the Krebs cycle or is glucose incompletely metabolized for other purposes?

Glucose luminal supply is mainly the proportion of dietary starch that escapes rumen fermentation and reaches the small intestine where it is digested and could represent about 0.25 of starch intake (Owens et al., 1986; Kreikemeier et al., 1991; Huntington, 1997). Arterial supply on the other hand is glucose that is absorbed from the small intestine in addition to glucose from gluconeogenesis. Glucose absorption from the lumen of the small intestine represents a smaller supply relative to that from the arterial blood (Janes et al., 1985). For example, in sheep fed a maize diet glucose luminal supply (i.e. glucose absorption) was 23 mmol/h whereas for the same animals arterial supply was 333 mmol/ h.

The ability of ruminants to efficiently digest starch in the small intestine and their ability to absorb glucose has been a subject of many reports. Some have suggested that digestion in the small intestines is the limiting process in starch utilization (Huntington, 1997) whereas others have proposed that the rate of glucose absorption (transport) places the greater limitation on its availability (Kreikemeier et al., 1991; Kreikemeier and Harmon, 1995; Harmon and McLeod, 2001).

Intestinal starch digestion and absorption

Shifting the site of starch digestion from the rumen to the intestine has been suggested to be more energetically beneficial to ruminants. This is based on theoretical grounds that more energy from glucose (starch) is lost as heat during rumen fermentation than energetic losses during tissue metabolism of glucose (Owens et al., 1986; Harmon and McLeod, 2001). It is estimated that 0.18-0.42 of dietary starch may escape rumen fermentation (Owens et al., 1986) this provides sheep (95g/d; Janes et al., 1985), beef (500 g/d; gSindt et al., 2006), and dairy cows (1-3 kg/d: Knowlton et al., 1994; Rémond et al., 2004) small intestine with large amounts of starch.

Digestion. In ruminants, starch is digested in the small intestine as it is in monogastric animals through a series of enzymatic hydrolysis steps. Pancreatic α-amylase secreted in the duodenum is the first enzyme acting upon starch molecules, hydrolyzing amylose and amylopectin at α-(1, 4) glycosidic linkages to maltose and dextrin moieties. Maltose comprises two glucose molecules linked by α-(1, 4) linkages whereas dextrins are glucose molecules linked at α-(1, 6) branch points. Following α-amylase hydrolysis, maltose and dextrins are further hydrolyzed before entering intestinal cells. Maltose and dextrins are hydrolyzed to glucose by brush border membrane α-glycosidases, mainly maltase and isomaltase in ruminants (Harmon 1992; Harmon 1993 Bauer; 1995).

Various reports seem to agree that ruminants have a limited ability to digest starch entering the small intestine (Huntington, 1997) and that this ability decreases with increasing intake (Kreikemeier et al., 1991). Pancreatic α-amylase activity and secretions have been shown to decrease with increasing postruminal starch supply (Walker and

Harmon. 1995). This decrease in α-amylase activity and secretion may explain the reduced efficiency with post-ruminal starch supply. Reports indicate that this decrease could be prevented by increasing AA supply post-ruminally. In beef cattle, casein infusion has been shown to increase α-amylase activity in the small intestine (Swanson et al., 2002; Richards et al., 2003). It was suggested that postruminal AA supply counteracts the negative response of starch through changes in mRNA expression (Swanson et al., 2003) possibly mediated through insulin. In contrast, others have suggested that the reduced efficiency of starch utilization with in the small intestines relates to limitations in glucose transport and absorption (Ørskov et al., 1971; Shirazi-Beechey et al., 1991).

Absorption. Glucose transport into intestinal cells is another factor that could influence glucose appearance in the blood. Glucose is transported (**Figure 1.2**) by two processes, paracellular diffusion and sodium-glucose transporter (SGLT1), the former being only a minor route (<10%) of total glucose absorption (Shirazi-Beechey et al., 1996; Harmon and McLeod, 2001). The highest SGLT1 transporter activity has been observed in pre-ruminants which declined to negligible levels in ruminant lambs (Shirazi-Beechey et al., 1991). However, the decline in glucose transporter activity in ruminant sheep has been shown to be reversible by infusion of glucose into the small intestines (Shirazi-Beechey et al., 1991). This adaptation to increased glucose supply to the small intestines was first reported in sheep infused with increments (20g/d) of glucose (Ørskov et al., 1971). In that study, sheep were able to cope with glucose loads up to 300 g/d, and this amount was considered the maximum without causing post-ileal spillage. Later, it was reported that part of the adaptive response to increased glucose availability in the small intestines was related to a 40-80 fold increase in SGLT1 transporter activity

(Shirazi-Beechey et al., 1991). Furthermore, the SGLT1 response has been linked to a glucose sensing mechanism in the small intestines (Harmon and Mcleod, 2001).

In vivo studies relevant to starch digestion and glucose absorption from the lumen of the small intestine provide a wealth of information. However such results should be interpreted with caution and interpreted in relation to the experimental conditions imposed. For example, short term infusions of starch and/or glucose (Kreikemeier et al., 1991) have provided estimates of the upper limits to digestive and absorptive capacities of ruminants relative to their particular physiological and dietary status. Further, such short term infusion regimes will not reflect the long-term adaptative mechanisms that ultimately will determine the practicality of supplying greater amounts of starch to ruminants (Janes et al., 1985; Shirazi-Beechey et al., 1991).

Despite the controversy surrounding starch utilization in ruminants it is clear that the GIT remains, despite it's relatively small size, a major site of extensive glucose metabolism from the arterial blood (Balcells et al., 1995; Cappelli et al., 1997) and luminal supplies (Cappelli et al., 1997; Seal and Parker, 1994; van der Schoor et al., 2001; Stoll et al., 1999).

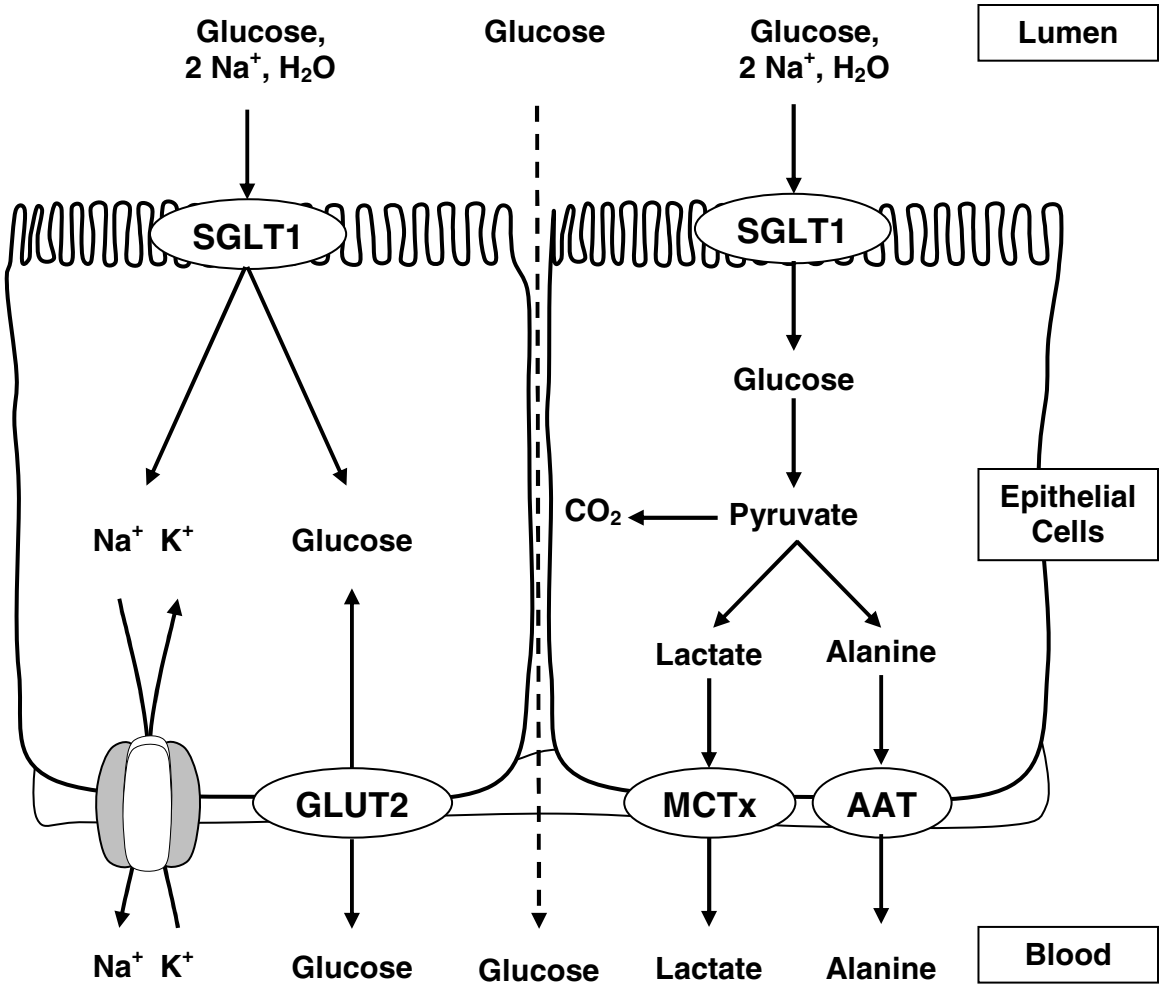

Figure 1.2. Glucose transport and metabolism in the gastrointestinal mucosa.

Gastrointestinal tract utilization

In ruminants, nutrients are supplied to the gastrointestinal tract mainly from the arterial blood supply, especially for the rumen and the hindgut tissues which have limited, if any, capacity to absorbed glucose and amino acids from their luminal aspects (Rémond et al., 2000). In situations such as feeding high grain diets the proportion of glucose that is unaccounted for in the portal blood is assumed to have been utilized during absorption.

The contribution of absorbed glucose to body requirements could vary depending on diet. Absorbed glucose represents 10% of whole body requirements when ruminant are fed high forage diets, but 60% when high levels of concentrates are fed (Bergman, 1973; van der Walt et al., 1983; Janes et al., 1985). This limited contribution of absorbed glucose to whole body requirements reflects in part its metabolism (0.25-0.35 of whole body irreversible loss) by the GIT tissues (Huntington et al., 1980, Huntington, 1982; Balcells et al., 1995; Cappelli et al., 1997).

Glucose serves a variety of metabolic functions (Figure 1.3) including precursors for nucleotide synthesis and intermediary metabolites, lactate and alanine, and energy production. Several studies have reported that epithelial and mucosal tissues have the ability to oxidize glucose for energy production both in monogastric (Windmueller 1974; Stoll et al., 1998) and ruminant species (Okine et al., 1995; Seal and Parker, 1996; Oba et al., 2004; Harmon, 1986; Baldwin and McLeod 2000). In ruminants, glucose catabolism by enterocytes depends upon the presence of other metabolites such as glutamine (Okine et al., 1995) or propionate (Harmon, 1986; Oba et al., 2004). Knowing the fuels selected

for energy generation and the flexibility of the GIT to utilize an array of substrates has implications on the prediction of nutrients to peripheral tissues for anabolism.

In this respect the response of the GIT to glucose supply has been inconsistent, while in some studies, glucose infusion into the jugular vein (Balcells et al., 1995) or the abomasum (Harmon et al., 2000) decreased AA acid absorption by the PDV. Others however, reported an increase in total NEAA absorption in response to intrajugular and intraduodenal glucose. It could be suggested that glucose would spare AAs from catabolism, therefore increased their appearance in PDV. This however, is based on the grounds that glucose and AAs share the same catabolic pathways.

Oxidation

Glucose contributes carbon to two oxidative pathways: the Krebs cycle and the oxidative arm of the pentose phosphate pathway. In the Krebs cycle oxidation is considered complete only if glucose enters at acetyl-CoA since all glucose-carbons are net lost as CO_2, while glucose oxidation in the pentose phosphate pathway is incomplete (partial) due to the loss of only one carbon atom from glucose molecule and the subsequent formation of ribose. Before glucose entry to the Krebs cycle it undergoes glycolysis. Glycolysis in the GIT and muscle does not commit glucose carbon to oxidation since the three-carbon intermediates lactate and alanine can be formed and recycled to glucose in the liver. However, if pyruvate enters into the Krebs cycle via oxaloacetate or malate through anaplerosis acetyl-CoA this leads to a net loss of glucose carbon because for every pyruvate molecule entering the cycle one carbon is lost at pyruvate dehydrogenase and two more carbon atoms are lost at isocitrate dehydrogenase and α–ketoglutarate dehydrogenase steps.

Adaptations in glucose metabolism can occur in response to dietary (Harmon et al., 1986) and physiological status (Okine et al., 1995). Rumen epithelial tissues from steers fed a high concentrate diet utilized more glucose by also produced more lactate and CO_2 than tissues from roughage fed steers (Harmon, 1986). Also in enterocytes lactate and CO_2 production from glucose were found to be higher in early-lactation cows as compared to mid and late-lactation cows (Okine, 1995).

One aspect that remains unresolved is the extent that glucose is oxidized for energy production. In most studies, [U-^{14}C]glucose tracer was used which does not allow differentiating between complete oxidation, i.e. Krebs cycle metabolism, or partial oxidation as it is the case in the pentose phosphate pathway. However, using [1-^{14}C]glucose and [6-^{14}C]glucose tracers it was demonstrated that pig enterocytes partial oxidation of glucose via the pentose phosphate pathway dominated over Krebs cycle metabolism (Wu et al., 1996). In the latter study, however, glucose was the only substrate included in the incubation media, and this would never be the situation in vivo. In other reports (Harmon, 1986; Okine et al., 1995; Oba et al., 2004), where a more complete array of substrates are present, there seems to be agreement that glucose catabolism is reduced when substrates such as SCFA (Harmon et al., 1986; Oba et al., 2004) or AA (Okine et al., 1995) are added to the incubations as treatments.

Intermediary metabolism

Lactate and alanine are the most important metabolic end products of glucose metabolism in GIT. Most lactate originates from glycolysis rather than propionate metabolism in sheep (Weeks and Webster 1975; Reynolds et al., 1991). Between 0.40-0.45 of lactate produced by the small intestines of sheep derives from arterial glucose

whereas on a whole body basis glucose contributes only 0.28-0.39 of lactate production (Janes et al., 1985). Similarly, in pregnant ewes, >50% of lactate produced by the PDV derives from arterial glucose (van der Walt et al., 1983; Reynolds et al., 1991), with the remainder derived from substrates that contain at least 3 carbons.

It was suggested that the high rates of glycolysis in rapidly growing cells is therefore likely to occur to maintain high levels of glucose-6-phosphate required for ribose synthesis (Newsholme et al., 1985). Data from pig studies support this view, as it has been shown that glucose oxidation by the pentose phosphate pathway is about 10 × higher than by the Krebs cycle (Wu et al., 1996). The end product of glycolysis, pyruvate, is reduced to lactate as a salvage mechanism and recycled to the liver for resynthesis of glucose.

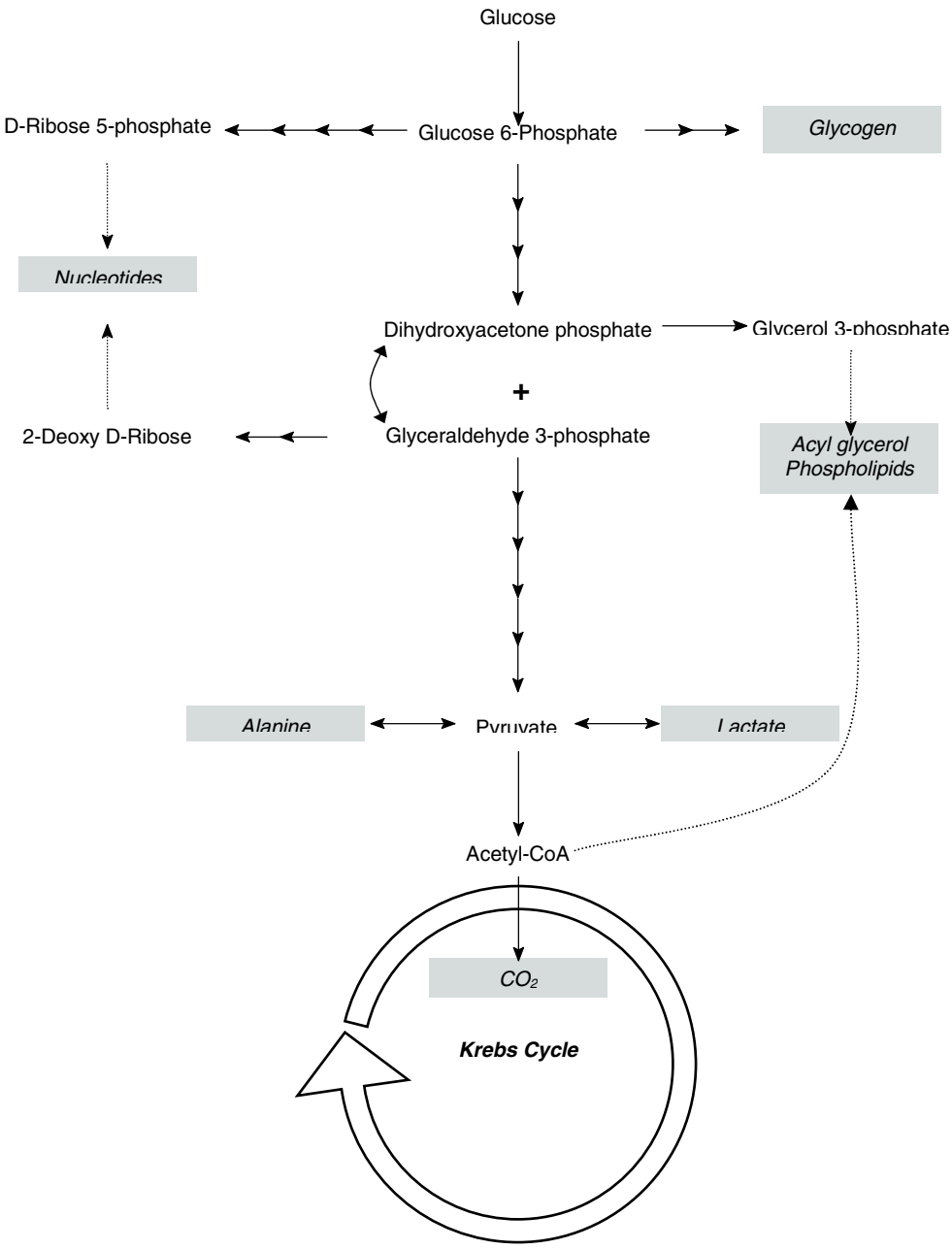

Figure 1.3. Overview of glucose catabolic and anabolic pathways.

Gluconeogenesis

Gluconeogenesis is the process by which glucose is formed from "non-hexose" precursors (Nelson and Cox, 2000). Therefore, gluconeogenic precursors, by definition, are those metabolic intermediates whose carbon skeleton does not originate from glucose. Where glucose is synthesized from glucose metabolic end-products, this process is termed "glucose recycling".

In general, the most important gluconeogenic precursors include those whose metabolism results in a net gain of carbon atoms in glucose. Thus, substrates metabolized via acetyl-CoA do not make a net carbon contribution because the resulting acetyl-CoA condenses with a molecule of oxaloacetate to yield a molecule of citrate. Subsequently, the carbons from acetyl-CoA are conserved after one turn of the cycle but at the same time two molecules of carbon dioxide are generated at the isocitrate dehydrogenase and α-ketoglutarate dehydrogenase steps. In consequence, the net balance of carbon resulting from inputs into the acetyl-CoA pool is neither a gain nor a loss of carbon from the Krebs cycle.

Although most amino acids, glycerol and lactate contribute net carbon to gluconeogenesis, in ruminants propionate (Leng et al., 1967) and alanine (Wolff and Bergmann, 1972) are quantitatively the most important. Liver removal of propionate from portal blood accounts for up to 90% of propionate absorbed from the rumen (Armentano, 1992). These removal rates, combined with a high hepatic propionyl-CoA activity, explain the large contribution of propionate to gluconeogenesis (Leng et al., 1967; Chow and Jesse, 1992). The additional benefit of feeding grains to ruminants is the increased contribution of propionate and that of absorbed glucose to glucose turnover rate

(0.08 vs. 0.60; Janes et al., 1985). Other substrates also contribute to gluconeogenesis for which alanine accounts for 10%, lactate 2 to 9%, and amino acids other than alanine 17-24% (Reynolds et al., 1991). However, lactate and alanine utilization for gluconeogenesis derive, in part, from glucose catabolism, representing a portion of glucose carbon recycling.

The rate of gluconeogenesis decreases significantly when glucose absorption from the small intestines is increased by feeding high grain diets. Corn supplementation to dried grass diets increased the contribution of absorbed glucose to glucose entry rate from 8% to 60% in sheep (Janes et al., 1985). It is important however for the additional starch source to escape rumen fermentation. If this is not the case, then rumen fermentation will ultimately increase hepatic glucose production through gluconeogenesis from propionate (Leng et al., 1967).

Amino acids are oxidized by the GIT, and this oxidation occurs via entry to the Krebs cycle. Glucose oxidation in the cycle via acetyl-CoA (i.e. sparing Leu, Lys) or via oxaloacetate (i.e. sparing AA entering prior to oxaloacetate) should lead to a reduction in AA catabolism, consequently increase AAs post-absorptively. Whether dietary changes leading to increased glucose absorption and/or increasing gluconeogenesis will supply more glucose to the GIT, hence have a sparing effect on AA utilization for oxidative purposes, is conditional upon glucose being oxidized for energy production via the Krebs cycle.

Short chain fatty acids

Short chain fatty acids (SCFA) are produced mainly in the gastrointestinal tract of ruminants and simple stomached animals as by-products of microbial fermentation of dietary carbohydrates (van Houtert, 1993). Although dietary proteins digested in the rumen contribute to SCFA production, this contribution is less significant in ruminants fed forage only diets (Bergman 1990; Britton and Krehbiel 1993; France and Siddons, 1993; van Houtert 1993).

Combined, SCFA represent about 0.75 of the metabolizable energy derived from fermentation of feed carbohydrates, and are comprised of mainly acetate, propionate and butyrate (0.95 of total SCFA; Bergman 1990; Britton and Krehbiel 1993; van Houtert 1993). Despite being by-products of rumen fermentation, SCFA are essential as they contribute 0.60-0.80 to ruminants' energy needs (Bergman, 1990; van Houtert, 1993). Short chain fatty acids are produced in large amounts during rumen fermentation that their constant removal and transport are important for the survival of the animal.

Production and absorption

The molar ratios of SCFA vary as a function of rumen supply of dietary ingredients. Diets that are high in forages (90%) generally produce a higher proportion of acetate to total SCFA produced (70:15:10) when fermented in the rumen because long forages favor the growth of acetate producing bacteria whereas concentrate (90%) based diets favor the production of propionate (55:30:12) at the expense of acetate (Harmon et al., 1991). Feeding time, quality of the dietary forage, level of intake and dietary additives affect this ratio and the total concentration and production rate of SCFA production (Bergman et al., 1990; France and Siddons, 1993).

Normally the pH of the rumen ranges between 6-7 at which most SCFA (weak acids; pKa ~4.8) are present in the dissociated form in the rumen fluid (Bergman, 1990; van Houtert, 1993). Short chain fatty acids are absorbed in the dissociated form across the apical aspect of rumen epithelial cells through a bicarbonate/acid exchanger (Gäbel et al., 2002). Under normal feeding conditions only a small proportion (<0.10) of ruminally produced SCFA reach the small intestines (Bergman, 1990; Gäbel et al., 2002). Once absorbed into the rumen epithelial cells, SCFA can be oxidized to CO_2 or metabolized to ketone bodies, or they are absorbed and released into the venous blood draining the gastrointestinal tract.

Metabolism of short chain fatty acids

The carbon skeleton of SCFA are used either for energy production in the Krebs cycle or they are metabolized to lactic acid and the ketones acetoacetate and β-hydroxybutyrate (**Figure 1.5**). The first step in SCFA metabolism involves the activation of the fatty acid by conjugation with coenzyme A (CoA). Acyl-CoA synthetase is the limiting step for SCFA catabolism and oxidative degradation (**Figure 1.4**) and it is believed this enzyme serves as the point of metabolic control (Cook et al., 1969; Ash and Baird, 1973).

