The Effects of Oral Mannohexulose Supplementation on Canine Energetics and Macronutrient Utilization

by
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THE EFFECTS OF ORAL MANNOHEPTULOSE SUPPLEMENTATION ON CANINE ENERGETICS AND MACRONUTRIENT UTILIZATION

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In companion animals there is a growing interest in the use of nutraceuticals for weight management and healthy longevity. A nutraceutical can broadly be considered a food or a part of a food that provides a health benefit. The overall goal of this work was to evaluate the efficacy of mannoheptulose (MH), a seven carbon sugar found in avocados, as a novel nutraceutical for canines.

The metabolic effects of MH are ascribed to its ability to competitively inhibit hexokinases. When given at doses greater than 1 g/kg intravenously or intra-arterially, MH transiently elicits profound hyperglycaemia and hypoinsulinemia. These findings suggest that doses greater than 1 g/kg are supra-physiological. The ability of MH to transiently lower the insulin to glucagon ratio could be of benefit to overweight animals as it would promote lipid oxidation. However, few studies have examined the metabolic effects of low doses of MH on metabolism.

Three experiments were undertaken to assess the effects of low oral doses of MH (2 mg/kg, Chapter 3; 4 mg/kg, Chapters 4 and 5; 8 mg/kg, Chapter 2) on fasting and post-prandial energy expenditure and macronutrient metabolism in healthy adult dogs. Overall, oral MH supplementation, at any dose, did not significantly affect macronutrient metabolism. A pilot study using a dietary dose of 2 mg/kg MH did not find any statistically significant effect of MH on plasma glucose kinetics or energy expenditure. At a dietary dose of 4 mg/kg, MH decreased voluntary physical activity as measured by an accelerometer, and transiently decreased dietary thermogenesis and respiratory quotient. In contrast, MH fed at a dose of 8 mg/kg in the presence of a high carbohydrate relative to fat diet (54% carbohydrate,
11% fat) transiently increased dietary thermogenesis. These findings suggest that oral MH has differential
effects on energy expenditure depending on the administered dose and macronutrient composition of the
diet.

Irrespective of MH, these experiments are the first to assess post-prandial glucose and lipid
kinetics in canines using stable isotope tracer and indirect calorimetry techniques. Such information is
pertinent and provides a basis for quantitative measures of macronutrient metabolism in dogs.
DEDICATION

I wish to dedicate this dissertation to my son, Nixon William Taylor.

“You have brains in your head.
You have feet in your shoes
You can steer yourself
any direction you choose.”

- Dr. Seuss
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LIST OF ABBREVIATIONS

Acetyl-CoA carboxylase
5' adenosine monophosphate-activated protein kinase
Area under the curve
Body condition score
Body weight
Carbohydrate
Cyclic AMP
Energy expenditure
Energy restriction
Energy restriction mimic
Epigallocatechin gallate
Free fatty acids
Intra-arterial
Intravenous
Mannoheptulose
Peroxisome proliferator-activated receptors
Peroxisome proliferator-activated receptor-γ co-activator 1α
Protein kinase A
Rate of plasma appearance
Rate of plasma disappearance
Respiratory quotient
Resting energy expenditure
Subcutaneous
Thermic effect of food

ACC
AMPK
AUC
BCS
BW
CHO
cAMP
EE
ER
ERM
ECGC
FFA
i.a.
i.v.
MH
PPAR
PGC1α
PKA
R_a
R_d
RQ
REE
s.c.
TEF
Volume of CO₂

Volume of O₂
CHAPTER 1
REVIEW OF THE LITERATURE

1.1. Canine obesity

Epidemiological data indicate that up to 55 % of dogs in developed countries are overweight or obese (Courcier et al., 2010, Lund et al., 2006, Ward 2011). Clinically, an animal is considered overweight if its current body weight is 5 – 20 % greater than its ideal body weight and obese if its current weight is greater than 20 % of its ideal body weight. In general, ideal body weight is estimated using a body condition scoring (BCS) system (Laflamme et al., 1994; 1997). BCS determinations are based on body silhouette and palpation of adipose tissue depots and do not assess muscle mass. While body condition scoring is a practical method, its precision is debatable, especially in very obese animals. Furthermore, BCS has been shown to be a poor predictor of target weight in obese dogs undergoing weight loss (German et al., 2009). The inaccuracy of using BCS to estimate ideal weight, especially in obese dogs, makes weight loss strategies all the more difficult to implement.