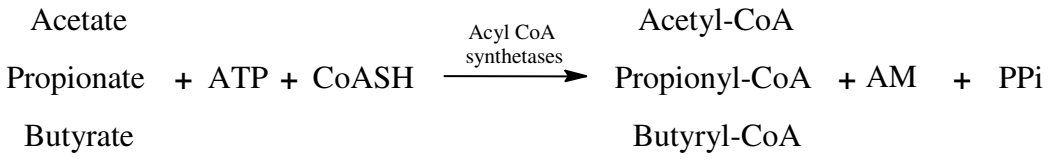

Figure 1.4. Schematic representation of short chain fatty acid activation.

Differences exist with respect to tissue acyl-CoA synthetase activity and the specificity of the acyl-CoA towards a SCFA. acetyl-CoA and propionyl-CoA synthetase activity in rumen epithelial tissue of calves are 0.10 and 0.64 that of butyryl-CoA (Harmon et al., 1991). In that study, the addition of butyrate to incubation media decreased the acetyl-CoA and propionyl-CoA synthetase activities by 0.63 and 0.82, whereas acetate and propionate had no effect on butyryl-Co synthetase activity. The significance of the distribution of –CoA synthetases relates to the role of the GIT and liver in regulating the appearance and delivery of the SCFA to peripheral tissues (e.g. muscle, mammary, adipose).

It has been proposed that the overall result of acyl-CoA synthetase activity and its specificity in the rumen and liver is to ensure that acetate is removed by peripheral tissues, that butyrate is metabolized to β-hydroxybutyrate by the rumen epithelium. that propionate is removed by the liver and that acetate reaches the peripheral tissues (Ash and Baird, 1973). The metabolism of butyrate to β-hydroxybutyrate is believed to be a protective mechanism against the hyperglycemic effect of butyrate, which causes the secretion of glucagon from the pancreas, while acetate, propionate and β-hydroxybutyrate have a lesser effect (see Cook et al., 1969). Ketogenesis in the rumen wall serves other functions such as the creation of a favorable concentration gradient for butyrate absorption across the rumen epithelium and for conserving most of the energy of the parent SCFA (Henning and Hird, 1972).

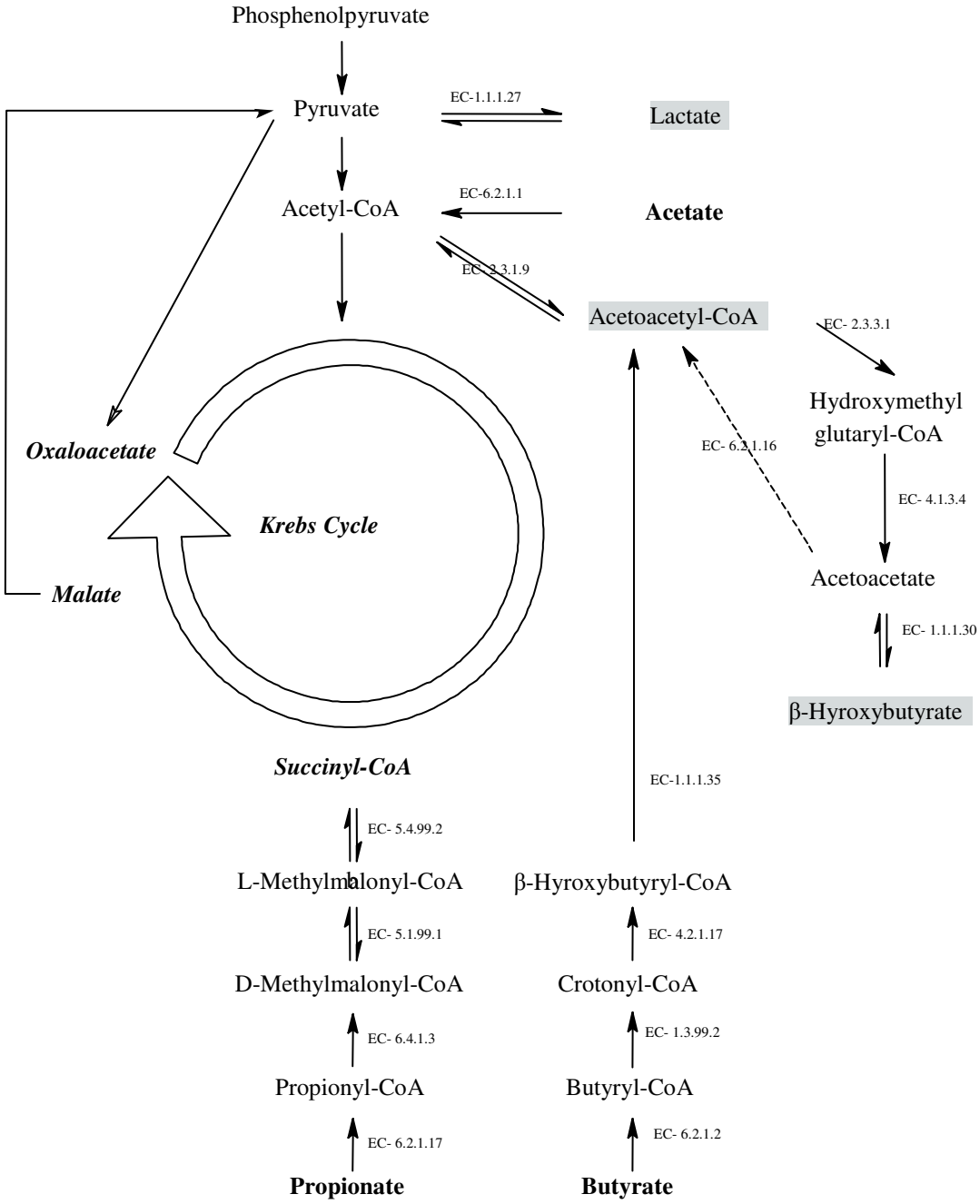

Figure 1.5. Schematic representation of short chain fatty acid metabolism.

It is widely accepted that in ruminants 0.30 of acetate, 0.50 of propionate and 0.90 of butyrate produced in the rumen is metabolized by stomach tissues (Bergman, 1990). The extents that acetate and propionate are metabolized by rumen tissues is controversial. Some studies have reported extensive metabolism of acetate and propionate (0.30-0.50, for reviews see Bergman, 1990; Britton and Krehbiel, 1993; van Houtert, 1993; Rémond et al., 1995) while others have reported very little degradation by rumen tissues (0.10-0.15, Kristensen et al., 2000b).

Recently, the washed rumen technique has been employed to distinguish between metabolism of SCFA by rumen tissues versus that by bacteria adhering to the rumen epithelium. Here, it was concluded that microbial metabolism accounts for most of the "loss" of SCFA between the rumen tissues and the blood. The results of a series of experiments (Kristensen et al., 1996; Kristensen et al., 2000ab; Kristensen and Harmon, 2004ab) provides evidence that a small proportion of propionate (0.05-0.10) is metabolized during absorption whereas acetate is not metabolized and acetate infused in the rumen is recovered across the rumen wall in the portal blood drainage. In that study, only butyrate was found to be metabolized by the rumen tissues with 0.18-0.82 catabolized.

While the metabolic fates of acetate and propionate have been questionable, the metabolic fates of butyrate have so far been conclusive in that a large (0.70-0.90) proportion of ruminal butyrate is metabolized to β-hydroxybutyrate by rumen wall (Bergman, 1990; Kristensen et al., 2000b; Nozière et al., 2000). The different techniques

employed to quantitate SCFA metabolism may explain some of the differences observed in the literature and this creates difficulties for making comparisons among studies.

Employing carbon (^{14}C, ^{13}C) tracers, it is possible to determine the fates of the carbon skeletons of SCFA, their catabolism to CO_2, lactate and ketone bodies. This is an advantage over non-tracer methods where the net inputs and outputs are measured and does not allow accounting for the exchanges and interconversion of carbon skeletons between metabolites in animal tissues.

In ruminants, small intestinal cells are exposed to SCFA escaping the rumen and those derived from the arterial blood (Bergman, 1990; Gäbel et al., 2002). The amounts of SCFA presented to the small intestine are lower (7-14 vs 84-109 mmol/L) than those presented to rumen epithelial cells (Rupp et al., 1994), yet they have been shown to alter enterocytes metabolism. Propionate and butyrate have been shown to reduce glucose and glutamine oxidation in isolated sheep enterocytes (Oba et al., 2004).

In summary, studies in the literature raise a question: is the concept of SCFA metabolism by the rumen wall valid or is it an artifact of the techniques used? Clearly the contribution of microbial metabolism leads to an overestimate of the tissue's metabolism, and therefore, an overestimate of the energetic requirements of the animal. However, when microbial metabolism is taken into account rumen metabolism of propionate (0.10) and butyrate (0.5-0.7) still occurs (Kristensen and Harmon, 2004ab). Furthermore, the supply of SCFA alters enterocytes metabolism and has an influence on glucose and AA utilization by the GIT (Harmon, 1986; Oba et al., 2004). Whether SCFA metabolism occurs for energy production requires further elucidation.

CHAPTER 2

GLUCOSE METABOLISM BY THE GASTROINTESTINAL TRACT OF SHEEP AS AFFECTED BY PROTEIN SUPPLY

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ABSTRACT

Gastrointestinal (GIT) metabolism of glucose was investigated in sheep (n=6, 35 ± 2.0 kg) fed a low protein diet (100 g CP/kg) to 1.6× maintenance. Catheters were fitted for duodenal infusion and flux measurements by the portal-drained viscera. Animals were given 10-d duodenal infusions of either glucose (50 g/ d, Control) or Glucose (50 g/ d) + Casein (60 g/d) in a cross-over design. On days 7 and 10, [1-^{13}C]glucose was infused (6 h) into either a jugular vein or the duodenum to determine glucose utilization by the GIT. The GIT utilized 48-51% of glucose available from absorption and gluconeogenesis. Most of the glucose used by the GIT derived from the arterial circulation (65-82%) compared to that from the GIT lumen(18-35%). Compared to Control, Casein + Glucose increased ($P<0.05$) the contribution of luminal (first-pass) and decreased ($P<0.05$) that of arterial glucose utilization by the GIT, but total GIT utilization remained the same. Casein + Glucose infusion increased ($P<0.05$) total glucose availability, however, this derived mainly from an increase in gluconeogenesis (+27%).

Keywords: glucose, gastrointestinal, sheep

INTRODUCTION

Gastrointestinal tract (GIT) metabolism by ruminants places a major drain on amino acid (AA) and glucose for growth. Metabolism of intestinal essential AA supply is high (0.25-0.60, MacRae et al., 1997b), as is non-essential AA metabolism (80-100%, Heitmann and Bergman, 1981). Similarly, a significant proportion (0.40-0.60) of intestinal glucose is removed by the GIT (Cappelli et al., 1997). Reducing the catabolic losses of AA and glucose by the GIT is a goal towards improving N and energy efficiency of ruminants. In this respect, our aim is to determine the optimal pattern of substrates for productive processes, and this requires knowledge of the fuels selected by the GIT for metabolism.

Based on previous studies with ruminal and intestinal cells, where metabolic flexibility was demonstrated for AA and glucose oxidation (Okine et al., 1995; Oba et al., 2004), we hypothesized that if AA, and not glucose, are the preferential energy substrates of the GIT, then intestinal infusion of casein would lead to a reduction in glucose use by the GIT.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at the University of Maryland approved all animal use procedures. Six sheep (4 Katahdin and 2 Dorset x Polypay, 35 ± 2.0 kg) were fitted with chronic catheters placed into the duodenum, an artery, the hepatic-portal and two mesenteric veins. After recovery, sheep were placed in metabolic crates and fed a pelleted diet by automatic feeder (12 × 2-h intervals). The diet was fed to 1.6 × maintenance energy requirements (NRC, 1985), and was low in protein (100 g

CP/kg) but energy adequate (7.5 MJ metabolizable energy/kg). Animals were arranged in a balanced cross over design with 10-d treatment periods. Treatments were duodenal infusion of either glucose (50 g/ d, Control) or Glucose(50g/d) + Casein (60 g/d).

On days 7 and 10 of each period, [1-^{13}C]glucose was infused (200 mg/h for 6 h) into either a jugular vein or the duodenum to determine the rate and proportion of glucose entry utilized by the GIT and to distinguish between luminal and arterial utilization of glucose. Plasma flow was determined by infusion (15 mg/ min, pH 7.4) of p-aminohippuric acid into the distal mesenteric vein. During the last 4 h, blood was continuously withdrawn from each vessel over 1-h periods into sealed syringes submerged in an ice bath. Samples were mixed and processed for analysis.

Plasma glucose enrichment and concentration were determined by gas chromatography-mass spectrometry under electrical impact mode (Hannestad and Lundblad, 1997, Calder et al., 1999). Plasma concentrations of p-aminohippuric acid were determined as previously described (McRae et al., 1997b). Gravimetric procedures were used throughout to reduce error and increase precision.

Data were analyzed using the MIXED procedure of SAS (2003), with sheep, period, breed, and treatment order considered as random effects. Differences were considered significant at $P \leq 0.05$.

Plasma glucose entry rate was calculated for jugular vein (GE_{jv}) and duodenal (GE_{duo}) [1-^{13}C]glucose infusions employing standard isotope dilution principles. Fractional splanchnic removal was calculated as: 1-(GE_{jv}/ GE_{duo}). Net flux (absorption or removal) of glucose by the PDV was calculated as the product of plasma flow (PF) and

arterio-venous concentration difference. Fractional utilization of arterial glucose (f_a) was based upon jugular infusion of [1-^{13}C]glucose calculated by:

$$fu_a = \frac{[A] \times A_E \times BF - [V] \times V_E \times BF}{[A] \times A_E \times BF}$$

where E is enrichment, and [A] and [V] are concentrations of glucose in artery (A) and portal vein (V). Arterial glucose utilization was calculated as: [A] × PF × fractional utilization of arterial glucose. Luminal use of glucose (i.e. first-pass) was calculated from recovery of [1-^{13}C]glucose infused into the duodenum after correction for second-pass arterial removal, and converted to an absolute rate based on unlabeled glucose infusion rate into the duodenum:

$$1 - \left(\frac{([V] \times V_E) - ([A] \times A_E)] + ([A] \times A_E \times f_a) \times PF}{\text{duodenal [1-}^{13}\text{C]glucose infusion rate}} \right) \times \text{duodenal glucose infusion rate}$$

RESULTS

There were no significant differences detected in glucose metabolism between Katahdin and Dorsett × Polypay sheep. Plasma glucose (**Table 2.1**) and glucose entry rates based on jugular and duodenal tracer infusion were not significantly affected by Casein + Glucose infusion compared to infusion of glucose alone (Control). Portal plasma flow tended ($P<0.08$) to be lower with Casein + Glucose infusion. Arterial use of glucose by the GIT accounted for 65-82% and luminal use 18-35% of total GIT use of glucose. Total utilization of glucose by the GIT was not affected; however, infusion of Casein + Glucose shifted the proportion of total glucose use by the GIT leading to a

reduced (P<0.05) contribution from the arterial supply and an increased (P<0.05) contribution from the gut lumen (first-pass). First-pass metabolism of glucose by the GIT accounted for 33 to 62% of the glucose infused into the duodenum, and this was reflected in a tendency (P<0.08) for [1-^{13}C]glucose recovery across the PDV to be lower with Casein + Glucose. Total glucose availability (gluconeogenesis + luminal absorption) was greater (P<0.05) with Casein + Glucose infusion, and after correction for luminal glucose absorption, indicated that gluconeogenesis increased with Casein + Glucose. Utilization of glucose by the GIT (arterial + luminal) accounted by 48 to 51% of glucose available from absorption plus gluconeogenesis, and this was not altered by treatment.

DISCUSSION

Many previous studies in ruminants have demonstrated that GIT metabolism places a major drain on AA and glucose availability for peripheral tissue anabolism. In this respect, there are two questions with regards to AA and glucose metabolism by the GIT: Is GIT metabolism of these nutrients obligate? To what extent are these nutrients metabolized from the gut lumen versus metabolism from the arterial circulation? We (Oba et al., 2004) and others (Okine et al., 1995) have demonstrated with ruminal and duodenal cells in vitro that the gut tissues of ruminants have some metabolic flexibility to oxidize either glucose or AA (glutamate, glutamine) for energy. In the present study, we tested, by increasing the supply of protein (casein) to the small intestines of sheep fed a marginally low protein but energy adequate diet, whether glucose metabolism by the GIT is 'spared' by provision of AA. Our results indicate that total GIT use of glucose is not affected by additional luminal AA supply, and so under these conditions glucose metabolism by the GIT was obligatory. However, this disguised the fact that casein

infusion increased the amount and proportion of glucose use by the GIT that was derived from the gut lumen supply (3.9 vs. 7.2 mmol/h, 18% vs. 35%) and decreased the proportional use from the arterial supply (82% vs 65%). Further, casein infusion on this low protein diet increased total glucose availability from luminal absorption and gluconeogenesis, which after correction for luminal absorption indicated that casein infusion increased gluconeogenesis by 27%.

Nutrients are delivered to the GIT from luminal and arterial supplies, and thus the factors regulating use from these sources will differ. Our results indicate that glucose use by the ruminant GIT derives mainly (65-82%) from the arterial circulation, which is probably under hormonal control and suggests that the GIT may be in competition with peripheral tissues (eg. muscle, mammary gland) for glucose supplies. Partition predominantly from the arterial circulation has also been observed for essential AA, where for most AA ~80% of GIT use of AA derived from the arterial circulation MacRae et al., 1997a). This pattern of glucose and AA use also corresponds with the known distribution of luminal absorptive capacity of the ruminant GIT with only ~20% of the luminal surface of the GIT capable of glucose and AA transport and absorption.

TABLE 2.1.

Whole body and gastrointestinal tract metabolism of glucose in sheep given intraduodenal infusions of glucose (Control) or Glucose plus Casein

Item	Treatment		SEM	P<
	Control (Glucose)	Glucose + Casein		
Plasma glucose flux and metabolism				
Arterial glucose (mM)	3.84 (6)	3.94 (6)[1]	0.490	0.59
Glucose entry rate (mmol/h)				
Jugular [1-^{13}C]glucose infusion (A)	37.6 (6)	43.4 (6)	3.01	0.13
Duodenal [1-^{13}C]glucose infusion (B)	45.0 (6)	48.2 (6)	5.75	0.47
Fractional splanchnic removal (1-(A/B))	0.21 (5)	0.17 (5)	0.100	0.83
Gastrointestinal glucose metabolism				
Portal plasma flow (g/min)	1880 (5)	1270 (6)	217	0.08
Glucose utilization				
Net flux[2] (mmol/h)	81.3 (5)	-19.3 (6)	55.00	0.16
Arterial use (mmol/h) (C)	13.6 (5)	16.3 (5)	3.72	0.62
Luminal use (mmol/h) (D)	3.9 (4)	7.2 (5)	0.79	0.01
Total gut use (mmol/h) (E)	21.0 (4)	23.0 (5)	5.49	0.78
Fractional luminal tracer recovery	0.65	0.45	0.134	0.15
Fractional arterial use of total gut use[4] (C/E)	0.82 (4)	0.65 (5)	0.071	0.03
Fractional luminal use of total gut use (D/E)	0.18 (4)	0.35 (5)	0.071	0.03
Total glucose availability[5] (TGA; mmol/h)	37.1 (5)	47.7 (5)	5.11	0.04
Luminal use as % of TGA	11.2 (4)	15.6 (5)	2.78	0.08
Arterial use as % of TGA	34.9 (5)	34.0 (5)	8.78	0.94
Total gut use as % of TGA	51.0 (4)	48.0 (5)	13.14	0.81

[1] Number of observations (n).
[2] Negative values indicate net removal and positive values net absorption.
[3] For both treatments, fractional arterial use was greater than fractional luminal use (at least P<0.05).
[4] The fractional contribution to total gut glucose use from arterial removal was significantly greater (P<0.01) than that from luminal use.
[5] TGA = glucose entry rate (jugular [1-^{13}C]glucose infusion) + first-pass luminal use.

CHAPTER 3

INTESTINAL PROTEIN SUPPLY ALTERS AMINO ACID, BUT NOT GLUCOSE, METABOLISM BY THE SHEEP GASTROINTESTINAL TRACT

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ABSTRACT

This study aimed to establish the extent which amino acids (AAs) and glucose are net metabolized by the gastrointestinal tract (GIT) of ruminant sheep when intestinal protein supply is varied. Wether sheep (n = 4, 33 ± 2.0 kg) were fitted with catheters for measurement of net absorption by the mesenteric (MDV) and portal-drained (PDV) viscera, and a catheter inserted into the duodenum for casein infusions. Sheep received a fixed amount of a basal diet that provided adequate metabolizable energy (10.9 MJ/d) but inadequate metabolizable protein (75 g/d) to support 300 g gain per day. Four levels of casein infusion (0 (water), 35, 70, & 105 g/d), each infused for 5.5 d, were assigned to sheep according to a 4 × 4 Latin square design. [methyl-$^{2}H_3$]Leucine was infused (8 h) into the duodenum while [1-^{13}C]leucine plus [6-$^{2}H_2$]glucose were infused (8 h) into a jugular vein. With the exception of glutamate and glutamine, net absorption of AAs increased linearly ($P < 0.05$, R^2= 0.46 to 1.79 for MDV; $P < 0.05$, R^2 = 0.6 to 1.58 for PDV) with casein infusion rate. Net absorption by the PDV accounted for <100% of the additional supplies of leucine, valine and isoleucine (0.6 to 0.66, $P < 0.05$) from casein infusion, whereas net absorption by the MDV accounted for 100% of the additional essential AA supply. Glucose absorption (negative) and utilization of arterial glucose supply by the GIT remained unchanged. There was a positive linear ($P < 0.05$) relation between transfer of plasma urea to the GIT and arterial urea concentration (MDV, $P < 0.05$, $r = 0.90$; PDV, $P < 0.05$, $r = 0.93$). The ruminant GIT appears to metabolize increasing amounts of the branched-chain AAs and certain non-essential AAs when the intestinal supply of protein is increased.

Key words: sheep, gastrointestinal tract, amino acid, urea, glucose.

INTRODUCTION

In ruminants, metabolism by the gastrointestinal tract (GIT)[1] represents the single largest metabolic fate of amino acids (AAs), glucose and other energy substrates in the body. Depending upon the AA, the GIT metabolizes 5 to 100% of the AA supply disappearing from the small intestines, and for glucose 50 to 100% of its intestinal disappearance (MacRae et al., 1997a; MacRae et al., 1997b; Berthiaume et al., 2001). The nutritional significance of GIT metabolism is well known, yet there are still important aspects of GIT metabolism that remain largely unresolved. In regards to AA metabolism, it could be hypothesized that, at a minimum, net metabolism of essential AAs by the GIT occurs at a fixed rate and amount relative to their compositions in the endogenous proteins lost from the GIT. Thus, once this 'service cost' is satisfied, absorption of AAs across the GIT should approximate to100% of their additional intestinal supplies. Current information suggests that this may not be the case, however, in particular with regards to certain non-essential AAs (Caton et al., 2001; Reynolds et al., 1991; Stoll et al., 1999; Bos et al., 2003; van der Schoor et al., 2001; Windemueller and Spaeth, 1980).

Net absorption of AAs by the small intestines of ruminants has been determined from the product of net arterio-venous difference and blood flow measurements across the mesenteric-drained viscera (MDV). Most often, however, net metabolism by the whole GIT is determined from measurements across the portal-drained viscera (PDV), which represents the sum of MDV plus rumen and hind-gut metabolism. This measure

[1] Abbreviations used: AA, amino acid; PAH, p-amino-hippuric acid;APE, atom percent excess; *t*-BDMS, *t*-butyldimethylsilyl; BCAA, branched chain amino acids; F, flow; GC-MS, gas chromatography -mass spectrometry; GIT, gastrointestinal tract; MDV, mesenteric drained viscera; PDV, portal drained viscera.

accounts for intestinal metabolism as well as that occurring by the rumen and hind-gut tissues the latter of which derive AAs almost exclusively from the arterial blood supply (MacRae et al., 1997). Although this arterial use of AAs by the PDV is considered a consequence of GIT metabolism, this arterial use of AAs represents potential competition with peripheral tissues (e.g. muscle, mammary gland). Based on PDV net flux measurements (dairy cows: Berthiaume et al., 2001; Caton et al., 2001; Reynolds et al., 2001; sheep: MacRae et al., 1997) net recovery of infused casein or free AAs or absorbed essential AAs is generally found to be lowest for the branched chain AAs (BCAA: leucine, valine and isoleucine; 10-62%), followed variously by methionine (33-69%), histidine (39-92%), phenylalanine (36-96%), lysine (30-66%), tryptophan (31-88%) and threonine (41-63%). By contrast, intestinal supplies of the non-essential AAs glutamine and glutamate have been found to be almost completely metabolized by the GIT, even when the supplies of these AAs are increased (Caton et al., 2001; Reynolds et al., 1991; Stoll et al., 1999; Bos et al., 2003; van der Schoor et al., 2001; Windemueller and Spaeth, 1980). These studies examined PDV nutrient use in response to only one level of protein, AAs or feed intake, and so it has not been possible to establish response relationships of intestinal supply and net absorption of individual AAs. In consequence, it is not known whether GIT metabolism occurs at a fixed or variable rate for individual AAs, and nor is it known whether this GIT metabolism ultimately leads to differential supplies of AAs to the liver and beyond to peripheral tissues for anabolic use.

The aim of this study is to answer two central questions regarding AA utilization by the ruminant GIT: 1) are certain AAs preferentially utilized? and 2) are certain AAs metabolized at fixed or variable rates relative to changes in their intestinal supply? A

secondary aim relates to observations that the GIT of ruminants net metabolizes the intestinal supply of glucose (Reynolds et al., 1988, Reynolds et al., 1991). For this reason, the net balance of glucose across the GIT is often negative. In this respect, an aim was to determine whether glucose utilization is reduced or unaffected by changes in the intestinal supply and GIT utilization of AAs.

MATERIALS AND METHODS

Sheep and Surgery. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Maryland (College Park, MD). Four mixed-breed (Polypay × Dorsett, 33 ± 2.0 kg body weight, 5 to 6-mo-old) wether lambs were prepared under general anesthesia with vascular catheters (Silastic®, 0.64 mm i.d., 1.19 mm o.d.) placed in the distal and proximal mesenteric vein, the hepatic portal vein and a femoral artery. The tip of the proximal mesenteric vein blood sampling catheter was positioned ~5 cm from the ileo-cecal vein junction at the most proximal point where the venous drainage from proximal small intestine enters the mesenteric arcade (see Seal and Reynolds, 1993). The tip of the distal mesenteric vein catheter was positioned ~30 cm upstream of the proximal catheter for infusion of the blood flow marker para-aminohippuric acid (PAH). A catheter (polyvinyl chloride, 2.4 mm i.d. × 4.0 mm o.d.) was also inserted into the proximal duodenum, the tip ~10 cm from the pylorus, for infusion of casein. From 2 wk post-surgery, feed (**Table 3.1**) was delivered in equal portions every 2 h via an automatic feeder. Sheep were fed to two times energy maintenance intake (10.9 MJ/d, ~80 g dry matter/kg body weight$^{0.75}$) a pelleted ration that was adequate in energy (calculated 10.2 MJ metabolizable energy/kg dry matter) but which was low in protein (10.4% crude protein, dry matter) and provided only ~60% of

the metabolizable protein intake (~75 g/d) required to support 300 g of gain per day (AFRC, 1992).

Casein infusion. Sheep were placed in individual metabolic crates equipped with automatic feeders and fresh water was provided daily. Sheep were randomly assigned to four levels of casein infusion (0, 35, 70 and 105 g/d in 1.0 L water) according to a balanced 4 × 4 Latin Square design. Rates of casein infusion were designed to increase metabolizable protein supply from 60% (Control, 75g/d) to 110% (180 g/d, 105 g casein/d level) of that required to support 300 g/d gain (AFRC, 1992). Each period lasted 10 d, with casein infused during the last 5.5 d. On day 1 and 3 of each 10-d experimental period, sheep were allowed to exercise in floor pens for at least 2 h/d. In addition, a 1-wk rest period (for exercise) in floor pens separated periods 2 and 3**.**

Tracer infusion and blood sampling. On day 4 of casein infusions, a temporary catheter (polyvinyl chloride, 0.8 mm i.d., 1.20 mm o.d.) was inserted (10 cm) into a jugular vein. On the last day, a sterile solution containing PAH (0.1 mol/L) and heparin (235 IU/mL) was infused (8 h, 20 mL/h) into the distal mesenteric vein catheter, and into the jugular vein was infused (8 h, 20 mL/h) a sterile solution containing [1-^{13}C]leucine (1.5 mg/mL) and [6-$^{2}H_2$]glucose (5 mg/mL). At the same time, [methyl-$^{2}H_3$]leucine (30 mg/h) was infused into the duodenum along with water or casein infusions. Over the last 4 h, blood samples were continuously withdrawn (5 mL/h) over 1-h periods from the artery, proximal mesenteric vein and portal vein by peristaltic pump and collected into sealed syringes submerged in an ice-bath. Each syringe was mixed by gentle hand-rolling, and plasma separated by centrifugation (1000 ×g for 15 min at 4°C).

Plasma amino acid and urea concentration. These were determined by isotope dilution with gas chromatography-mass spectrometry (GC-MS) as previously described (Calder et al., 1999). To a known weight (0.5 g) of fresh plasma was added an equal known weight of a solution containing 0.2 mg hydrolyzed [U-^{13}C]algae protein powder (99 atoms %; Martek Biosciences Corp., Columbia, MD), 100 nmol [indole-$^{2}H_5$]tryptophan, 200 nmol [5-^{15}N]glutamine, 25 nmol [methyl-$^{2}H_3$]methionine, and 3 µmol [$^{15}N_2$]urea, and the samples stored frozen (-20°C). Thawed samples were deproteinized by addition (1 mL) of sulfosalicylic acid (15% w/v), the supernatant desalted by cation (AG-50, H^+ form) exchange, and AAs and urea eluted with 2 mol/L NH_4OH followed by water. For urea analysis, 20 µL of this eluate was dried under a stream of N_2, and urea converted to the *t*-butyldimethylsilyl (*t*-BDMS) derivative prior to GC-MS (HP 5973N Mass Selective Detector, Agilent, Palo Alto, CA). The remaining eluate was lyophilized to dryness, and AAs converted to their *t*-BDMS derivative. Under electron impact mode, the following ions (*m/z*) were monitored: urea 231, 233; alanine 260, 263; glycine 246, 248; valine 288, 293; isoleucine 302, 308; proline 286, 291; methionine 292, 295; serine 390, 393; threonine 404, 408; phenylalanine 234, 242; aspartate 302, 304; glutamate 432, 437; lysine 300, 306; histidine 440, 446; glutamine 168, 169; tyrosine 302, 304; and tryptophan 244, 249. For leucine isotope enrichment and concentration, ions at 302, 303 ([1-^{13}C]leucine), 305 ([$^{2}H_3$]leucine) and 308 ([$^{13}C_6$]leucine, internal standard) were monitored. Calibration curves were generated from gravimetric mixtures of labeled and unlabeled AA. For leucine, correction was also made for the spillover of [1-^{13}C]leucine (*m/z* 303) into [$^{2}H_3$]leucine (*m/z* 305), and

spillover of [$^{2}H_{3}$]leucine into [$^{13}C_{6}$]leucine (*m/z* 308). All enrichments were expressed as atoms percent excess (APE) relative to background natural abundance.

Glucose concentration and enrichment. To a known weight (0.150 g) of fresh plasma was added an equal known weight of a solution containing 0.6 mmol [$^{13}C_{6}$]glucose. The aldonitrile pentaacetate derivative of glucose was formed (Hannestad and Lundblad, 1997), and ions at 242, 244 ([$^{2}H_{2}$]glucose) and 247 ([$^{13}C_{6}$]glucose) monitored by GC-MS under electrical impact mode. This derivative of glucose loses carbon-1 under electron impact to yield *m/z* 247 which corresponds to the internal standard [$^{13}C_{6}$]glucose. Calibration curves were generated, and corrections made for spill-over of *m/z* 244 ([$^{2}H_{2}$]glucose) to *m/z* 247 ([$^{13}C_{6}$]glucose). Enrichments are expressed as APE above background.

PAH concentration. Plasma concentrations of PAH were determined in duplicate using 0.25 g plasma and employing gravimetric procedures (Bequette et al., 1996).

Calculation of net fluxes of amino acids. Net absorption or removal of an AA by the mesenteric (MDV; mostly small intestines) and portal (PDV; whole GIT) drained viscera was calculated as the product of plasma flow (F; (kg/(kg BW • h) and plasma veno-arterial concentration difference (μmol/kg plasma) as appropriate. The incremental recovery by the MDV and PDV of individual AAs infused as casein into the duodenum was determined from the slope of the regression of net flux rate against duodenal casein-AA infusion rate (μmol/(kg BW • h). The intestinal digestibility of casein was assumed to be 100% (Stoll et al., 1998)**.**

Calculation of leucine fluxes. Rate leucine of appearance (Leu R_a) was calculated from arterial plasma leucine enrichment (E_A) when the leucine tracer was

infused into the jugular vein ([^{13}C]leucine; systemic Leu R_a) or into the duodenum ([$^{2}H_3$]leucine; whole body Leu R_a):

$$\text{Leu } R_a = [(E_I / E_A)\text{-}1] \times IR$$

where E_I is the APE of the leucine tracer infused ([$^{2}H_3$] or [1-^{13}C]), and IR is isotope infusion rate per kg BW per h. The difference between whole body Leu R_a and systemic Leu R_a is leucine removed by the gut and liver during its first-pass. Fractional first-pass arterial utilization of leucine by the MDV or PDV was calculated from jugular infusion of [^{13}C]leucine:

$$\text{Fractional arterial utilization} = ([A] \times E_A - [V] \times E_V)/([A] \times E_A)$$

where [A] is arterial and [V] is MDV or PDV concentration of leucine as appropriate. First-pass arterial utilization by the MDV or PDV was calculated as:

$$\text{First-pass arterial utilization} = [A] \times F \times \text{fractional arterial utilization}$$

where F is plasma flow rate for the MDV or PDV as appropriate. Fractional first-pass intestinal utilization of leucine was computed from [$^{2}H_3$]leucine tracer balance across the PDV, corrected for [$^{2}H_3$]leucine recycled and sequestered on second-pass by the PDV from the arterial circulation (i.e. [^{13}C]leucine arterial removal):

Fractional first-pass intestinal utilization =

$$(([V] \times E_V) - ([A] \times E_A) + ([A] \times E_A \times \text{fractional arterial utilization} \times F))/IR$$

where IR is the rate of [$^{2}H_3$]leucine infusion into the duodenum.

Calculation of glucose utilization. Net removal of plasma glucose by the MDV and PDV was calculated as above for AAs. Glucose rate of appearance (gluconeogenesis,

glucose recycling and absorption) was calculated as described for Leu R_a. First-pass arterial utilization of glucose by the MDV and PDV was calculated as:

First-pass arterial glucose utilization = (([A]×E_A – [V]×E_V) / ([A]×E_A)) × [A] × F

where E is the enrichment (APE) of [$^2H_2$]glucose in the artery (A) and vein (V; MDV or PDV).

Statistical analysis. For all data, ANOVA assumptions were checked prior to analysis. Data were analyzed by three-way ANOVA for a 4×4 Latin square design using the MIXED procedure of SAS (version 8.0, SAS Institute, Cary, NC) in which the infusion level was the fixed effect and sheep and experimental period are random effects. The following linear mixed model was used:

$$Y_{ijk} = \mu + T_i + R_j + C_k + \varepsilon_{ijk}$$

Where Y_{ijk} is the observed value for the k^{th} sheep, the j^{th} period and the i^{th} treatment, μ is the grand mean, T_i is the treatment effect for the i^{th} treatment, R_j is the period effect for the j^{th} period, C_k is the sheep effect for the k^{th} sheep and ε_{ijk} is the random error associated with Y_{ijk}. When a significant treatment effect was detected, means were compared using Tukey-Kramer Multiple Comparison Test. Backward stepwise regression was performed in which a third order model was tried first, and if not significant the analysis was repeated with a lower order model until significance was reached. The 95% confidence intervals were calculated for the slopes of the regression equation. Significance of differences between first pass utilization and net absorption was assessed by Student's t test. Data are presented as least square means ± SEM and differences were considered significant at $P \leq 0.05$.

RESULTS

Sheep. Sheep consumed their feed allowances with no refusals throughout the experiment and over the period of experimentation (55 days) sheep gained ~75 g/d. The MDV measurements for one sheep were omitted due to catheter tip misalignment noted post-mortem, and for another sheep the PDV data were omitted for the last two treatment periods (35 and 105 g casein/d) due to PDV catheter blockage.

Plasma concentrations. Casein infusion significantly increased ($P < 0.05$) arterial concentrations (**Table 3.2**) of the BCAA, methionine, phenylalanine and proline whereas glycine concentration decreased ($P < 0.05$). Plasma urea also increased ($P < 0.001$) with level of casein infusion, with values doubling between the Control and the 105 g/d casein infusion level.

MDV net fluxes. Casein infusion significantly ($P < 0.05$) increased the net appearance in the MDV of all the essential AA (**Table 3.3**), except for tryptophan. Amongst the non-essential AAs, the net appearances of only alanine ($P < 0.05$) and proline ($P < 0.001$) increased with casein infusion level. The incremental efficiency of absorption (Δ net absorption $\div$ Δ casein-AA infusion) was computed based on the slope of regressing net AA absorption against the casein-AA infusion rate for that AA (see **Fig. 3.1**). The 95% confidence intervals were generated to test for unity, i.e. a slope of 1 equating to 100% recovery of the infused AA. Net MDV absorption (**Table 3.4**) of all essential and non-essential AAs, except for glutamate and glutamine, was linearly ($P < 0.05$) related to their casein-AA infusion rates. The incremental efficiency of absorption across the MDV fell within a narrow range (0.87 to 1.05) for all essential AA, excluding tryptophan. For the non-essential AA, values ranged from 0.46 for aspartate to 1.79 for

alanine. Because of the wide confidence intervals associated with each AA measurement, however, there was no statistical basis to suggest anything other than that net absorption across the MDV (small intestines) accounted for 100% of the additional casein-AA infused.

PDV net fluxes. Net PDV absorption (**Table 3.5**) of leucine, isoleucine, methionine, phenylalanine, alanine, proline, and aspartate increased ($P < 0.05$) in a linear relationship with their casein-AA infusion rates. Based on the regression slopes, the incremental efficiency of absorption (**Table 3.6**) across the PDV ranged from 0.60 for valine and isoleucine to 0.93 for phenylalanine, with a high of 1.58 for alanine. For the BCAA, the upper confidence limits were significantly less than 1 ($P < 0.05$), indicating that less than 100% of the additional supplies of these AAs was recovered in blood by the PDV.

For the MDV and PDV, net removal of urea from plasma was not affected by casein infusion, although numerically the values were higher. The correlation of urea removal with plasma urea concentration was significant for the MDV ($P = 0.0379$, $r = 0.90$) and PDV ($P = 0.0124$, $r = 0.93$).

Leucine metabolism. Rate of casein infusion increased ($P < 0.01$) systemic and whole body leucine R_a (**Table 3.7**). As a proportion of whole body leucine R_a, the MDV accounted for 0.16 ($\pm$ 0.059) whereas casein infusion increased the proportion (0.33 to 0.60 $\pm$ 0.060, $P < 0.033$) of whole body leucine R_a partition to the PDV. Although there net absorption of leucine by the PDV increased with casein infusion, there was also increased ($P < 0.05$) first-pass arterial utilization of leucine by the PDV. For the MDV, net absorption of leucine increased with casein infusion, but there was no change in the

rate of first-pass arterial utilization. Fractional first-pass intestinal utilization was computed from PDV, rather than MDV, flux measurements to avoid introduction of errors due to site of luminal tracer infusion and location of the downstream MDV sampling catheter. Fractional first-pass intestinal utilization of leucine was higher (Student's *t* test, $P < 0.05$) than fractional first-pass arterial utilization, but neither was altered by casein infusion.

Glucose metabolism. Plasma glucose concentration increased ($P < 0.05$) with casein infusion rate (**Table 3.8**) whereas plasma glucose R_a remained unchanged. The relationship of glucose R_a and casein infusion rate was linear ($P < 0.05$, $R^2 = 0.76$), with daily glucose R_a increasing by 0.188 g glucose/g casein infused. Intestinal casein infusion did not alter the amount and proportion of whole body glucose R_a partitioned to the PDV. The PDV accounted for ~0.38 of whole body glucose R_a whereas the MDV accounted for a smaller but increasing (0.11 to 0.24) proportion in response to casein infusion. Due to the higher rates of first-pass arterial utilization of plasma glucose, the net balances of glucose across the PDV and MDV were negative (i.e. arterial utilization > net absorption) and these were not significantly affected by casein infusion. First-pass arterial utilization of glucose by the PDV (Student's *t* test, $P < 0.001$) and MDV (Student's *t* test, $P < 0.0011$) both exceeded net glucose removal, indicating that glucose was absorbed from the small intestines only to be removed on second-pass from blood.

DISCUSSION

Despite the numerous studies in the literature, there remains a lack of information in ruminants and other farm species regarding the patterns of AA utilized by the GIT and whether this pattern of use fluctuates or remains unchanged with intestinal

protein supply. In part, the availability of such information has been limited by analytical and technical constraints of existing methodologies. Therefore, to address the questions we posed, the methods we employed for measurement of blood flow and substrate concentrations needed to be sufficiently accurate and sensitive for detection of biologically and statistically significant changes in the flux of AAs, urea and glucose by the GIT. Herein, gravimetric (i.e. weighing) procedures and isotope dilution with GC-MS was employed to quantify plasma AA, urea and glucose concentrations. Based on the isotope dilution technique, the coefficient of determination was 0.56-3.93% for individual AAs, 0.12% for urea and 0.33% for glucose. By contrast, conventional AA analyzers may reach a precision of only 1-4% (Qureshi and Qureshi 1989), and this variance is further propagated when measurements of both arterial and venous concentrations are combined for detection of often small (2-15 μmol/L) arterio-venous differences across tissue beds (19), such as in the present study.

It was also important that our measurements of GIT metabolism were made over a wide range of intestinal protein supplies, spanning from marginal to above estimated MP requirements for maintenance and growth for sheep of this body weight. This was achieved by infusion of casein into the duodenum at four levels (0, 35, 70 and 105 g/d). The highest level of protein supply was predicted to raise total protein supply (diet + casein infusion) to 110% of the MP supply required to support gain of 300 g/d for sheep of this size (AFRC, 1992). Furthermore, to acquire a more representative estimate of the efficiency of AA absorption, net fluxes for each AA were plotted against their respective casein-AA infusion rates (Fig. 3.1 and Tables 3.4 and 3.6). Regression slopes were derived that are analogous to the net efficiency of absorption above the basal level of

protein intake in this study. Confidence intervals (95%) were then computed from the slope relationships and tests against unity (i.e. 100%) were performed for each AA.

There are three observations from the slopes of the regression analysis that we wish to highlight. First, the net absorption data for each essential AA best fit a linear relationship ($P < 0.005$; Fig. 3.1 and Tables 3.5 and 3.6) in response to casein infusion. We had hypothesized that because the level of metabolizable protein intake (~75 g/d) of the basal ration was low relative to growth requirements, net AA requirements of the GIT may not have been met and, in consequence, net absorption would not increased until perhaps the 70 g casein infusion level. Even with the limited observations in this study (see Fig. 3.1), there appears to be no indication of an inflection in net absorption, but this will need to be further confirmed at lower protein intakes and with a larger number of observations.

Second, the 95% confidence intervals of the slopes indicated that essential AA absorption by the MDV (small intestines) was effectively 100% (i.e. slope = 1) whereas absorption of the BCAA by the PDV was significantly less than 100% (i.e. slope < 1). Also approaching significance were the slopes for threonine (slope 0.75, 95% confidence interval 0.36 to 1.10) and lysine (slope 0.78, 95% confidence interval 0.55 to 1.05) for the PDV. We interpret these data as demonstration that the small intestines (MDV) were in net balance with respect to essential AA utilization, irrespective of the rate of intestinal protein supplies above the maintenance requirement. The net efficiency of absorption of the BCAA by the PDV was less than 100% (slope ranges: 0.60-0.66, 95% confidence interval 0.31 to 0.91) across the protein infusion levels with, in consequence, greater net metabolism (e.g. lost as protein into the GIT or catabolized) of the BCAA as intestinal

protein supply increased. In fact, for leucine, as representative of the BCAA, the majority of the increased use by the GIT occurred from the arterial blood supply (Table 3.7), mainly by the non-MDV tissues (i.e. rumen, hind-gut). MacRae et al. (1997a, 1997b) made similar observations in sheep (~35 kg body weight) that were fed alfalfa pellets (15.6% crude protein) at two levels of intake (maintenance *vs*. 1.5× maintenance). In that report, recoveries of the essential AA in the MDV approximated to 100% (mean 106%, range 74-131%) whereas recoveries by the PDV were significantly less (mean 69%, range 45-96%) than the disappearance of the AAs from the small intestines (duodenum to ileum).

Thirdly, for both glutamate and glutamine, net absorption by the MDV and PDV were far less than their intestinal supplies and net absorption were not increased by casein infusion. We find it of interest that the BCAA, glutamate and glutamine were utilized by the whole GIT in greater amounts as the supply of protein to the small intestines increased. The primary fates of AAs utilized by the GIT are catabolism and incorporation into constitutive and secretory proteins (Stoll et al., 1998, Stoll et al., 1999). Except for the threonine-rich mucoproteins (van Klinken et al., 1995), the AA composition of GIT protein is not likely to fluctuate. In this respect, we consider it unlikely that the higher removals of the BCAA, glutamate and glutamine by the GIT relates to their increased incorporation into GIT proteins (and endogenous losses). If the additional removal of these AAs had been directed towards GIT protein synthesis, we should have also observed a corresponding increase in the net removal of other essential AAs, which we did not observe. The more likely explanation is that these AAs were catabolized either by

luminal microbes or by the gut tissues either from the luminal, arterial or both aspects of the GIT.

The BCAA have been shown to be oxidized by the GIT of ruminants (Lobley et al., 2003) and considerable evidence indicates extensive catabolism of glutamate and glutamine by the GIT of ruminants and monogastric species (Caton et al., 2001; Reynolds et al., 1991; Stoll et al., 1999; Bos et al., 2003; van der Schoor et al., 2001; Windemueller and Spaeth, 1980). For leucine and glutamate, their oxidation by the GIT fluctuates in direct relation to intestinal protein supply (dairy cattle: Lapierre et al., 2002; piglets: van der Schoor et al., 2001). To date, it remains unresolved whether catabolism of AA occurs directly by the GIT tissues or by microbes in the intestinal lumen. The present data indicate nearly complete metabolism of glutamate and glutamine by the MDV tissue (small intestines), but it was not possible herein to determine the extent of luminal vs. arterial use. However, based on out leucine kinetic data, most of the increased use of leucine by the GIT occurred from the arterial supply in response to casein infusion. Therefore, we suggest that the GIT tissues, but not luminal microbes, were responsible for metabolism of the BCAA.

Numerous studies in rats (see Windmueller et al., 1980) and piglets (Stoll et al., 1999: see also Wu et al., 1998), under conditions of adequate food intake, have shown that AAs are the major (50-70%) substrates contributing to oxidative energy generation by the intestinal mucosa. By contrast, glucose contributes relatively less (30-40%, Wolf et al., 1972). Similar information is not complete for ruminant animals, and to date investigations in vivo have been limited to glutamate and glutamine (Reeds et al., 1998), leucine (MacRae et al., 1999, Yu et al., 2000) and lysine, methionine and phenylalanine

(Lobley et al., 2003). Given that the GIT of the sheep appears to metabolize greater amounts of the BCAA, glutamate and glutamine as protein supply is increased, it was logical to consider whether metabolism of other nutrients would be reciprocally reduced, namely glucose. The majority of glucose supplied to the ruminant GIT is from the arterial blood, and so it is commonly observed that net balance of glucose across the MDV and PDV is negative. There is, of course, some glucose available to the small intestinal lumen from dietary starch escaping the rumen and from bacterial polysaccharides (Lou et al., 1997). It is apparent from the comparison of unidirectional and net removals of glucose (Table 3.8) that glucose was presented to the small intestinal lumen and absorbed into the MDV and PDV blood drainages (i.e. unidirectional > net removal).

Net removal and first-pass arterial utilization of glucose by the GIT remained constant across the levels of casein infusion, suggesting that glucose use by the PDV was not spared by the additional protein supply. This is perhaps not surprising for two reasons. First, Van der Schoor et al., (2001) observed that glucose oxidation by the PDV of piglets remained the same at two levels of protein intake (9.6 vs. 21.6 g/(kg • d)). And, second, catabolism of BCAA carbon skeletons occurs mainly via acetyl-CoA, and for these AA to spare glucose from complete catabolism necessitates that glucose is also metabolized via this route. In a study conducted with isolated sheep rumen epithelial and small intestinal mucosal cells (Bequette et al., 2004), we observed that glucose contributed to only 1-5% of acetyl- CoA flux, despite the fact that glucose made a significant contribution to pyruvate flux (8-38%). In this connection, it would not be expected that increased metabolism of the BCAA by the GIT would lead to sparing of glucose from complete catabolism. On the other hand, we have shown that sheep rumen

and intestinal cells partially catabolize glucose to pyruvate, and thence to alanine (Bequette et al., 2004), and that net production of alanine by intestinal cells is increased in the presence of glucose (Oba et al., 2005). In this connection, the high and increased net release of alanine by the PDV with casein infusion may reflect greater partition of glucose towards alanine synthesis and release.

In summary, AA and glucose metabolism by the MDV and PDV was examined in growing sheep in response to incremental increases in protein supply to the small intestines that spanned the range of metabolizable protein supplies from marginal to well above requirements for maintenance and growth. The efficiency of absorption above maintenance of all the essential AAs, except the BCAA, was effectively 100% and this remained fixed at levels of protein supply above maintenance. By contrast, the efficiency of absorption the BCAA, glutamate and glutamine were less than 100%, and for glutamate and glutamine high rates of net metabolized by the GIT were maintained even at upper levels of intestinal protein supply. Despite the excess metabolism of these AAs, glucose utilization by the GIT was not altered, and therefore not spared by AA supply. The basis for the greater metabolism the BCAA, glutamate and glutamine with increased intestinal protein supply remains uncertain. In practical terms, the nearly complete catabolism of these non-essential AAs and to some extent aspartate by the GIT necessitates that they be synthesized de novo from carbon skeletons and nitrogen that likely derive from absorbed essential AA.

TABLE 3.1

Ingredients and nutrient composition of the experimental diet[1]

	g/kg diet
Ingredient	
Alfalfa meal 17%	301
Beet pulp, dried	200
Wheat straw	467
Ammonium chloride	5
Vitamin mineral premix[2]	16
Sodium phosphate[3]	11
Nutrient composition	
Dry matter	911
Crude protein	95
Acid detergent fiber	358
Neutral detergent fiber	521
Starch	39
Crude fat	24
Total digestible nutrients	610
Net energy for maintenance, *MJ/kg*	5.44
Net energy for gain, *MJ/kg*	3.23

[1] As-fed.

[2] Shepherd's Pride®, provided per kg premix: calcium, 220 g; salt 160 g; sulfur, 31 g; phosphorus, 30 g; magnesium, 27 g; potassium, 24 g; iron 1,820 mg; zinc 2,700 mg; manganese 240 mg ; iodine 40 mg; cobalt 35 mg ; selenium 24 mg; vitamin A, 682,799 IU; vitamin D, 137,574 IU; vitamin E, 1,774 IU. (Renaissance Nutrition, Inc. Roaring Spring, PA 16673).

[3] XP-4®, sodium acid pyrophosphate and monosodium phosphate anhydrous, provided per kg premix: phosphorus, 260 g; sodium, 193 g. (Astaris LLC, 622 Emerson road, St. Louis, MO 63141).

TABLE 3.2

Plasma arterial concentrations (μmol/kg plasma) of amino acids and urea in sheep infused with increments (0, 35, 70 and 105 g/d) of casein into the duodenum[1]

	Casein infusion *(g/d)*					
	0	35	70	105	SEM	*P*-value
Essential AA						
Valine	204[c]	296[b]	370[b]	450[a]	18.6	0.0001
Leucine	153[c]	209[b]	244[ab]	281[a]	13.0	0.0007
Isoleucine	89[b]	118[a]	134[a]	139[a]	6.4	0.0002
Methionine	24[b]	33[ab]	38[ab]	40[a]	3.6	0.0431
Threonine	95	134	140	109	21.2	NS
Phenylalanine	48[b]	57[b]	61[ab]	72[a]	3.5	0.0044
Lysine	155	202	194	175	18.3	NS
Histidine	56	75	71	69	6.7	NS
Tryptophan	40	55	59	90	19.0	NS
Non-essential AA						
Alanine	169	170	144	131	13.4	NS
Glycine	533[a]	427[b]	361[bc]	313[c]	37.2	0.0003
Proline	84[c]	136[b]	142[b]	192[a]	8.6	0.0001
Serine	55	62	61	54	11.2	NS
Aspartate	16	20	17	17	2.4	NS
Glutamate	120	133	109	118	6.4	NS
Glutamine	275	284	259	277	20.2	NS
Urea, mmol/kg	6.35[c]	8.52[b]	10.36[b]	13.52[a]	0.604	0.0001

[1] Values are least-square treatment means, $n = 16$. [abc] Means with different superscripts within a row are significantly different from one another ($P < 0.05$). Abbreviations: SEM, pooled standard error of the means; NS, not significant ($P > 0.05$).

TABLE 3.3

Plasma flow and net absorption of amino acids across the mesenteric-drained viscera of sheep infused with increments (0, 35, 70 and 105 g/d) of casein into the duodenum.[1]

	Casein infusion (g/d)					
	0	35	70	105	SEM	*P*-value
Plasma flow, kg/(kg BW • h)	1.082	1.116	1.373	1.567	0.2330	NS
Net absorption, µmol/(kg BW • h)						
Essential AA						
Valine	43^{b}	45^{b}	95^{a}	87^{a}	11.4	0.0044
Leucine	51^{b}	61^{b}	129^{a}	116^{a}	10.5	0.0008
Isoleucine	35^{b}	39^{b}	74^{a}	70^{a}	7.0	0.0031
Methionine	13^{c}	15^{bc}	30^{ab}	32^{a}	3.3	0.0078
Threonine	35^{b}	42^{ab}	70^{a}	67^{ab}	9.0	0.0249
Phenylalanine	30^{b}	33^{b}	64^{a}	61^{a}	6.4	0.0051
Lysine	48^{b}	52^{b}	101^{a}	97^{a}	10.1	0.0043
Histidine	14^{b}	14^{b}	23^{ab}	34^{a}	5.8	0.0158
Tryptophan	7	2	25	33	6.9	NS
Non-essential AA						
Alanine	69^{b}	77^{ab}	129^{a}	125^{ab}	12.8	0.0220
Glycine	61	66	88	83	11.1	NS
Proline	31^{b}	46^{b}	102^{a}	105^{a}	7.7	0.0005
Serine	55	55	95	94	13.9	NS
Aspartate	3	9	11	14	3.4	NS
Glutamate	20	24	40	38	9.7	NS
Glutamine	-3	-4	45	25	24.1	NS
Urea	-175	-204	-373	-361	68.7	NS

[1] Values are least-square treatment means, $n = 14$. Positive values denote net release (absorption into blood) and negative values denote net removal from blood. [abc] Means with different superscripts within a row are significantly different from one another ($P < 0.05$). Abbreviations: SEM, pooled standard error of the means; NS, not significant ($P > 0.05$).

TABLE 3.4

Plasma flow and net absorption of amino acids across the portal drained viscera of sheep infused with increments (0, 35, 70 and 105 g/d) of casein into the duodenum[1]

	Casein infusion (g/d)					
	0	35	70	105	SEM	*P*-value
Plasma flow, kg/(kg BW • h)	2.795	2.515	2.488	3.055	0.3065	NS
Net absorption, µmol/(kg BW • h)						
Essential AA						
Valine	32	39	58	69	9.7	NS
Leucine	46^{c}	60^{bc}	86^{ab}	95^{a}	7.8	0.0043
Isoleucine	27^{c}	35^{bc}	49^{ab}	56^{a}	4.4	0.0035
Methionine	11^{c}	15^{bc}	27^{ab}	30^{a}	3.1	0.0043
Threonine	25	32	43	56	7.5	NS
Phenylalanine	31^{b}	38^{b}	51^{ab}	62^{a}	5.3	0.0113
Lysine	41	52	72	85	9.7	NS
Histidine	14	14	19	29	3.5	NS
Tryptophan	7	1	9	11	4.2	NS
Non-essential AA						
Alanine	61^{c}	80^{bc}	101^{ab}	119^{a}	7.8	0.0020
Glycine	39	56	47	75	9.7	NS
Proline	24^{c}	42^{bc}	77^{ab}	93^{a}	9.1	0.0010
Serine	61	60	87	81	22.9	NS
Aspartate	-2^{b}	4^{ab}	9^{ab}	22^{a}	7.1	0.0380
Glutamate	6	21	34	23	12.5	NS
Glutamine	-6	10	73	13	44.1	NS
Urea	-587	-625	-671	-1072	124.7	NS

[1] Values are least-square treatment means, $n = 14$. Positive values denote net release (absorption into blood) and negative values denote net removal from blood. [abc] Means with different superscripts within a row are significantly different from one another ($P < 0.05$). Abbreviations: SEM, pooled standard error of the means; NS, Not significant ($P > 0.05$).

TABLE 3.5

Linear mixed-effect model predictions of the intercept, slope and 95% confidence intervals describing the relationship between net mesenteric-drained viscera absorption (µmol/(kg BW • h) of amino acids and the rate of casein-amino acid infusion (0, 35, 70 and 105 g/d) into the duodenum of sheep.[1]

	Intercept (a)	SE_a	Slope (b)	SE_b	*P*-value[2]	R^2	95% CI for (b)
Essential AA							
Valine	39	10.7	0.87	0.223	0.0045	0.69	(0.55, 1.21)
Leucine	50	11.4	1.01	0.233	0.0014	0.65	(0.73, 1.28)
Isoleucine	33	6.3	0.88	0.191	0.0017	0.71	(0.43, 1.30)
Methionine	12	2.7	1.00	0.198	0.0005	0.72	(-0.02, 1.94)
Threonine	36	8.1	0.89	0.239	0.0073	0.73	(0.36, 1.44)
Phenylalanine	29	6.1	1.05	0.267	0.0041	0.65	(0.41, 1.66)
Lysine	45	9.5	0.99	0.236	0.0029	0.68	(0.62, 1.35)
Histidine	11	5.6	1.00	0.231	0.0024	0.81	(-0.10, 2.01)
Tryptophan	1	5.7	4.74	1.352	0.0056	0.55	(1.39, 7.84)
Non-essential AA							
Alanine	66	10.7	1.79	0.449	0.0025	0.56	(0.91, 2.63)
Glycine	62	10.2	0.96	0.391	0.0388	0.26	(-0.26, 2.23)
Proline	29	8.1	0.77	0.121	0.0001	0.78	(0.46, 1.07)
Serine	51	12.1	1.06	0.397	0.0280	0.42	(0.30, 1.80)
Aspartate	4	3.2	0.46	0.098	0.0051	0.29	(-0.94, 1.86)
Glutamate	20	7.5	0.23	0.131	NS	0.21	(-0.14, 0.58)
Glutamine	-2	19.8	0.47	0.418	NS	0.18	(0.02, 0.86)

[1] Values were derived from the model: $\hat{y} = a + bx$, where x is the casein-amino acid infusion rate in µmol/(kg BW • h), a the intercept and b the slope of the regression (n = 12). Abbreviations: SE_a, standard error of the intercept (a) estimate; SE_b, standard error of the slope (b) estimate; NS, Not significant (P > 0.05).

[2] Probability that the slope estimate = 0.

TABLE 3.6

Linear mixed-effect model predictions of the intercept, slope and 95% confidence intervals describing the relationship between net portal-drained viscera absorption (μmol/(kg BW • h) of amino acids and the rate of casein-amino acid infusion (0, 35, 70 and 105 g/d) into the duodenum of sheep.[1]

	Intercept (a)	SE_a	Slope (b)	SE_b	P-value[2]	R^2	95% CI for (b)
Essential AA							
Valine	30	7.4	0.60	0.159	0.0044	0.66	(0.37, 0.82)
Leucine	46	5.7	0.66	0.126	0.0002	0.69	(0.48, 0.86)
Isoleucine	27	3.6	0.60	0.109	0.0004	0.76	(0.31, 0.91)
Methionine	11	2.5	0.91	0.197	0.0010	0.66	(0.29, 1.64)
Threonine	24	6.1	0.75	0.186	0.0029	0.71	(0.36, 1.10)
Phenylalanine	29	4.0	0.93	0.175	0.0004	0.77	(0.55, 1.40)
Lysine	39	6.7	0.78	0.188	0.0021	0.62	(0.55, 1.05)
Histidine	12	2.7	0.67	0.225	0.0109	0.43	(0.02, 1.46)
Tryptophan	4	3.8	0.77	0.701	NS	0.56	(-1.26, 3.16)
Non-essential AA							
Alanine	60	5.2	1.58	0.223	<0.0001	0.82	(0.26, 2.94)
Glycine	39	7.7	1.20	0.484	0.0347	0.45	(-0.72, 3.17)
Proline	23	6.8	0.67	0.108	<0.0001	0.76	(0.20, 1.15)
Serine	58	18.4	0.61	0.532	NS	0.55	(-0.51, 1.82)
Aspartate	-3	7.2	0.95	0.161	0.0011	0.95	(-1.10, 3.27)
Glutamate	10	9.2	0.24	0.172	NS	0.14	(-0.31, 0.80)
Glutamine	-1	35.8	0.58	0.648	NS	0.43	(-0.17, 1.14)

[1] Values were derived from the model: $\hat{y} = a + bx$, where x is the casein-amino acid infusion rate in μmol/(kg BW • h), a the intercept and b the slope of the regression (n = 12). Abbreviations: SE_a, standard error of the intercept (a) estimate; SE_b, standard error of the slope (b) estimate; NS, not significant ($P > 0.05$).

[2] Probability that the slope estimate = 0.

TABLE 3.7

Whole body and gastrointestinal tract fluxes of leucine in sheep infused with increments of casein (0, 35, 70 and 105 g/d) into the duodenum[1]

	Casein infusion *(g/d)*					
	0	35	70	105	SEM	*P*-value
Leucine R_a						
Systemic R_a (A)	163[c]	187[bc]	216[ab]	244[a]	14.9	0.0075
Whole body R_a (B)	236[b]	251[b]	286[ab]	318[a]	19.6	0.0111
First-pass splanchnic Utilization (B – A)	73	64	64	79	11.5	NS
Fractional first-pass splanchnic utilization ((B – A)/B)	0.30	0.26	0.25	0.24	0.029	NS[3]
Portal-drained viscera metabolism						
Net absorption	46[c]	60[bc]	86[ab]	95[a]	7.8	0.0043
First-pass arterial utilization	53[b]	62[b]	70[b]	146[a]	7.7	0.0001
Proportion of whole body Ra	0.33[b]	0.34[b]	0.33[ab]	0.60[a]	0.060	0.033
Fractional first-pass arterial utilization	0.13	0.12	0.11	0.16	0.023	NS
Fractional first-pass intestinal utilization	0.32	0.32	0.20	0.27	0.060	NS
Mesenteric-drained viscera metabolism						
Net absorption	51[b]	61[b]	129[a]	116[a]	10.5	0.0008
First-pass arterial utilization	24	30	36	36	11.1	NS
Proportion of whole body R_a	0.15	0.17	0.17	0.15	0.059	NS
Fractional first-pass arterial utilization	0.16	0.13	0.10	0.10	0.028	NS

[1] Values are in units of μmol/(kg BW • h) unless otherwise indicated. Values are least-square treatment means for whole body (n =16), mesenteric-drained viscera (n =12) and portal drained viscera (n =14) fluxes. Positive values denote net release (absorption into blood) and negative values denote net removal from blood. [a-c] Means within a row not sharing a common superscript differ significantly ($P < 0.05$). Abbreviations: R_a, rate of appearance; SEM, pooled standard error of the mean; NS, not significant ($P > 0.05$).

TABLE 3.8

Whole body and gastrointestinal tract metabolism of glucose in sheep infused with increments of casein (0, 35, 70 and 105 g/d) into the duodenum.[1]

	Casein infusion (g/d)					
	0	35	70	105	SEM	P-value
Arterial concentration, (μmol/kg)	4.249[b]	4.283[b]	4.540[a]	4.567[a]	0.1086	0.0103
Plasma R_a	690	730	783	814	49.5	NS
Portal-drained viscera metabolism						
Net absorption	-110	-111	-128	-216	49.8	NS
First-pass arterial utilization	255	247	302	322	76.0	NS
Proportion of plasma R_a	0.37	0.35	0.41	0.38	0.101	NS
Fractional arterial utilization	0.02	0.02	0.03	0.02	0.007	NS
Mesenteric-drained viscera metabolism						
Net absorption	-25	-66	-37	-70	23.303	NS
First-pass arterial utilization	83	143	187	119	31.495	NS
Proportion of plasma R_a	0.11[b]	0.21[ab]	0.24[a]	0.14[ab]	0.041	0.022
Fractional arterial utilization	0.02	0.03	0.03	0.02	0.005	NS

[1] Values are in units of μmol/(kg BW • h) unless otherwise indicated. Values are least-square treatment means for whole body (n =16), mesenteric-drained viscera (n =12) and portal drained viscera (n =14) fluxes. Positive values denote net release (absorption into blood) and negative values denote net removal from blood. [ab] Means within a row not sharing a common superscript differ significantly ($P < 0.05$). Abbreviations: R_a, rate of appearance; SEM, pooled standard error of the mean; NS, not significant ($P > 0.05$).

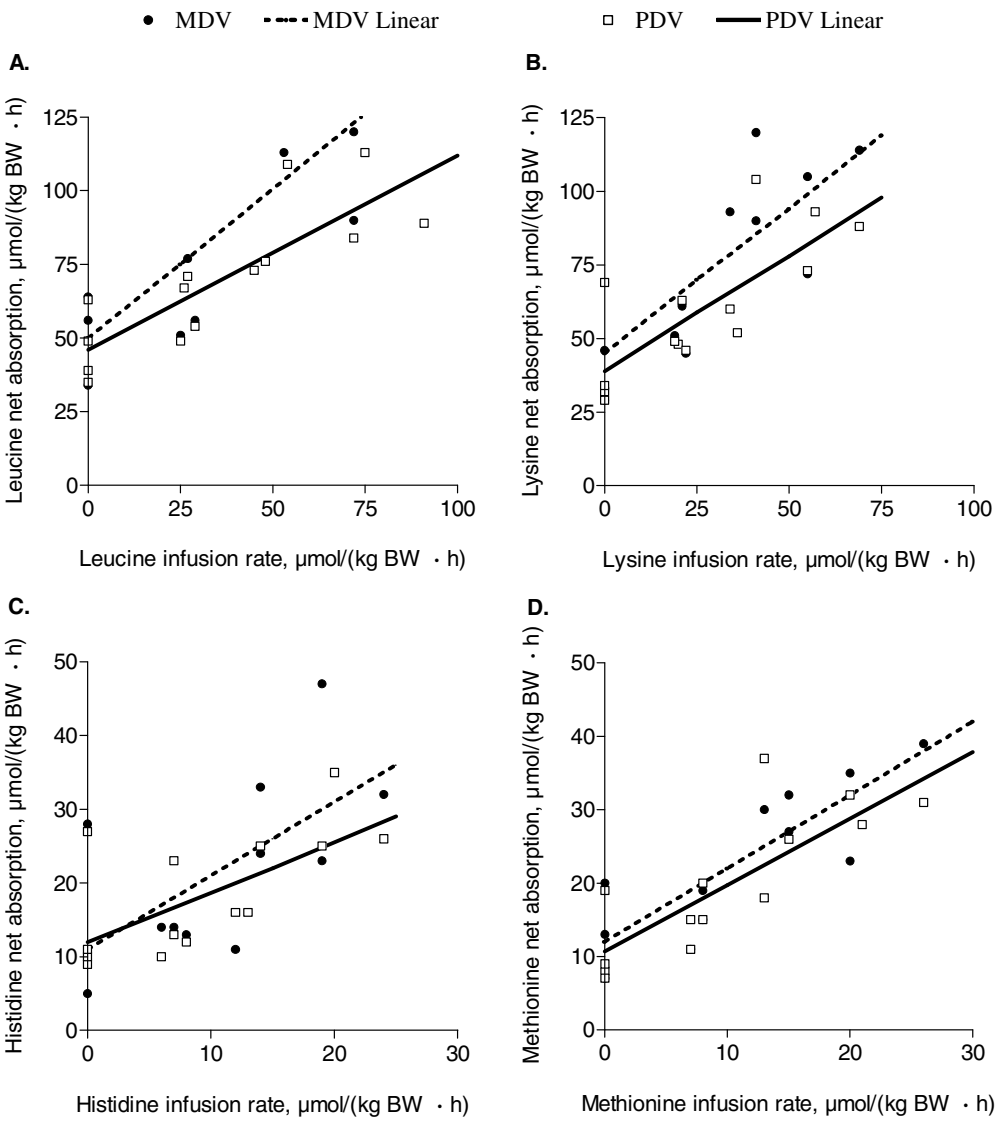

Figure 3.1. Plots of mesenteric (MDV; n = 12) and portal (PDV; n = 14) net absorption rates of leucine (A), lysine (B), histidine (C) and methionine (D) against their rates of infusion as casein into the duodenum of sheep. (see Tables 3.5 and 3.6 for linear regression analysis).

CHAPTER 4

METABOLIC PROFILING OF SUBSTRATE METABOLISM IN BEEF RUMEN EPITHELIAL AND DUODENAL MUCOSAL CELLS

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ABSTRACT

The aim of this study was to determine the flux and contributions to overall Krebs cycle metabolism of primary substrates normally available to the rumen and small intestinal tissues. An additional aim was to determine whether the type of diet fed to steers altered the use and selection of these substrates for metabolism. To meet these aims, rumen epithelial (REC) and duodenal mucosal cells (DMC) were isolated from two groups of Angus bulls (5 per group, 391 ± 34 kg) that were fed either a high forage (75% Orchard grass silage) or a high concentrate (75% concentrate mix) diet for 4 weeks prior to slaughter at which time cells were isolated for incubations. Cell incubations were conducted with media containing all amino acids (AAs), glucose, and the three primary short chain fatty acids (acetate, propionate and butyrate; SCFAs) produced in the rumen. [1-^{13}C] or [U-^{13}C] tracer forms of glucose, acetate, propionate, butyrate, glutamate, glutamine, valine and leucine were individually added to triplicate incubations to determine the extent and routes of their metabolism in the Krebs cycle of REC and DMC. Our results indicated that glucose contributed 25% of lactate flux in REC from bulls fed high concentrate and in DMC from bulls fed both diets. By contrast, in REC form bulls fed the high forage diet glucose contributed only 12% of lactate flux. Furthermore, the flux of leucine and valine to ketoisocaproic and ketoisovaleric acid increased with leucine (17-63%) and valine (19-82%) supplies, but neither entered Krebs cycle intermediates. Glutamate was the largest contributor to α-ketoglutarate flux and this contribution increased (9-41%) with glutamate supply. By contrast α-ketoglutarate flux from glutamine did not exceed 3%. These data suggest that the partial catabolism of glucose to lactate and

possibly alanine may play a role in preserving 3-carbon units from glucose for gluconeogenesis. Furthermore, increasing the supply of glutamate to REC and DMC increase the flux of Krebs cycle intermediates from glutamate, thereby reducing the entry of other substrates entering at or beyond α-ketoglutarate. Glutamine catabolism by GIT cells has been previously demonstrated, but in the current study glutamine entry to the Krebs cycle was limited. Therefore, glutamine catabolic pathway(s) merit further investigation

Key words: gastrointestinal, amino acids, glucose, short chain fatty acids

INTRODUCTION

In ruminants, the gastrointestinal tract (GIT) represents less than 10% of empty body weight (McLeod and Baldwin, 2000) yet its metabolic activity accounts for 25 to 35% of whole body protein synthesis (Lobley et al., 1994) and 30% of whole body oxygen consumption (Burrin et al., 1989). Furthermore, studies have shown that, across all species, metabolism by the GIT is the single largest fate of AAs, glucose and several short chain fatty acids (SCFA). Evidence in the rat and pig suggests that, in addition to AA metabolism for protein synthesis, the GIT also catabolizes essential (EAAs) and non essential (NEAAs) AAs for energy production (Windmueller and Spaeth, 1974, 1978, 1980; Stoll et al., 1998; Stoll et al., 1999; van Goudoever et al., 2000; van der Schoor et al., 2001). In this respect, the catabolism of AAs and glucose by the GIT has been shown to be highly correlate with their availability in the small intestines and in the blood supply (Stoll et al., 1998; Stoll et al., 1999; van Goudoever et al., 2000; van der Schoor et al., 2001). In ruminants however, studies that describe the extent that AAs are catabolized for energy production are scarce.

Studies measuring EAA oxidation by the ruminant GIT have mostly been carried out using leucine as a tracer (MacRae et al., 1997; Cappelli et al., 1997; Yu et al., 2000). Diet alterations have been reported to affect EAA oxidation in dairy cows where increasing the dietary supply of metabolizable protein led to an increase in leucine oxidation from 16 to 22% of small intestinal utilization (Lapierre et al., 2002). In this connection, it remains unclear whether this oxidation represents an obligate

requirement or whether oxidation can be reduced by providing alternative energy substrates.

Glutamine and glutamate have been shown to contribute to energy production in isolated rumen epithelial (REC) and duodenal mucosal (DMC) cells (Harmon et al., 1986; Okine et al., 1995; Oba et al., 2004). In these studies, REC and DMC incubated in the presence of glucose produced less CO_2 from glutamine, however the presence of glucose did not affect CO_2 production from glutamate (Oba et al., 2004). This metabolic flexibility was also observed for short chain fatty acids (SCFA), which decreased glutamine and glucose catabolism in REC and DMC when added to the media (Harmon, 1986; Oba et al., 2004). Combined, these observations seem to suggest that glutamate catabolism is obligate whereas catabolism of glutamine may not be obligate and that its catabolism may depend upon the availability of other complimentary substrates. The results also suggest that glucose and SCFAs can replace the need for certain AAs to be catabolized, and this raises the question of why this replacement phenomenon is not common to all AAs.

Glucose removal by the GIT represents a significant (0.30-0.35) proportion of whole body utilization (Balcells et al., 1995; Cappelli et al., 1997). In some studies glucose infusion increased AA net absorption across the PDV (Huntington and Reynolds, 1986). Similarly, SCFAs are also metabolized by GIT tissues and propionate infusion into the rumen of beef cattle led to an increase in the net absorption of AA across the GIT (Seal and Parker, 1994; 1996). With both glucose and propionate, the net flux data seem to suggest that either these substrates replaced the need to metabolize AA or that other metabolic signals (e.g. insulin) initiated by

glucose and propionate had regulated (reduced) AA catabolism. If the former is correct, then the mechanism(s) by which glucose and SCFAs reduce AA catabolism presumably occurs because these substrates are metabolized via the Krebs cycle at pyruvate, acetyl-CoA and succinyl-CoA, points of entry that nearly all AAs must eventually flow through for their catabolism.

Much remains to be elucidated about the regulation of substrate catabolism by the GIT of ruminants. Our hypothesis was that REC and DMC possesses metabolic flexibility in substrate selection for catabolism by the Krebs cycle and that this metabolism is dependent upon diet conditions and the mix of substrates availability to the tissues. The objectives of this study were to determine the routes and extents of metabolism of acetate, butyrate, propionate, glucose, glutamate, glutamine, leucine and valine by REC and DMC in vitro. Metabolic measurements were made by incubating isolated GIT cells in media containing [1-^{13}C] or [U-^{13}C] tracer forms of the latter substrates and by use of mass spectrometry and ^{13}C-isotopomer distribution in the Krebs cycle and keto-acid intermediates, their routes of catabolism were determined.

MATERIALS AND METHODS

Animals and diets. The experimental protocol was approved by the Beltsville Area Animal Care and Use Committee at the Beltsville Agricultural Research Center and the Institutional Animal Care and Use Committee at the University of Maryland. Twelve Angus bulls were housed in individual stalls and adapted to their environment and fed orchard grass silage for 1 wk prior to the initiation of the experiment.

Animals were divided into two groups based on body weight so that the average weight per group was 391 ± 34 kg. Two dietary treatments were randomly assigned to the groups in a completely randomized design. The two diets (**Table 4.1**), comprised of high forage (HF; 75% orchard grass silage and 25% concentrate) or high concentrate (HC; 25% orchard grass silage and 75% concentrate), were fed to meet the requirements (NRC, 2000) for dry matter intake and energy to support 0.5 kg body weight gain per d.

Fresh water was available ad libitum using automatic waterers and feed was offered daily in the morning. Feed refusals were weighed. The bulls were weighed on weekly basis throughout the duration of the experiment, and feed intake adjusted accordingly.

Isolation of REC and DMC. Bulls were stunned with a captive-bolt gun and immediately exsanguinated. The gastrointestinal tract was removed from the abdominal cavity within 15 min. A 15×30 cm piece of rumen wall was taken from the cranial sac. The piece was rinsed with warm tap water to remove feed particle and debris followed by warm isotonic buffer[2] (KRB-HEPES). The epithelial layers were stripped off the musculature, cut into 2×5 cm strips and transported to the lab in isolated containers containing trypsin/$CaCl_2$ (5%; 0.016%) in warm KRB-HEPES.

Rumen tissues were allowed to digest in a forced-air orbital shaker (Model 3527 LabLine Instruments, Melrose Park, IL) at 37°C for 30 min. Following the first digestion cycle, epithelial tissues were filtered through a 1000-µm polypropylene mesh (Spectra/Mesh, Spectrum Laboratory Products, Los Angeles, CA) and the

[2] Krebs-Ringer salts, plus 25 mM HEPES, pH 7.4, 37°C, freshly aerated with with O_2:CO_2 (95:5%).

filtrate was discarded. The tissues were redigested in fresh trypsin/$CaCl_2$ (5%; 0.016%) in KRB-HEPES for two more cycles. After each subsequent digestion the solution was sequentially filtered through a 1000-µm and a 300-µm polypropylene meshes. The filtrates, recovered from the second and third cycles, were centrifuged (Centra-MP4R, International Equipment Company, Neeham Heights, MA) at $60 \times g$ for 6 min. The supernatant containing the enzyme and cell debris was discarded and the cell pellet resuspended in warm KRB-HEPES that contains no enzyme for two wash cycles after which the cell pellets were combined and suspended in a minimal volume of buffer to allow for cell concentration adjustments. Cell yield and viability were assessed using trypan blue dye exclusion method (Baldwin and McLeod, 2000).

Duodenal mucosal cells were collected from a segment taken 1 to 2 m distal to the pyloric sphincter. The duodenal segment was emptied of digesta, rinsed with warm tap water followed by rinsing with KRB-HEPES and cut longitudinally. Mucosa was scraped off the underlining musculature using a glass microscope slide and minced to 2×5 mm pieces. Scraped duodenal mucosa were transported to the lab in isolated containers containing collagenase/dispase I/$CaCl_2$ (90CDU[3]/ml; 0.6U[4]/ml; 0.14mg/ml) dissolved in warm KRB-HEPES. Cell isolation was done in a fashion similar to rumen epithelia with some modifications; mucosa was digested for 45min in a forced-air orbital shaker at 37°C followed by a sequential filtration through a 1000-µm and a 300-µm polypropylene mesh. Subsequent centrifugation, washing

[3] CDU: collagenase digestion unit. One collagen digestion unit liberates peptides from collagen equivalent in ninhydrin color to 1.0 µmole of leucine in 5 hr at pH 7.4 at 37 °C in the presence of calcium ions.

[4] One unit is the enzyme activity which liberates under assay conditions (37° C, casein as substrate, pH 7.5) within 1 min Folin-positive amino acids and peptides corresponding to 1 µmol tyrosine.

with KRB-HEPES to remove enzymes and determination of cell yield and viability was done as described for REC.

Incubations. Incubations were performed in 25-ml Erlenmeyer flasks containing an amino acid basal mixture (Table 4.2) in addition to either [$^{13}C_6$]glucose, [$^{13}C_2$]acetate, [$^{13}C_3$]propionate, [1-$^{13}C_1$]butyrate, [$^{13}C_6$]leucine, [$^{13}C_5$]valine, [$^{13}C_5$]glutamate or [$^{13}C_5$]glutamine plus KRB-HEPES and water. Labeled substrates were added at a final concentration of 0.25, 0.5, 1 or 2.5 times the basal concentration (Table 4.3) except for the volatile fatty acids in the rumen cell incubation media, where the concentration reflected those found in rumen fluid. All incubations were run in triplicates.

Incubation flasks were gassed for 20 s with O_2:CO_2 (95:5%) and incubation initiated by the addition of 0.5ml cell suspension to the incubation flask. After the addition of cells to the media, flasks were sealed with a rubber serum cap, and placed in a reciprocal water bath (Precision Model 50, Jouan, Cedex, France) set at 37°C for 90min. Incubations were terminated by the addition of 0.2 mL perchloric acid (70% w/w) followed by neutralization with 0.4 ml potassium carbonate (5.8 mol/ L). Flask contents were transferred to borosilicate tubes and centrifuged at 2,500 × *g* for 10 min, and the supernatant decanted to clean tubes and stored at -20^{o}C for analysis.

Metabolite analysis Isotopic enrichment of Krebs cycle intermediates were analyzed (Des Rosiers et al., 1995) by gas chromatography-mass spectrometry (GC-MS) followed by isotopomer distribution analysis (Fernandez et al., 1995). To the

thawed samples was added sulfosalicylic acid to a final concentration of 10% (w/w) to precipitate proteins, mixed by vortexing and centrifuged at 4000 rpm for 5 min. The supernatant was decanted to clean screw-cap tubes, 5 mmol of hydroxylamine-hydrochloride added and the pH adjusted to 7-8 with 4 mol/L potassium hydroxide. The samples were sonicated for 15 min with heat and then allowed to react at 65^{o}C for 1 h on a heating block. Hydrochloric acid (6 mol/L) was used to bring the pH down to <2 and the solution saturated with NaCl and vortexed for 1 min to precipitate sulfosalicylic acid. Samples were then extracted twice with 4 ml ethyl acetate for 15 min each time using a multi-tube vortexer. Samples were then centrifuged for 5 min at 2,500 rpm and the organic phase collected into V-vials. Ethyl acetate was dried under a stream of N_2 gas at 40^{o}C and 30 µL of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) + 1% *tert*-butyldimethylsilylchlorosilane (DMCS) plus ethyl acetate (50:50) added, capped and allowed to react for 1 h at 65°C on a heating block.

Krebs cycle intermediates were separated by gas chromatography (HP 6890; Agilent, Palo Alto, CA) using a fused silica capillary column (HP-5; 30 m × 0.25 mm i.d., 1µm Hewlett-Packard) with helium as carrier gas (1.0 mL/ min). Chromatography was carried out using a temperature gradient (100 ^{o}C for 2 min; 30 ^{o}C to 160 ^{o}C; 10^{o}C/ min to 250^{o}C/ min). The GCMS (HP 5973N Mass Selective Detector, Agilent, Palo Alto, CA) was operated in the electron ionization mode, and selected ion monitoring was performed on the $[M-57]^+$ fragments of the tert-butyldimethylsilyl TBDMS derivatives from [M] to [M+x], where x is the number of C atoms in the analyte. Ions (mass-to-charge) monitored were for lactate 261-264,

pyruvate 274-277, β-hydroxybutyrate 275-278, ketoisovaleric and ketoisocaproic acid 316-322, succinate 289-293, malate 419-423, oxaloacetate 432-436 and α–ketoglutarate 446-451.

Mass isotopomer distributions were corrected for the difference between theoretical and measured values using the correlation matrix approach (Fernandez et al., 1995). Enrichments (MPE) were expressed as tracer-tracee ratios (TTR, % moles tracer/100 moles tracee). The molar fraction (MF) of ^{13}C-labeled isotopomers was calculated from enrichments as:

$$MF = [M+ i] / ([M]+ \sum_{n}^{1}[M + n]$$

where [M+i] is the enrichment of the ^{13}C-labeled isotopomer, [M] is the enrichment of the unlabeled isotopomer and $\sum_{n}^{1}[M + n]$ is the sum of all ^{13}C-labeled isotopomers from 1 to n carbons.

Precursor product relationships (FC; fractional contribution) were calculated (Des Rosiers et al., 1994; Khairallah et al., 2004) using the general equation:

$$FC_{precursor \rightarrow product} = MF_{product} / MF_{precursor}$$

The FC of glucose to pyruvate and lactate was calculated using the [M+3] enrichment in lactate or pyruvate incubated with [$^{13}C_6$]glucose:

$$FC_{glucose \rightarrow pyruvate} = [M+3]pyruvate / [M+6]glucose$$

$$FC_{glucose \rightarrow lactate} = [M+3]lactate / [M+6]glucose$$

When glutamate or glutamine are the only source of 13C then their contribution to the Krebs cycle could be measured from the [M+5]α-ketoglutarate and [M+4]succinate malate and oxaloacetate due to the loss of one carbon at α-

ketoglutarate dehydrogenase. The FC of glutamate and glutamine to Krebs cycle intermediates was calculated as:

$$FC_{glutam(x)\rightarrow\alpha\text{-ketoglutarate}} = [M+5]\alpha\text{-ketoglutarate} / [M+5]glutam(x)$$

$$FC_{glutam(x)\rightarrow succinate} = [M+4]succinate / [M+5]glutam(x)$$

$$FC_{glutam(x)\rightarrow malate} = [M+4]malate / [M+5]glutam(x)$$

$$FC_{glutam(x)\rightarrow oxaloacetate} = [M+4]oxaloacetate / [M+5]glutam(x)$$

where glutam(x) represents either glutamate or glutamine.

The FC of leucine and valine to KIC and KIV was calculated as:

$$FC_{leucine\rightarrow KIC} = [M+6]KIC / [M+6]leucine$$

$$FC_{valine\rightarrow KIC} = [M+5]KIV / [M+5]valine$$

Statistical analysis. For all data, ANOVA assumptions were checked prior to analysis. Data were analyzed by ANOVA for a 3-way factorial design using the MIXED procedure of SAS (version 8.0, SAS Institute, Cary, NC). The following linear mixed model was used:

$$Y_{ijk} = \mu + T_i + S_j + L_k + TS_{ij} + TL_{ik} + SL_{ik} + TSL_{ijk} + \varepsilon_{ijk}$$

Where Yijk is the observed value for the i^{th} dietary treatment j^{th} substrate and k^{th} substrate level, μ is the grand mean, T_i is the treatment effect for the i^{th} treatment, S_j is the substrate effect for the j^{th} substrate, TS_{ij} TL_{ik} SL_{ik} and TSL_{ijk} are the interaction effects and ε_{ijk} is the random error associated with Y_{ijk}. When a significant effect was detected, means were compared using Tukey-Kramer Multiple Comparison Test. Data are presented as least square means ± SEM and differences were considered significant at $P \leq 0.05$.

RESULTS

The isotopomer distributions of metabolic intermediates at physiologic levels were statistically analyzed (Figure **4.1**, **4.2**, **4.3**, **4.4**, and **4.5**). When the enrichment was significantly different from zero, a further statistical analysis was conducted to test for concentration effect and for $FC_{precursor \rightarrow product}$.

Substrate contributions to pyruvate and lactate flux. There was a significant ($P<0.05$) tissue × level interaction for the fractional contribution of glucose to pyruvate flux ($FC_{glucose \rightarrow pyruvate}$). The $FC_{glucose \rightarrow pyruvate}$, averaged across diets and glucose concentrations, was 1.3% for REC and 4.1% for DMC, and for both tissues this contribution increased with glucose concentration ($P<0.05$).

There was a diet × tissue interaction ($P<0.05$) for the contribution of glucose to lactate flux ($FC_{glucose \rightarrow lactate}$). For REC from bulls fed the high concentrate diet and for DMC from bulls fed both diets, glucose contributed 25% to lactate flux compared to 12% in REC form bulls fed the high forage diet.

Metabolism of Valine and Leucine. For REC and DMC, leucine was deaminated to ketoisocaproic acid (KIC), and KIC enrichment (M+6/M+0) had a significant ($P<0.05$) diet × tissue interaction (**Table 4.4**). The enrichment of KIC from REC incubations was significantly higher than for DMC from bulls fed the high concentrate diet. Leucine contributed to 18, 28, 43, and 62% of KIC flux at 0.06, 0.12, 0.24, and 0.60 mmol/L [$^{13}C_6$]leucine, respectively. For both diets, the contribution of [$^{13}C_5$]valine to ketoisovaleric acid flux (KIV) was higher for REC (58%) than for DMC (40%) (**Table 4.5**). The average contribution of [$^{13}C_5$]valine to

KIV in REC and DMC incubations increased from 25 to 73% at the highest [$^{13}C_5$]valine concentration ($P<0.05$).

Substrate contributions to Krebs cycle intermediate fluxes. Catabolism of glutamate to α-ketoglutarate increased with [$^{13}C_5$]glutamate concentration for both cell types and the contribution of glutamate to α-ketoglutarate flux was higher ($P<0.05$) for REC (31%) than for DMC (12%). For REC, glutamate accounted for a higher proportion (37% vs. 25%, $P < 0.05$) of α-ketoglutarate flux when bulls were fed the high concentrate diet. By contrast, there was no effect of diet on glutamate deamination by DMC (12%). For both cell types the proportion of α-ketoglutarate flux derived from glutamine catabolism increased (0.1 to 2.7) with [$^{13}C_5$]glutamine concentration in incubation media and there were no differences between cell types or diets (**Table 4.7**).

The contribution (**Table 4.8**) of α-ketoglutarate to succinate (5 and 10%) and to malate (9 and 34%) flux was lower for REC than for DMC ($P<0.05$). Because the enrichment in succinate was unexpectedly lower compared to malate (**Figure 4.5**), it was not possible to calculate the fractional contribution of succinate to malate. Instead the fractional contribution of α-ketoglutarate to malate was calculated. The contribution of α-ketoglutarate to malate flux had a significant diet × tissue interaction ($P<0.05$). Also, as the concentration of [$^{13}C_5$]glutamate increased, the contribution of α-ketoglutarate to malate flux decline (26 to 17%). Diet and glutamate level had no significant effect on the oxaloacetate formed form malate, however there was a significant difference between REC and DMC in which 31 and 64% of oxaloacetate formed from malate ($P<0.05$).

DISCUSSION

Although the current knowledge suggests that the high metabolic demand for AA catabolism by the GIT of ruminants occurs in part towards energy production (MacRae et al., 1997; Cappelli et al., 1997; Yu et al., 2000; Lapierre et al., 2002; Lobley et al., 2003), the response of AAs, glucose and SCFA metabolic pathways are not fully understood. It has been shown that diet and nutrient supply could influence AA use for energy production (Harmon et al., 1986; Oba et al., 2005). In the current study, intermediary metabolic pathways of macronutrient use were detailed in REC and DMC by use of specific ^{13}C-labelled substrates normally available to these tissues in vivo. We set out to test whether REC and DMC possessed metabolic flexibility for substrate catabolism. We had hypothesized that flexibility to metabolism certain substrates and thus the ability of other substrates to spare them from catabolism was dependent upon their initial point(s) of entry to the Krebs cycle.

The results indicated that REC from bulls fed the high concentrate diet metabolized more glucose to lactate than those from animals fed a high forage diet, therefore reducing the contribution of other substrates to lactate production. Previous reports have shown that adaptation in tissue demand for glucose uptake and catabolism occurred in response to starch feeding (Harmon, 1986). What was interesting in the present study was that glucose catabolism did not results in entry of carbon into the Krebs cycle, and thus most of glucose catabolized by the isolated REC and DMC cells yielded lactate or possibly alanine (Not determined). Lactate may represent a salvage mechanism for glucose catabolized in glycolytic pathway. There is evidence to suggest that the high rate of lactate production in fast growing

cells such as GIT, immune and cancer cells occurs to support ribose production through the pentose phosphate pathway (Newsholme et al., 1985). Our data also suggest that CO_2 production form glucose (Harmon, 1986; Okine et al., 1995; Seal and Parker, 1996) could have occurred in the oxidative arm of the pentose cycle. There are no data in ruminants that have measure glucose oxidation by the pentose cycle, however in pig enterocytes glucose oxidation accounted for 90% of CO_2 production via the pentose cycle (Wu, 1996). It is possible that a similar mechanism occurs in ruminants GIT given lack of labeling in Krebs cycle intermediates measured in our study.

Leucine (17-63%) and valine (19-82%) contribution to ketoisocaproic and ketoisovaleric acid fluxes increased with increasing concentrations. For KIC, there was no difference between the proportion of KIC produced from leucine between REC and DMC. However, no detectable incorporation of leucine carbon into Krebs cycle intermediates occurred in REC and DMC. Increasing the supply of dietary metabolizable protein has been reported to increased leucine oxidation from 16 to 22% of GIT utilization in dairy cows (Lapierre et al., 2002), and that KIC production by the GIT increased with feed intake (Pell et al., 1986; Lapierre et al., 1999). Furthermore, oxidation of leucine by the GIT of sheep accounted for 12-22% of arterial sequestration and only a limited amount of leucine was oxidized from the luminal aspect of the small intestines of sheep (0 to 0.05; Cappelli et al., 1997; MacRae et al., 1997b; Yu et al., 2000). These data are in agreement with the present results employing isolated luminal cells for incubations and where leucine was not found to be metabolized via the Krebs cycle.

Most of what is known about EAA metabolism in ruminants in vivo has been gleaned from the use of [^{13}C] or [^{14}C]leucine tracers, mainly on the grounds of cost. Whilst the metabolism of leucine may give some clues to the general metabolism of other EAA, this assumption is probably incorrect and requires validation. Herein, valine was found to be metabolized to KIV by REC and at a higher rate (31-80%) than for DMC (20-65%). As was the case with KIC production from leucine, KIV derived from valine was not channeled to the Krebs cycle for complete catabolism.

Glutamate was the single most important contributor to Krebs cycle intermediates, and its carbon accounted for 4-54% of α-ketoglutarate. Glutamate contribution to α-ketoglutarate increased with increased supply and was higher in REC (13-54%) than in DMC (4-25%). By contrast, glutamine's contribution to α-ketoglutarate flux did not exceed 3% for REC and DMC.

Previous studies in sheep have shown that glucose addition to REC and DMC incubation media reduced CO_2 production from glutamine (Harmon et al., 1986; Okine et al., 1995; Oba et al., 2004). By contrast, the addition of glucose to DMC incubations had no effect on reducing CO_2 production from glutamate (Oba et al., 2004). These authors suggested that DMC could have an obligate requirement for glutamate oxidation or that the small differences noted were outwith the sensitivity of their measurements. We had expected that glutamine would contribute substantially to α-ketoglutarate flux in DMC, given that CO_2 production (6 vs 1.8 nmoles) from glutamine exceeded those from glutamate at similar concentrations (Oba et al., 2004). We can only speculate that the limited metabolism of glutamine in the Krebs cycle, as

is observed in rats (Watford, 1994), relates to some form of compartmentation of glutamine and glutamate catabolic pathways.

The proportion of succinate flux (3-15%) derived via α-ketoglutarate was lower than we had anticipated, and this suggests that flux through succinate also involves contributions from other unlabelled substrates catabolized for entry into the cycle at succinyl-CoA. However, we did observe a large contribution of α-ketoglutarate to malate flux (4-40%), which follows succinyl-CoA in the Krebs cycle pathway.

In conclusion, the approach we used in this study has been used in profiling cardiac metabolism in the working heart muscle (Des Rosier et al., 1994), and to our knowledge this is the first attempt to provide a more complete profile of major substrates metabolized by cells lining the lumen of the ruminant GIT. These data provide evidence that glutamate is the single most important (9-41%) substrate contributing to the Krebs cycle intermediates, a process referred to as anaplerosis because of the importance of this process in ensuring replenishment of intermediates that leave the Krebs cycle via cataplerosis. By contrast, glutamine's contribution to Krebs cycle flux via α-ketoglutarate did not exceed 3% of its flux. Despite the large contribution of glucose to lactate flux (8-31%), there was no detectable entry of glucose carbon skeletons into the Krebs cycle. We believe that this process serves a role in preserving glucose carbon skeletons, in particular 3-carbon skeletons, from catabolism by the GIT and for subsequence recycling to the liver for glucose synthesis.

TABLE 4.1

Ingredients and nutrient composition of the experimental diets[1]

	High forage	High concentrate
Ingredient	g/kg dry matter	
Orchardgrass, silage	750	250
Ground corn	167	554
Soybean meal	59	172
Calcium carbonate	12	14
Dicalcium phosphate	2	-
Vitamin mix	5	5
Mineral mix	5	5
Nutrient composition (calculated)		
Dry matter	305	680
Crude protein	137	174
Acid detergent fiber	332	140
Neutral detergent fiber	553	245
Crude fat	38	38
Metabolizable energy, MJ/kg	8.8	11.7

[1] Mineral mix provided per kg premix: calcium, 220 g; salt 160 g; sulfur, 31 g; phosphorus, 30 g; magnesium, 27 g; potassium, 24 g; iron 1,820 mg; zinc 2,700 mg; manganese 240 mg ; iodine 40 mg; cobalt 35 mg ; selenium 24 mg.
Vitamin mix provided per kg premix: vitamin A, 682,799 IU; vitamin D, 137,574 IU; vitamin E, 1,774 IU.

TABLE 4.2

Composition of media used in primary cell incubations

	Calculated concentration	
	Duodenal Mucosa	Rumen epithelia
	mmol/L	
Experimental substrates		
Glucose	4.000	4.000
Acetate	0.880	64.000
Propionate	0.040	23.000
Butyrate	0.009	15.000
Leucine	0.230	0.230
Valine	0.330	0.330
Glutamine	0.150	0.150
Glutamate	0.270	0.270
Basal amino acid mixture		
Histidine	0.085	0.085
Isoleucine	0.144	0.144
Lysine	0.141	0.141
Methionine	0.032	0.032
Phenlalanine	0.074	0.074
Threonine	0.124	0.124
Tryptophan	0.022	0.022
Alanine	0.275	0.275
Arginine	0.097	0.097
Aspartate	0.040	0.040
Asparagine	0.042	0.042
Cysteine	0.081	0.081
Glycine	0.411	0.411
Proline	0.136	0.136
Serine	0.117	0.117
Tyrosine	0.075	0.075

TABLE 4.3

Concentration of [13C]tracers used in primary cell incubations

Tracer substrates	Calculated concentration			
Level	1	2	3	4
Duodenal Mucosa[1]	mmol/L			
[$^{13}C_6$]Glucose	1.000	2.000	4.000	10.000
[$^{13}C_2$]Acetate	0.220	0.440	0.880	2.190
[$^{13}C_3$]Propionate	0.010	0.020	0.040	0.090
[1-$^{13}C_1$]Butyrate	0.002	0.004	0.009	0.021
[$^{13}C_6$]Leucine	0.060	0.120	0.230	0.580
[$^{13}C_5$]Valine	0.080	0.160	0.330	0.810
[$^{13}C_5$]Glutamine	0.040	0.070	0.150	0.360
[$^{13}C_5$]Glutamate	0.070	0.130	0.270	0.660
Rumen epithelia[2]				
[$^{13}C_6$]Glucose	1.000	2.000	4.000	10.000
[$^{13}C_2$]Acetate	16.000	32.000	64.000	160.000
[$^{13}C_3$]Propionate	6.000	12.000	23.000	58.000
[1-$^{13}C_1$]Butyrate	4.000	8.000	15.000	38.000
[$^{13}C_6$]Leucine	0.060	0.120	0.230	0.580
[$^{13}C_5$]Valine	0.080	0.160	0.330	0.810
[$^{13}C_5$]Glutamine	0.040	0.070	0.150	0.360
[$^{13}C_5$]Glutamate	0.070	0.130	0.270	0.660

[1] Incubation media with [$^{13}C_6$]glucose [$^{13}C_6$]acetate [$^{13}C_6$]propionate and [$^{13}C_6$]butyrate contained 25 moles [$^{13}C_6$]tracer per 100 moles unlabelled tracee.
[2] Incubation media with [$^{13}C_6$]glucose contained 25 moles [$^{13}C_6$]tracer per 100 moles unlabelled tracee.

Table 4.4

Fractional contribution of glucose to pyruvate flux (FCGlucose → Pyruvate) in rumen epithelial (REC) and duodenal mucosal cells (MDC) isolated from bulls fed high concentrate (HC) or high forage (HF) diets, and incubated in the presence of [13C6]glucose[1].

Diet	Tissue	Glucose (mmol/L)	$FC_{Glucose \rightarrow Pyruvate}$, %
HC	REC	1	1.3
HC	REC	2	1.7
HC	REC	4	1.9
HC	REC	10	2.4
HC	DMC	1	2.2
HC	DMC	2	2.5
HC	DMC	4	3.4
HC	DMC	10	7.3
HF	REC	1	0.4
HF	REC	2	0.9
HF	REC	4	0.9
HF	REC	10	1.1
HF	DMC	1	2.5
HF	DMC	2	3.7
HF	DMC	4	4.8
HF	DMC	10	6.7
	SEM		1.22

Effect	P-value
Diet	NS
level	0.0007
Tissue	0.0001
Diet × level	NS
Diet × tissue	NS
Tissue × level	0.0213
Diet × tissue × level	NS

[1] The incubation contained 25 moles [$^{13}C_6$]glucose per 100 moles unlabelled glucose. Therefore, the contribution of glucose to lactate flux was calculated as pyruvate [M+3]/25 molar % [$^{13}C_6$]glucose.
[2] NS= not significant (P>0.05); SEM= pooled standard error of the means.

Table 4.5

Fractional contribution of glucose to lactate flux (FCGlucose → Lactate) in rumen epithelial (REC) and duodenal mucosal cells (MDC) isolated from bulls fed high concentrate (HC) or high forage (HF) diets, and incubated in the presence of [13C6]glucose[1].

Diet	Tissue	Glucose (mmol/L)	$FC_{Glucose \rightarrow Lactate}$, %
HC	REC	1	22.0
HC	REC	2	25.7
HC	REC	4	26.3
HC	REC	10	30.9
HC	DMC	1	22.0
HC	DMC	2	25.0
HC	DMC	4	27.6
HC	DMC	10	29.7
HF	REC	1	8.1
HF	REC	2	11.0
HF	REC	4	12.9
HF	REC	10	15.4
HF	DMC	1	21.0
HF	DMC	2	25.2
HF	DMC	4	27.1
HF	DMC	10	28.2
	SEM		3.77

Effect	P-value
Diet	NS
level	0.0001
Tissue	0.0001
Diet × level	NS
Diet × tissue	0.0001
Tissue × level	NS
Diet × tissue × level	NS

[1] The incubation contained 25 moles [$^{13}C_6$]glucose per 100 moles unlabelled glucose. Therefore, the contribution of glucose to lactate flux was calculated as lactate [M+3]/25 molar % [$^{13}C_6$]glucose.
[2] NS= not significant (P>0.05); SEM= pooled standard error of the means.

Table 4.6

Fractional contribution of leucine to [M+6]ketoisocaproic acid (FCleucine → ketoisocaproic) in rumen epithelial (REC) and duodenal mucosal cells (MDC) isolated from bulls fed high concentrate (HC) or high forage (HF) diets, and incubated in the presence of [13C6]leucine[1].

Diet	Tissue	Leucine (*mmol/L*)	$FC_{leucine \rightarrow ketoisocaproic}$, %
HC	REC	0.06	19.8
HC	REC	0.12	34.7
HC	REC	0.24	55.4
HC	REC	0.60	68.0
HC	DMC	0.06	15.5
HC	DMC	0.12	25.8
HC	DMC	0.24	38.8
HC	DMC	0.60	59.7
HF	REC	0.06	15.7
HF	REC	0.12	25.1
HF	REC	0.24	38.2
HF	REC	0.60	57.9
HF	DMC	0.06	19.3
HF	DMC	0.12	28.2
HF	DMC	0.24	41.0
HF	DMC	0.60	63.1
	SEM		6.97

Effect	P-value
Diet	NS[2]
level	0.0001
Tissue	NS
Diet × level	NS
Diet × tissue	0.0068
Tissue × level	NS
Diet × tissue × level	NS

[1] NS= not significant (P>0.05); SEM= pooled standard error of the means.

Table 4.7

Fractional contribution of valine to [M+5]ketoisovaleric acid (FCvaline → ketoisovaleric) in rumen epithelial (REC) and duodenal mucosal cells (MDC) isolated from bulls fed high concentrate (HC) or high forage (HF) diets, and incubated in the presence of [13C5]valine[1].

Diet	Tissue	Valine (*mmol/L*)	$FC_{valine \rightarrow ketoisovaleric}$, %
HC	REC	0.08	38.5
HC	REC	0.16	55.7
HC	REC	0.33	71.7
HC	REC	0.81	85.8
HC	DMC	0.08	24.4
HC	DMC	0.16	37.0
HC	DMC	0.33	55.3
HC	DMC	0.81	74.1
HF	REC	0.08	26.5
HF	REC	0.16	43.9
HF	REC	0.33	61.0
HF	REC	0.81	78.0
HF	DMC	0.08	13.8
HF	DMC	0.16	27.0
HF	DMC	0.33	36.6
HF	DMC	0.81	53.0
	SEM		7.57
Effect			P-value
Diet			NS
level			0.0001
Tissue			0.0001
Diet × level			NS
Diet × tissue			NS
Tissue × level			NS
Diet × tissue × level			NS

[1] NS= not significant (P>0.05); SEM= pooled standard error of the means.

Table 4.8

Fractional contribution of glutamate to [M+5] α-ketoglutarate (FCGlutamate → α-ketoglutarate) in rumen epithelial (REC) and duodenal mucosal cells (MDC) isolated from bulls fed high concentrate (HC) or high forage (HF) diets, and incubated in the presence of [13C6]glutamate[1].

Diet	Tissue	Glutamate (*mmol/L*)	$FC_{Glutamate \rightarrow \alpha\text{-ketoglutarate}}$, %
HC	REC	0.07	15.6
HC	REC	0.13	28.0
HC	REC	0.27	41.2
HC	REC	0.66	62.5
HC	DMC	0.07	2.8
HC	DMC	0.13	5.9
HC	DMC	0.27	8.9
HC	DMC	0.66	17.9
HF	REC	0.07	10.3
HF	REC	0.13	17.2
HF	REC	0.27	26.8
HF	REC	0.66	46.2
HF	DMC	0.07	4.3
HF	DMC	0.13	7.6
HF	DMC	0.27	16.8
HF	DMC	0.66	31.7
	SEM		3.502
Effect			P-value
Diet			NS
level			0.0001
Tissue			0.0001
Diet × level			NS
Diet × tissue			0.0001
Tissue × level			0.0001
Diet × tissue × level			0.0074

[1] NS= not significant (P>0.05); SEM= pooled standard error of the means.

Table 4.9

Fractional contribution of glutamine to [M+5] α-ketoglutarate (FCGlutamine → α-ketoglutarate) in rumen epithelial (REC) and duodenal mucosal cells (MDC) isolated from bulls fed high concentrate (HC) or high forage (HF) diets, and incubated in the presence of [13C6]glutamine[1].

Diet	Tissue	Glutamine (*mmol/L*)	$FC_{Glutamine \rightarrow \alpha\text{-ketoglutarate}}$, %
HC	REC	0.04	0.5
HC	REC	0.07	0.7
HC	REC	0.15	1.2
HC	REC	0.36	2.3
HC	DMC	0.04	0.1
HC	DMC	0.07	0.8
HC	DMC	0.15	1.5
HC	DMC	0.36	2.7
HF	REC	0.04	0.2
HF	REC	0.07	0.4
HF	REC	0.15	0.7
HF	REC	0.36	1.6
HF	DMC	0.04	0.3
HF	DMC	0.07	0.6
HF	DMC	0.15	1.2
HF	DMC	0.36	2.2
	SEM		0.332
Effect			P-value
Diet			NS
level			0.0001
Tissue			NS
Diet × level			NS
Diet × tissue			NS
Tissue × level			NS
Diet × tissue × level			NS

[1] NS= not significant (P>0.05); SEM= pooled standard error of the means.

Table 4.10

Fractional contribution of α-ketoglutarate to succinate (FC α-KG →Suc), α-ketoglutarate to malate (FC α-KG →Mal), malate to oxaloacetate (FC Mal →Oaa) in rumen epithelial (REC) and duodenal mucosal cells (MDC) isolated from bulls fed high concentrate (HC) or high forage (HF) diets, and incubated in the presence of [13C6]glutamate[1]

Diet	Tissue	Glutamate (*mmol/L*)	$FC_{\alpha\text{-KG} \rightarrow \text{Suc}}$	$FC_{\alpha\text{-KG} \rightarrow \text{Mal}}$	$FC_{\text{Mal} \rightarrow \text{Oaa}}$
HC	REC	0.07	2.6	6.3	22.2
HC	REC	0.13	2.6	5.4	24.7
HC	REC	0.27	3.1	4.6	26.7
HC	REC	0.66	4.1	4.3	32.1
HC	DMC	0.07	7.7	42.0	43.7
HC	DMC	0.13	7.8	37.8	67.4
HC	DMC	0.27	5.9	37.1	79.6
HC	DMC	0.66	4.6	30.5	79.7
HF	REC	0.07	6.0	15.7	28.5
HF	REC	0.13	6.3	15.4	31.4
HF	REC	0.27	6.5	11.9	37.2
HF	REC	0.66	7.1	10.3	41.6
HF	DMC	0.07	15.0	39.5	59.5
HF	DMC	0.13	15.4	35.0	57.9
HF	DMC	0.27	13.7	28.2	61.3
HF	DMC	0.66	12.8	24.0	63.6
	SEM		4.099	4.742	9.210
Effect			P-value		
Diet			NS	NS	NS
level			NS	0.0025	NS
Tissue			0.0001	0.0001	0.0001
Diet × level			NS	NS	NS
Diet × tissue			NS	0.0001	NS
Tissue × level			NS	NS	NS
Diet × tissue × level			NS	NS	NS

[1] NS= not significant (P>0.05); SEM= pooled standard error of the means.

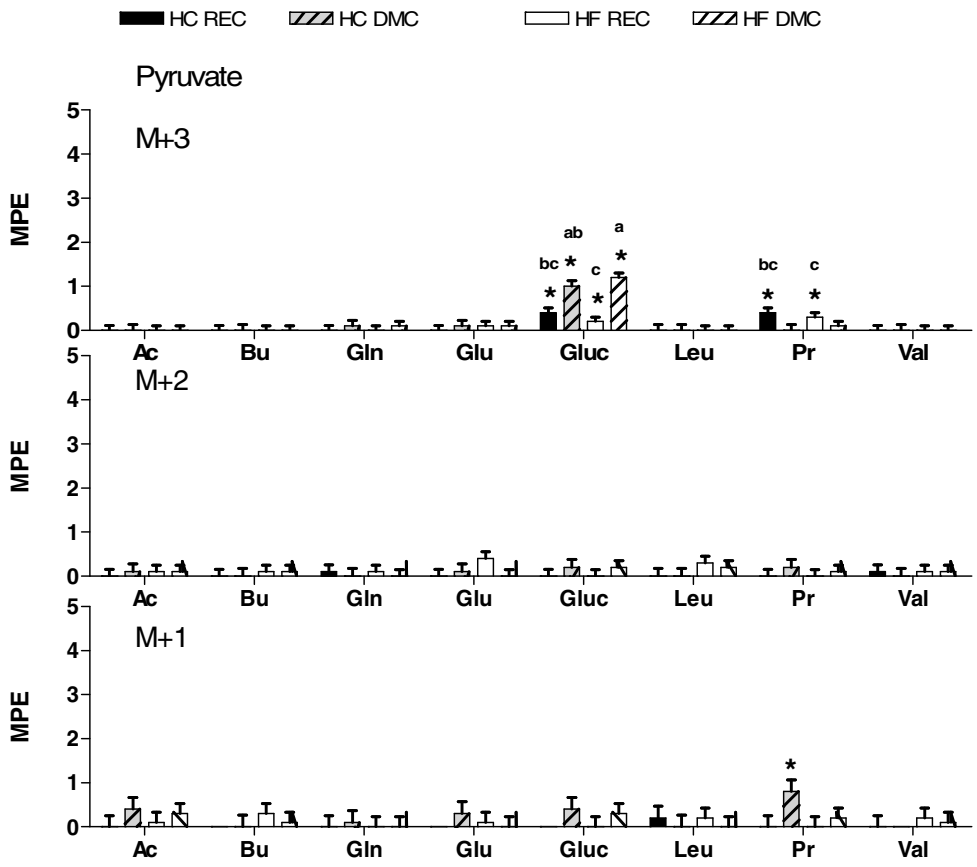


Figure 4.1. Mass isotopomer distribution of pyruvate from rumen epithelial (REC) and duodenal mucosal (DMC) cells isolated from bulls fed high forage (HF) or high concentrate (HC) diet and incubated with [$^{13}C_2$]acetate, [1-$^{13}C_1$]butyrate, [$^{13}C_5$]glutamine, [$^{13}C_5$] glutamate, [$^{13}C_6$] glucose, [$^{13}C_6$]leucine, or [$^{13}C_5$]valine. * denotes values that are significantly different from 0 ($P<0.05$). ^{abc} values not sharing a common letter differ significantly ($P<0.05$).

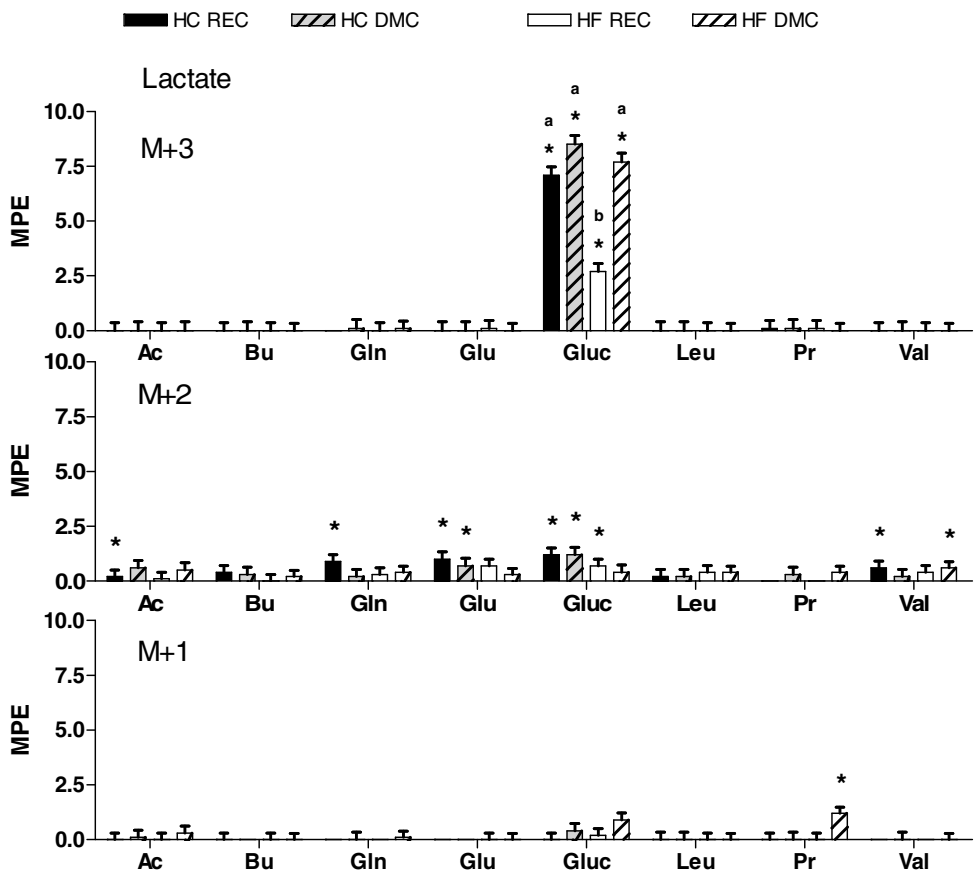


Figure 4.2. Mass isotopomer distribution of lactate from rumen epithelial (REC) and duodenal mucosal (DMC) cells isolated from bulls fed high forage (HF) or high concentrate (HC) diet and incubated with [$^{13}C_2$]acetate, [1-$^{13}C_1$]butyrate, [$^{13}C_5$]glutamine, [$^{13}C_5$] glutamate, [$^{13}C_6$] glucose, [$^{13}C_6$]leucine, or [$^{13}C_5$]valine. * denotes values that are significantly different from 0 ($P<0.05$). ^{abc} values not sharing a common letter differ significantly ($P<0.05$).

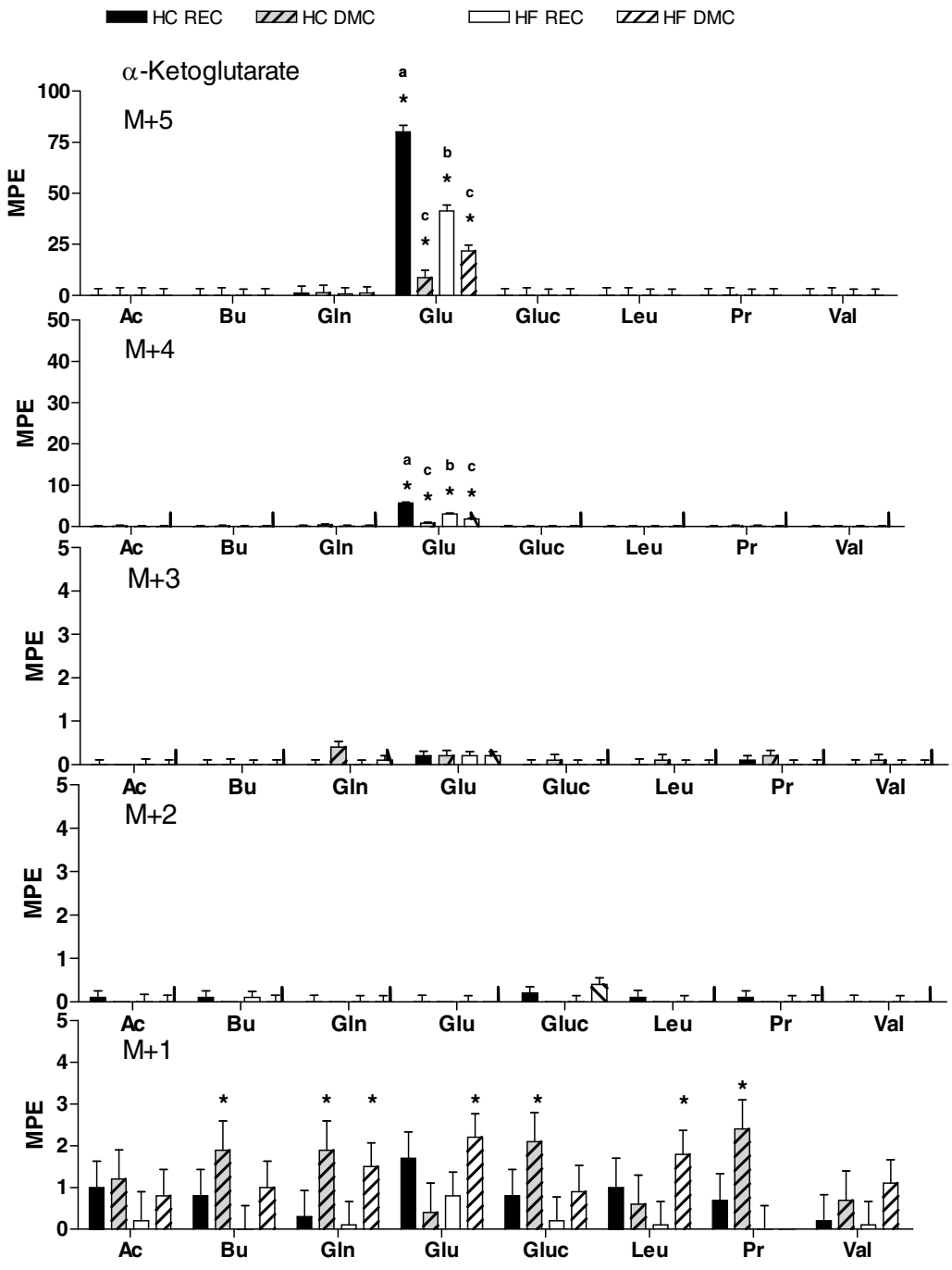


Figure 4.3. Mass isotopomer distribution of α-ketoglutarate from rumen epithelial (REC) and duodenal mucosal (DMC) cells isolated from bulls fed high forage (HF) or high concentrate (HC) diet and incubated with [$^{13}C_2$]acetate, [1-$^{13}C_1$]butyrate, [$^{13}C_5$]glutamine, [$^{13}C_5$] glutamate, [$^{13}C_6$] glucose, [$^{13}C_6$]leucine, or [$^{13}C_5$]valine. * denotes values that are significantly different from 0 ($P<0.05$). ^{abc} values not sharing a common letter differ significantly ($P<0.05$).

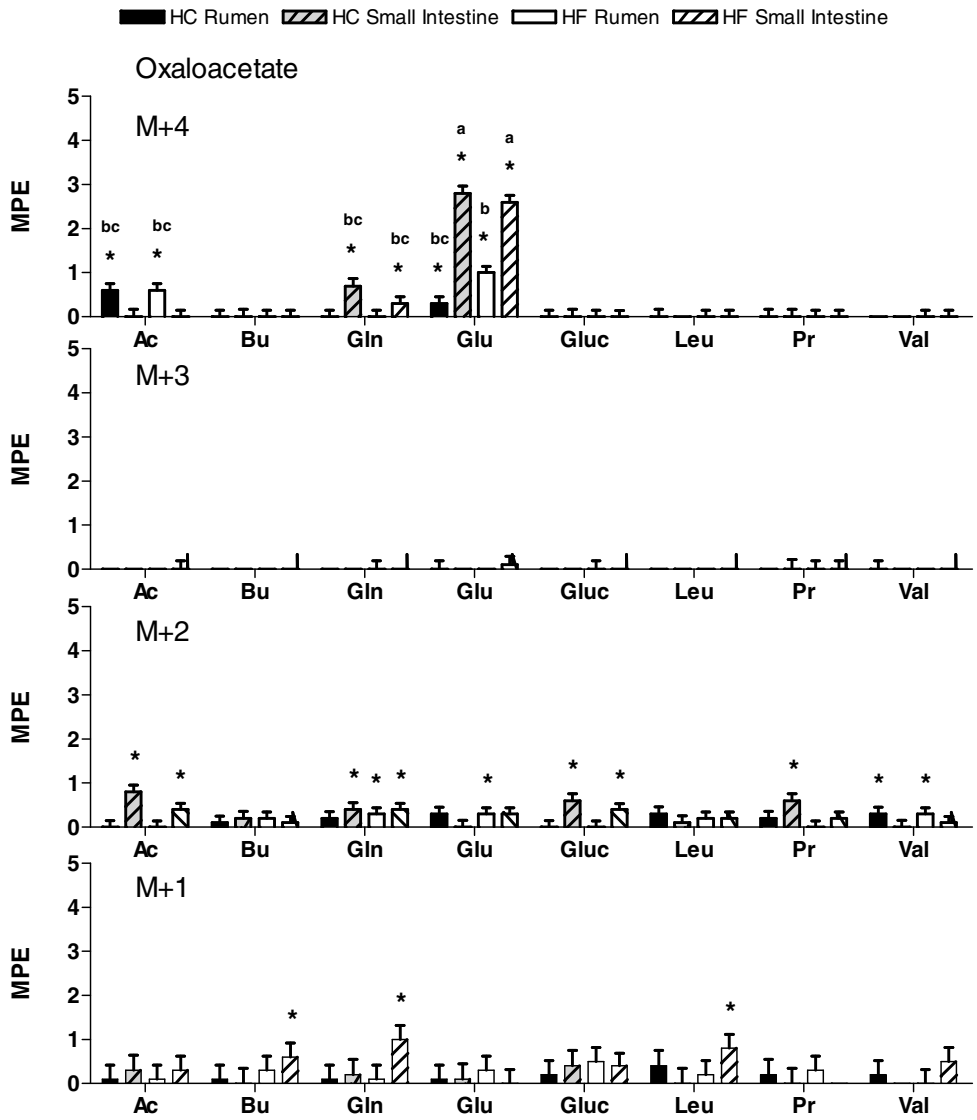


Figure 4.4. Mass isotopomer distribution of oxaloacetate from rumen epithelial (REC) and duodenal mucosal (DMC) cells isolated from bulls fed high forage (HF) or high concentrate (HC) diet and incubated with [$^{13}C_2$]acetate, [1-$^{13}C_1$]butyrate, [$^{13}C_5$]glutamine, [$^{13}C_5$] glutamate, [$^{13}C_6$] glucose, [$^{13}C_6$]leucine, or [$^{13}C_5$]valine. * denotes values that are significantly different from 0 ($P<0.05$). ^{abc} values not sharing a common letter differ significantly ($P<0.05$).

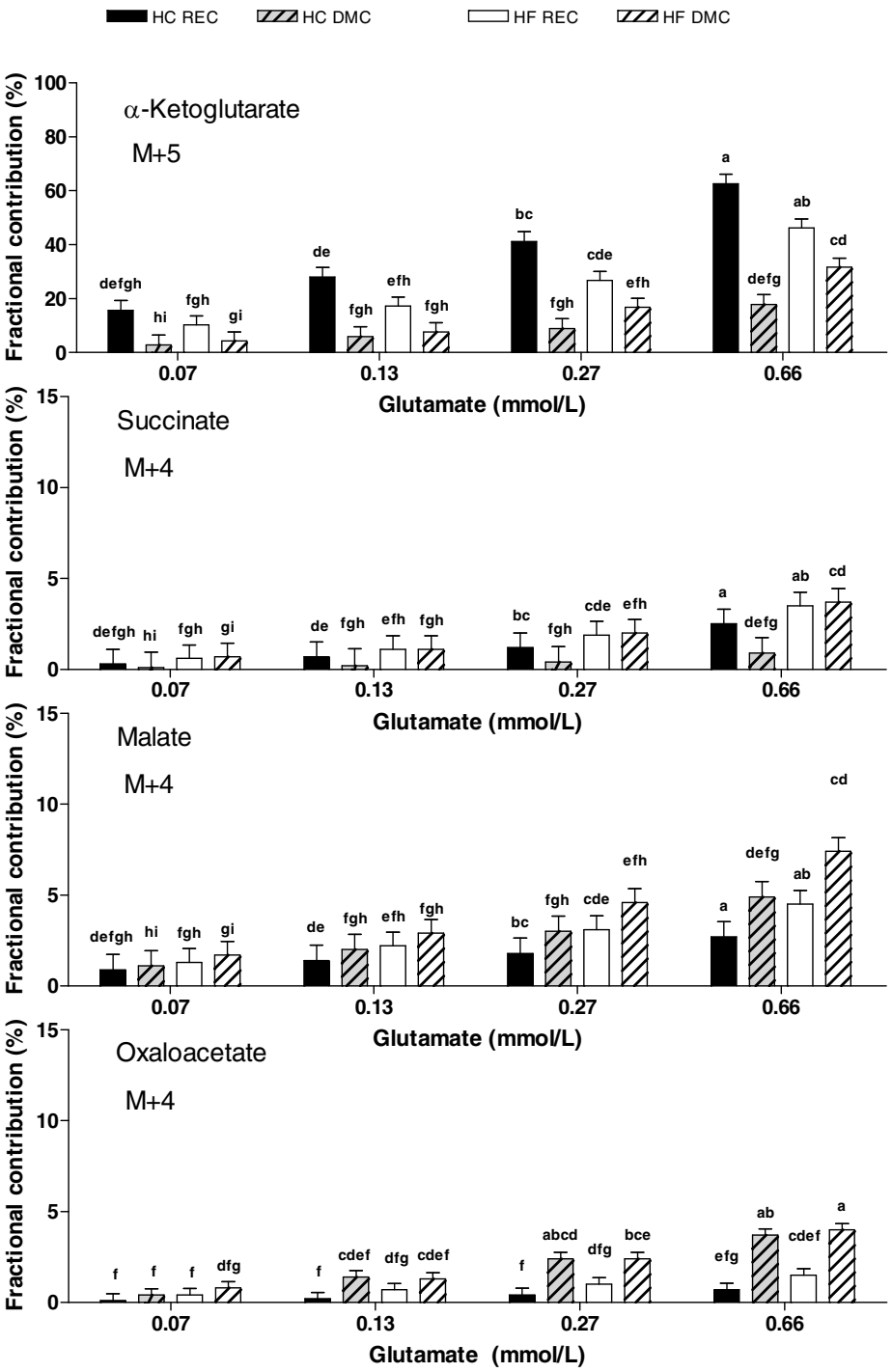


Figure 4.5. Fractional contribution of glutamate to α-ketoglutarate, succinate, malate, and oxaloacetate from rumen epithelial (REC) and duodenal mucosal (DMC) cells isolated from bulls fed high forage (HF) or high concentrate (HC) diet and incubated with [$^{13}C_5$] glutamate. ^{abc} values not sharing a common letter differ significantly ($P<0.05$).

SUMMARY

The gastrointestinal tract (GIT) plays a significant role in determining the efficiency by which amino acids (AAs) and energy are deposited in productive tissues. The GIT not only dictates nutrient delivery to productive tissues from luminal supplies, but also competes with these tissues for nutrients from blood supplies. The high metabolic demand for AAs by the GIT relates to protein synthesis and energy production. One area susceptible to manipulation is AA utilization for energy production, and decreasing it might provide a means to enhancing the efficiency of AA utilization in productive tissues.

The overall hypothesis of this dissertation research was that the GIT of ruminant animals catabolizes AA preferentially. We sought to determine whether this catabolism represents an obligate requirement, and whether this requirement stems from the need to generate energy or support other metabolic demands.

Glucose metabolism by the GIT was investigated in sheep fed a low protein diet and given duodenal infusions of glucose or glucose/casein. Small intestinal protein supplies increased (18 vs 35%) the contribution of luminal (first-pass) and decreased (82 vs 65%) that of arterial glucose utilization by the GIT. Despite this shift, total glucose utilization remained unchanged and the GIT utilized 48-51% of glucose available from absorption and gluconeogenesis, mainly glucose derived from the arterial circulation. We had hypothesized that if AAs, and not glucose, are the preferential energy substrates of the GIT, then intestinal infusion of casein would lead to a reduction in glucose use by the GIT. Our results indicated that total GIT use of

glucose was not affected by additional luminal AA supply, and therefore under these conditions, glucose metabolism by the GIT was obligatory.

The second study aimed at establishing the extent by which AAs are net metabolized by the GIT of growing sheep, and at determining the extent by which AAs are used from arterial and luminal supplies in response to increased protein supply to the small intestine. The efficiency of absorption across the GIT of all the essential AAs, except the BCAAs, was 100% and this remained fixed at all levels of protein supply, whereas the efficiency of absorption of BCAAs was 60-66%. By contrast, the high removal rates of glutamate, glutamine, and serine remained constant even at the upper levels of protein supply. The ruminant GIT appears to metabolize increasing amounts of the BCAAs and certain non-essential AAs when the intestinal supply of protein is increased. The basis for this disproportionate metabolism of some AAs with increased intestinal protein supply could suggest their utilization for energy production.

The flux and contribution of glucose, AAs, and SCFAs to overall Krebs cycle metabolism was determined in rumen epithelial (REC) and duodenal mucosal (DMC) cells of beef cattle. Our results indicated that despite the high flux of glucose to lactate (8-31%), leucine to ketoisocaproic acid (17-63%), and valine to ketoisovaleric (19-82%), there was no detectable entry of glucose leucine and valine to Krebs cycle intermediates. In contrast, glutamate contributed 9-41% of α-ketoglutarate flux, while glutamine contribution did not exceed 3%. Our hypothesis was that REC and DMC are metabolically flexible in substrate selection for catabolism in the Krebs cycle, and that this metabolism is affected by diet and substrate availability. The results of this

study suggest that only glucose metabolism by REC is affected by dietary manipulations. Moreover, the supplies of glutamate and glutamine increased their contribution to Krebs cycle intermediates, and as a result decreased the contribution of other substrates entering the cycle.

The results of these studies underline the extent by which the GIT metabolizes macronutrients. In this respect, the GIT seems to have an obligate requirement for macronutrients. The basis for this catabolism remains uncertain for certain substrates. Firstly, glucose utilization from arterial and luminal supplies represents a large proportion of glucose available form absorption and gluconeogenesis and the large removal of glucose by GIT cells occurs towards lactate production and possibly alanine. Glucose catabolism to lactate and alanine may serve as a mechanism to conserve 3 carbon units from catabolism, while maintaining the supply of glycolytic intermediates to support other metabolic demands. In this regard, additional research is needed to determine the significance of glucose partial catabolism to 3 carbon intermediates by the GIT.

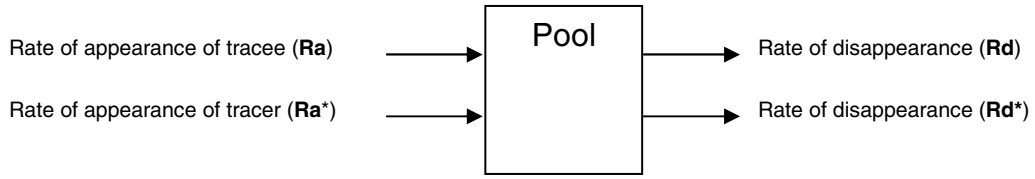
Secondly, although previous studies have reported branched chain AA (BCAA) oxidation to CO_2, our results indicated that they do not enter Krebs cycle intermediates. Hence, their catabolism may represent microbial degradation in the lumen of the GIT. However, our results raise a new question about the significance of the large amounts of BCAAs removed by the GIT and the fate of their amino-nitrogen.

Thirdly, the large removal of glutamate and glutamine by the GIT would imply that the requirements for these AAs in the liver and peripheral tissues must be

met through de novo synthesis, possibly from essential AAs and glucose. While glutamate catabolism occurs primarily via α-ketoglutarate, which subsequently enters the Krebs cycle, the fate of glutamine remains unclear. These observations suggest that increasing the supplies of glutamate would decrease the contribution of other substrates entering Krebs cycle intermediates at or beyond α-ketoglutarate. Our results also suggest that the requirement for glutamine may occur to meet other metabolic demands. In this respect investigating the catabolic pathway(s) of glutamine is important to understand the fate and the role its catabolism serves in the GIT.

Appendix A

Isotope dilution calculations



Three assumptions are needed for tracer studies:

1. The system cannot differentiate between tracer and tracee.
2. Steady state conditions.
3. Tracer and tracee share the same entry sites to body pools.

When a tracer is infused into a pool, it is considered that after a time *t*, the pool will be in isotopic equilibrium with the input and the rate of disappearance.

$$E_{in} = E_{pool} = E_{out} \quad (1)$$

Where E_{in} is the enrichment of the tracee entering the pool, E_{pool} the enrichment in the pool and E_{out} the enrichment in the tracee exiting the pool. The enrichment is calculated as mole tracer per mole of tracee.

$$E_{in} = (\text{mol tracer / mol tracee})_{in} \quad (2)$$

$$E_{pool} = (\text{mol tracer / mol tracee})_{pool} \quad (3)$$

Substituting equations (2) and (3) in equations (1) gives equation (4):

$$(\text{mol tracer / mol tracee})_{in} = E_{pool} \quad (4)$$

Because the tracer and tracee enter the pool simultaneously, then if we divide the numerator and the denominator on one side of the equation by the time *t*, equation (4):

$$(\text{mol tracer}/\text{ t})/(\text{ mol tracee}/\text{ t})_{in} = E_{pool} \quad (5)$$

Where, $(\text{mol tracer}/\text{ t})$ is the isotope infusion rate (IR) in mol/ min, and $(\text{mol tracee}/\text{ t})$ is the rate of appearance (Ra) of the tracee in the pool. Equation (5) can be written as:

$$IR/R_a = E_{pool} \quad (6)$$

Rearrange (6),

$$R_a = IR/E_{pool} \quad (7)$$

The tracer is not 100% enriched, at a given label position, so a correction is needed that takes into account this incomplete labeling:

$$IR_{true} = IR \times E_{tracer} \quad (8)$$

Where IR_{true} is the true infusion rate of the tracer (*mol/ min*), Substituting equation (8) in equation (7) gives:

$$R_a = IR \times E_{tracer}/E_{pool} \quad (9)$$

If we consider a case where no tracee is added to the pool then the enrichment of the infused solution and the enrichment of the pool will be equal and Ra will be equal to the rate of infusion of the tracer.

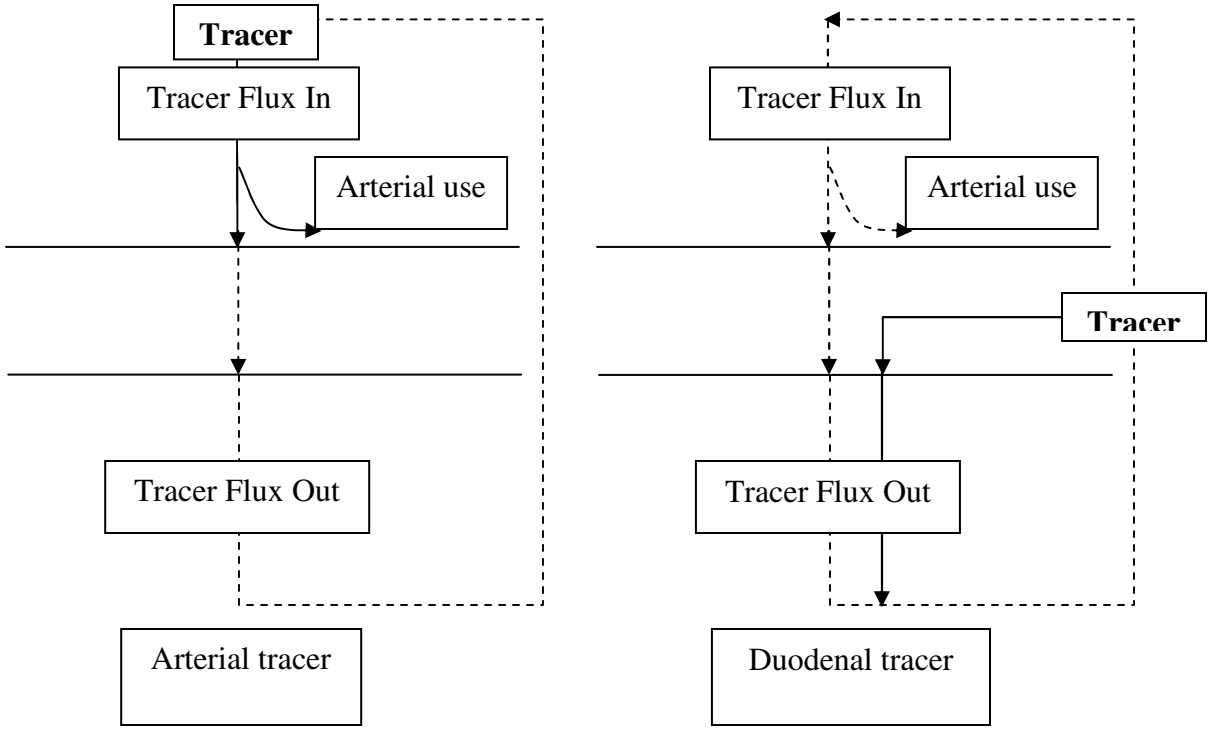
This is why another correction is required in equation (9) to account for the tracer infused into the pool by an amount equal to the infusion rate IR (*mol/ min*)

$$R_a = (IR \times E_{tracer}/E_{pool}) - IR \quad (10)$$

Rearranging (10) gives,

$$R_a = ((E_{tracer}/E_{pool}) - 1)IR \quad (11)$$

Arterial and duodenal removal



Net flux (absorption or removal) of tracer is calculated from plasma flow and arterio-venous concentration difference. The amount of tracer supplying and leaving the tissue is calculated from the concentration and enrichment of the tracee.

$$\text{Tracer in} = [A] \times A_E \times PF \quad (8)$$

$$\text{Tracer out} = [V] \times V_E \times PF \quad (9)$$

Where $[A]$ and $[V]$ are the arterial and venous concentrations, A_E and V_E are the arterial and venous enrichments, and PF is the plasma flow. The net rate at which the metabolite is removed from the arterial blood or added to the venous blood is the net flux:

$$\text{Net flux} = \text{Tracer out} - \text{Tracer out} \quad (10)$$

Replacing equations (8) and (9) in (10) gives:

$$\text{Net flux} = [A] \times A_E \times PF - [V] \times V_E \times PF \quad (11)$$

Taking PF as a common factor;

$$\text{Net flux} = ([A] \times A_E - [V] \times V_E) \times PF \quad (12)$$

The fractional use of the arterial tracer is the tracer net flux calculated as a proportion to the amount flowing in (12) divided by (8):

$$\text{fua} = \frac{([A] \times A_E - [V] \times V_E}{[A] \times A_E} \quad (13)$$

Arterial use of the tracee is calculated from the arterial supply of the tracer (tracee in) multiplied by the fractional use of the arterial tracer (13);

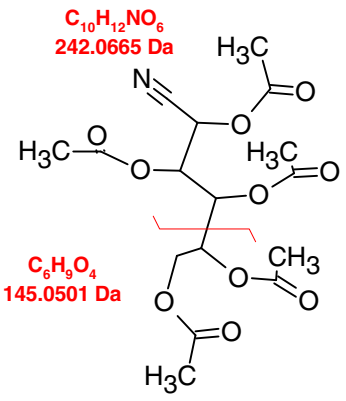
$$\text{Arterial utilization} = [A] \times PF \times \text{fua} \quad (14)$$

Luminal (i.e. first-pass) use of the tracer is calculated from the recovery of duodenal tracer. Given that some recycling (appearance in arterial blood) of the luminal tracer will occur, luminal use will be overestimated by an amount equal to the tracer removed from arterial circulation.

$$(([V] \times E_V) - ([A] \times E_A) + ([A] \times E_A \times \text{fua} \times PF))/IRd \quad (15)$$

Where $[A]$ and $[V]$ are the arterial and venous concentrations, A_E and V_E are the arterial and venous enrichments, PF is the plasma flow, fua is the fractional arterial utilization and IR_d is the luminal tracer infusion rate.

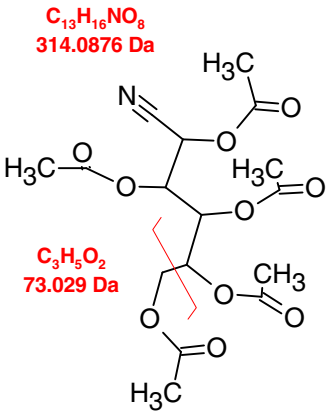
Appendix B



Molecular Formula = $C_{16}H_{21}NO_{10}$

Formula Weight = 387.33864

Glucose-aldonitrile pentaacetate



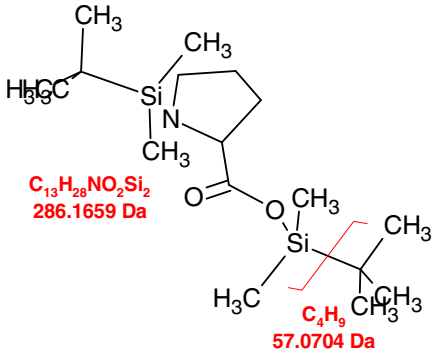
Molecular Formula = $C_{16}H_{21}NO_{10}$

Formula Weight = 387.33864

Glucose-aldonitrile pentaacetate

Fragmentation of glucose aldonitrile derivative under electric ionization.

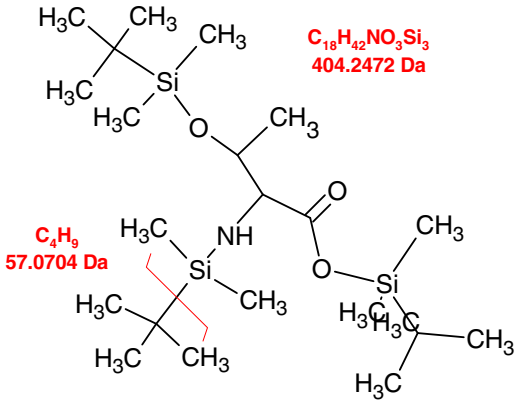
Appendix C



Molecular Formula = $C_{17}H_{37}NO_2Si_2$

Formula Weight = 343.65218

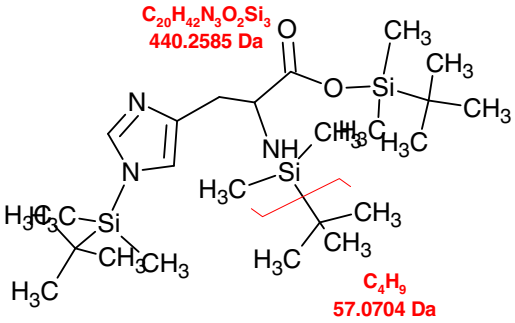
Proline TBDMS derivative



Molecular Formula = $C_{22}H_{51}NO_3Si_3$

Formula Weight = 461.90174

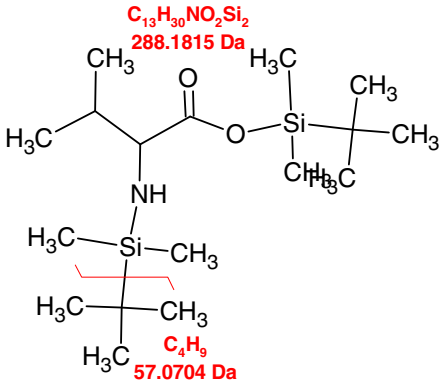
Threonine TBDMS derivative



Molecular Formula = $C_{24}H_{51}N_3O_2Si_3$

Formula Weight = 497.93714

Histidine TBDMS derivative

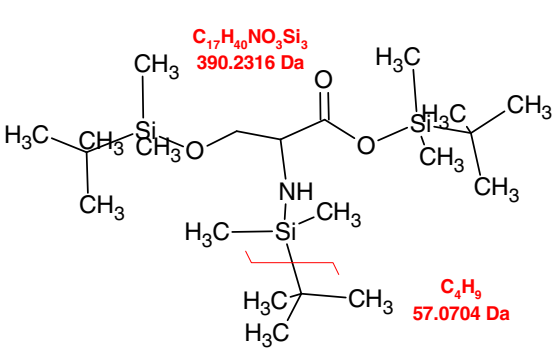


Molecular Formula = $C_{17}H_{39}NO_2Si_2$

Formula Weight = 345.66806

Valine TBDMS derivative

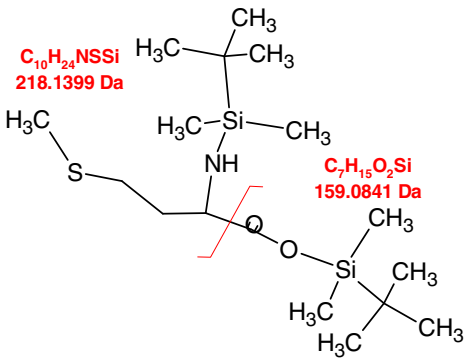
Fragmentation of proline, threonine, histidine and valine *t*-butyldimethylsilyl (TBDMS) derivatives under electric ionization



Molecular Formula = $C_{21}H_{49}NO_3Si_3$

Formula Weight = 447.87516

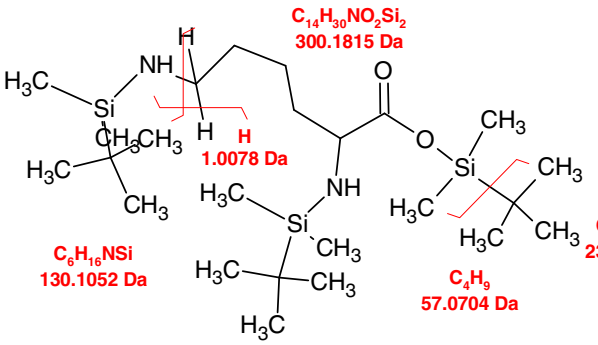
Serine TBDMS derivative



Molecular Formula = $C_{17}H_{39}NO_2SSi_2$

Formula Weight = 377.73306

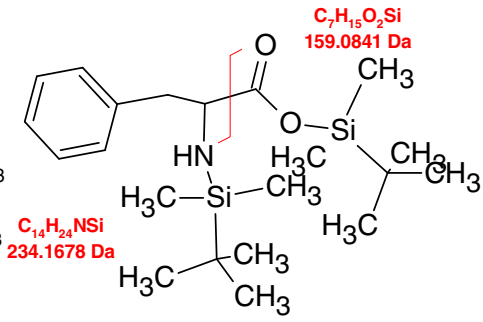
Methionine TBDMS derivative



Molecular Formula = $C_{24}H_{56}N_2O_2Si_3$

Formula Weight = 488.97014

Lysine TBDMS derivative

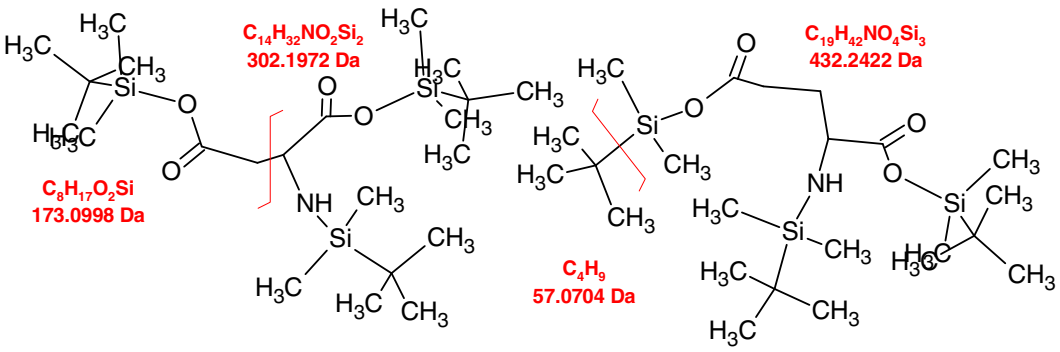


Molecular Formula = $C_{21}H_{39}NO_2Si_2$

Formula Weight = 393.71086

Phenylalanine TBDMS derivative

Fragmentation of Serine, methionine, lysine and phenylalanine *t*-butyldimethylsilyl (TBDMS) derivatives under electric ionization



Molecular Formula = $C_{22}H_{49}NO_4Si_3$

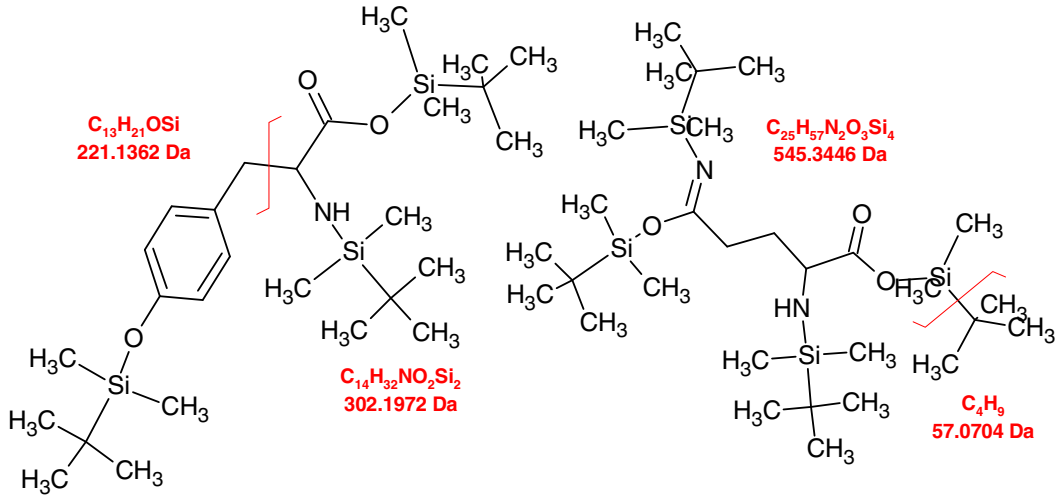
Formula Weight = 475.88526

Aspartate TBDMS derivative

Molecular Formula = $C_{23}H_{51}NO_4Si_3$

Formula Weight = 489.91184

Glutamate TBDMS derivative



Molecular Formula = $C_{27}H_{53}NO_3Si_3$

Formula Weight = 523.97112

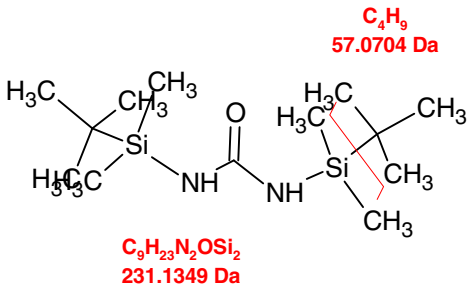
Tyrosine TBDMS derivative

Molecular Formula = $C_{23}H_{52}N_2O_3Si_3$

Formula Weight = 488.92708

Glutamine TBDMS derivative

Fragmentation of aspartate, glutamate, tyrosine and glutamine *t*-butyldimethylsilyl (TBDMS) derivatives under electric ionization



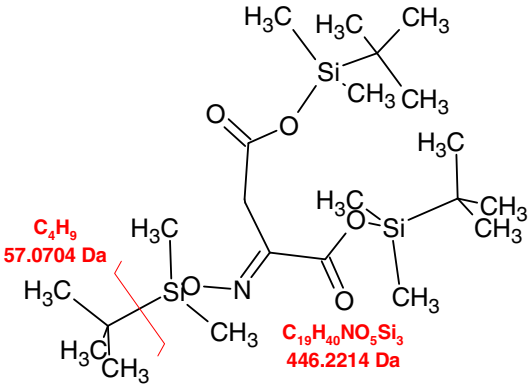
Molecular Formula = $C_{13}H_{32}N_2OSi_2$

Formula Weight = 288.57698

Urea TBDMS derivative

Fragmentation of urea *t*-butyldimethylsilyl (TBDMS) derivatives under electric ionization

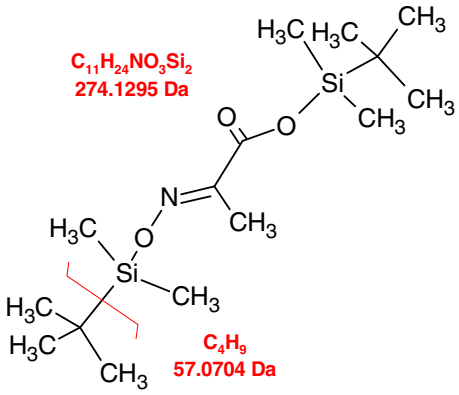
Appendix D



Molecular Formula = $C_{23}H_{49}NO_5Si_3$

Formula Weight = 503.89536

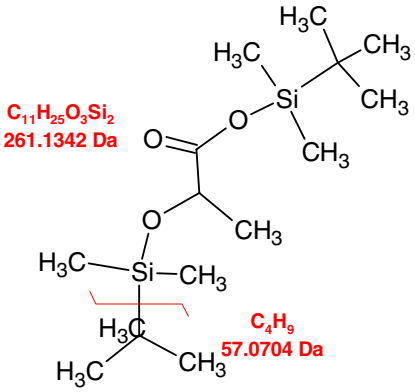
α-Ketoglutarate-oxime TBDMS derivative



Molecular Formula = $C_{15}H_{33}NO_3Si_2$

Formula Weight = 331.59842

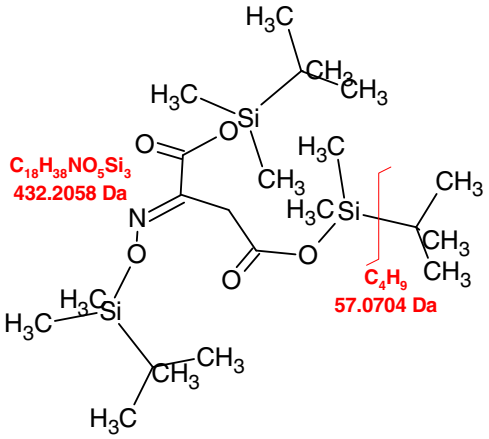
Pyruvate-oxime TBDMS derivative



Molecular Formula = $C_{15}H_{34}O_3Si_2$

Formula Weight = 318.59966

Lactate TBDMS derivative

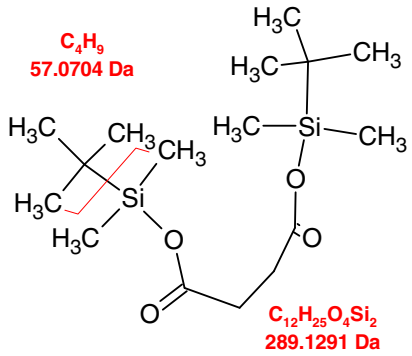


Molecular Formula = $C_{22}H_{47}NO_5Si_3$

Formula Weight = 489.86878

Oxaloacetate-oxime TBDMS

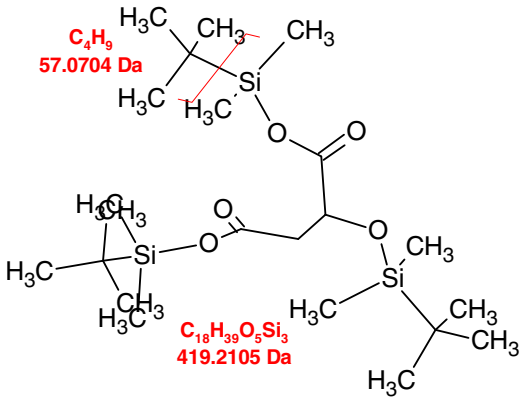
Fragmentation of α-ketoglutarate-oxime, pyruvate-oxime, lactate and oxaloacetate *t*-butyldimethylsilyl (TBDMS) derivatives under electric ionization



Molecular Formula = $C_{16}H_{34}O_4Si_2$

Formula Weight = 346.60976

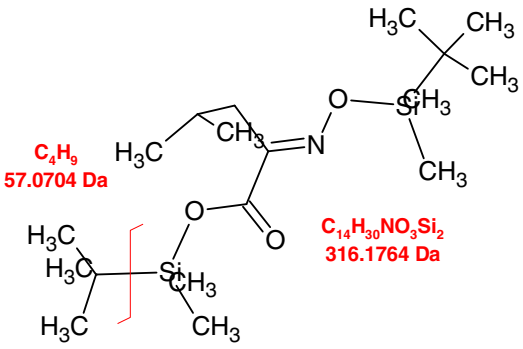
Succinate TBDMS derivative



Molecular Formula = $C_{22}H_{48}O_5Si_3$

Formula Weight = 476.87002

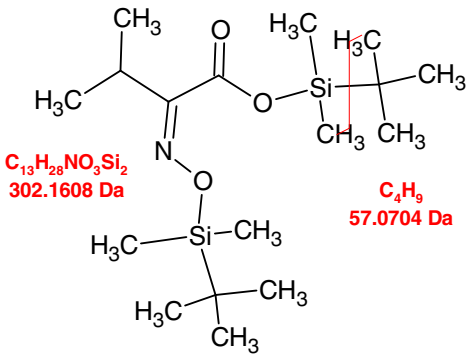
Malate TBDMS derivative



Molecular Formula = $C_{18}H_{39}NO_3Si_2$

Formula Weight = 373.67816

KIC-oxime TBDMS derivative

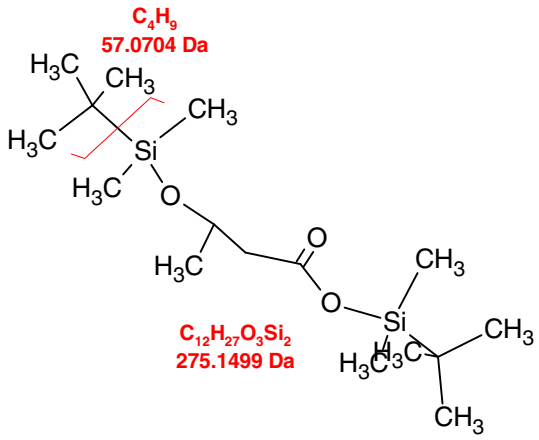


Molecular Formula = $C_{17}H_{37}NO_3Si_2$

Formula Weight = 359.65158

KIV-oxime TBDMS derivative

Fragmentation of succinate, malate, ketoisocaproic-oxime (KIC), and ketoisovaleric-oxime (KIV) *t*-butyldimethylsilyl (TBDMS) derivatives under electric ionization.



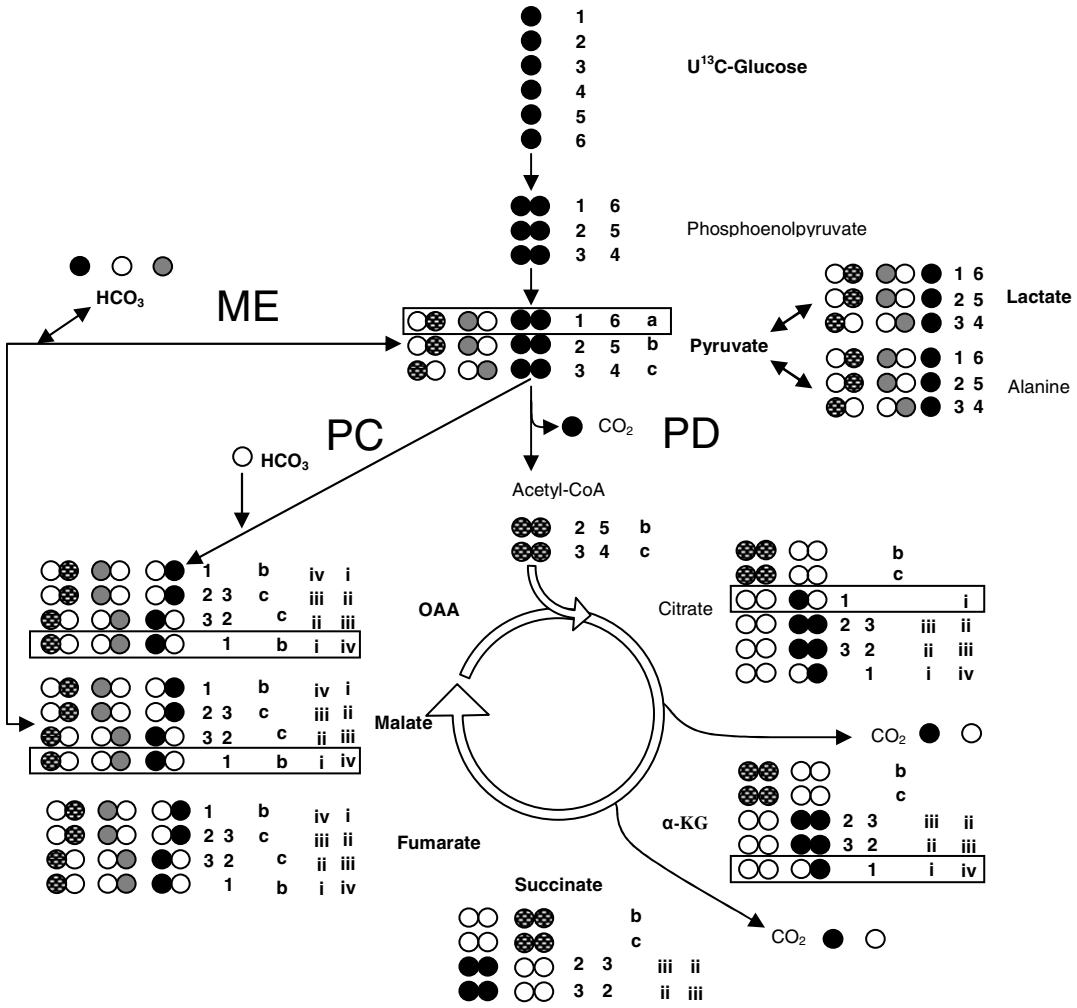
Molecular Formula = $C_{16}H_{36}O_3Si_2$

Formula Weight = 332.62624

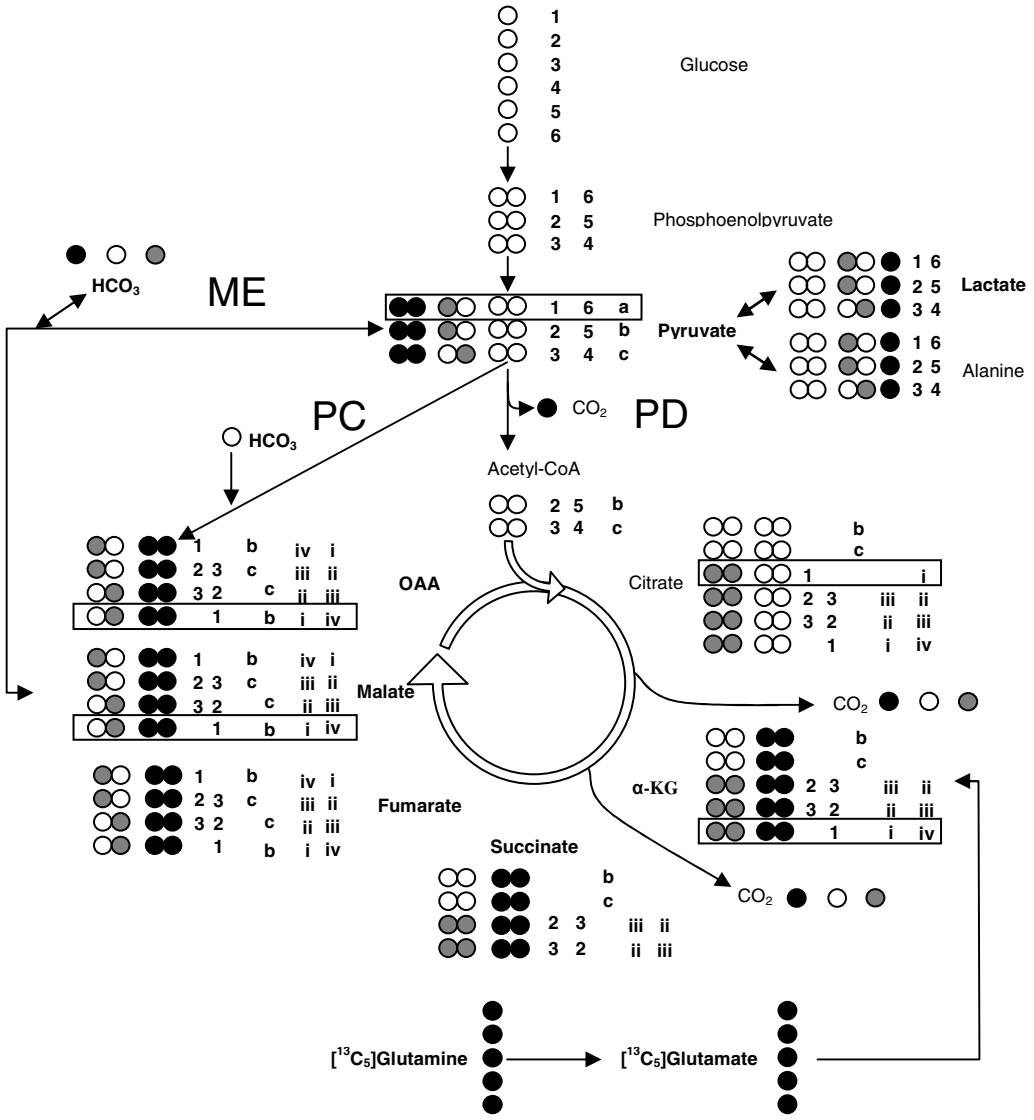
β-Hydroxybutyrate TBDMS derivative

Fragmentation of succinate β-hydroxybutyrate *t*-butyldimethylsilyl (TBDMS) derivatives under electric ionization

Appendix E



Schematic representing ^{13}C-labeling in glycolysis and Krebs cycle intermediates at the end of the first turn in the cycle with [$^{13}C_6$]glucose tracer.



Schematic representing ^{13}C-labeling in glycolysis and Krebs cycle intermediates at the end of the first turn in the cycle with [$^{13}C_5$]glutamine or [$^{13}C_5$]glutamine tracers.

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