The aetiology of obesity is multi-factorial. Several risk factors have been identified, including breed pre-disposition, age and spay/neuter status. Lund et al. (2006) found a higher prevalence of obesity in purebred dogs, particularly Dachshunds, Beagles, Cocker Spaniels and Golden Retrievers, compared to mixed breed dogs. The authors also found overweight and obese animals were more likely to be between the ages or 5 and 10 years. Additionally, neutering was identified as an important risk factor for weight gain. Excessive energy intake and sedentary lifestyle are also implicated in canine obesity. Lifestyle factors including excessive energy intake and sedentary lifestyle also contribute to obesity (Laflamme, 2012).
The pathophysiology of obesity is not fully understood. The expansion of subcutaneous adipose tissue by hyperplasia and hypertrophy is presumed to exceed a certain threshold whereby the vasculature cannot support the tissue creating hypoxia and cell death (Sorisky, 2008). As such, obesity is characterized by an inflammatory state and is associated with osteoarthritis, joint stress, musculoskeletal pain and reduced immune function (German, 2006; Zoran, 2010; Laflamme, 2012). Obese animals also deposit fat in other tissues including liver and skeletal muscle (Bachmann et al., 2001; Belfort et al., 2005). In healthy tissues, leptin released from adipocytes signals endogenous energy storage to the brain in addition to playing a primary role in fat metabolism adipose and skeletal muscle. Obese animals have increased circulating leptin, despite dysregulations in fat metabolism, suggesting that the cells are resistant to the effects of leptin (Lin et al, 1998). Similarly, the accumulation of fat in skeletal muscle interferes with insulin signaling creating insulin resistance and impaired glucose uptake (recently reviewed by Chow et al., 2010). Due to these dysregulations in nutrient metabolism, obesity is associated with metabolic diseases including diabetes and hypertension (German, 2006; Laflamme, 2012). Obesity also reduces health-related quality of life in dogs. Specifically, owners of obese dogs that underwent weight loss reported improved vitality and decreased emotional distress and pain with weight loss (German et al., 2012).

1.1.1. Factors affecting energy intake.

Obesity can be fundamentally considered a positive energy imbalance. However, many factors influence energy balance in canines. The human-animal relationship has a large influence on canine obesity. Specifically, energy availability is dictated by the owner. In a large survey of dog owners, Laflamme and colleagues (2008), found the majority of dogs in the United States and Australia were fed an extruded (dry) commercial diet (90 %) once (~40 %) or twice (~40 %) daily. Table scraps and commercial treats also comprise a significant
portion of the dog’s diet. Specifically, 20 % of dogs were fed table scraps daily and 40 % received treats. The feeding practices of owners of overweight and obese dogs have been shown to be different to that of owners of normal weight dogs (Kienzle et al., 1998). Not surprisingly, owners of overweight and obese dogs offer more meals, snacks and table scraps than owners of normal weight dogs. Furthermore, these owners spent more time watching their pet consume food (25 % of owners, >0.5 h) than normal weight owners (10.6 % of owners). This finding would suggest that owners of overweight animals consider feeding to be a positive experience. Interestingly, owners of obese dogs are more likely to be obese themselves (Kienzle et al., 1998).

Another important factor concerning energy intake is palatability. Palatability refers broadly to subjective food preferences. More specifically, palatability represents the integration of behaviours (i.e. learning, motivation), sensory cues (i.e. smell, sight, taste), environmental cues (i.e. physical location of the meal), and diet properties (i.e. macronutrient composition) related to the acquisition and consumption of food. As animals cannot communicate preferences, palatability remains inherently difficult to measure and interpret. Nevertheless, Hewson-Hughes et al. (2012) found that when given the opportunity to self-select, dogs consistently selected for diets offering ~30 % protein, 63 % fat and 7 % carbohydrate. These findings suggest that dietary fat is highly palatable. From an evolutionary perspective, selection of fat and protein over carbohydrate is not surprising. Ancestors of domestic dogs and wolves are social hunters that consume large mammals (higher fat to protein ratio than smaller prey). Furthermore, wolves kill prey every few days and would require the high energy density from fat to sustain prolonged fasting. Indeed, Acevedo et al. (2006) found canine muscle fibres (type II) express “unusually high” oxidative capacity in comparison to other mammals, including humans, horses, goats, rats, pigs. In addition, circulating free fatty acid (FFA) concentrations are higher in dogs than other species.
(McLelland et al. 1994). Together these findings support the notion that dogs are highly adapted to fat metabolism. Despite the dog’s unique adaptation to fat metabolism, analyses of 2,208 commercial foods manufactured by 204 companies, found that dry extruded diets contained approximately 30 % protein, 12 % fat and 43 % carbohydrate (measured as nitrogen free extract) (Hill et al., 2009). Whether the relatively high ratio of dietary carbohydrate to fat found in commercial diets leads to metabolic abnormalities and obesity in dogs is unknown. However, Axelsson et al. (2013) demonstrated that the domestication of dogs was accompanied by an epigenetic shift towards starch digestion, suggesting that dietary carbohydrates are available to dogs.

Given the multitude of commercial diets on the market, understanding the influence of individual dietary macronutrient intake on the health of companion animals is difficult. Experimentally, studies have used dogs as a model for humans, examining the influence of dietary fat content on obesity development. The consumption of high dietary fat (44% Kcal from fat) over 12 wk significantly increased subcutaneous and visceral adipose depots in dogs fed to weight maintenance. Increased adiposity was accompanied by hepatic insulin resistance in these animals (Kim et al., 2003). Similarly, Roccohini et al. (1997) observed peripheral insulin resistance, but no change in body weight, after 6 wk of high fat (54 % Kcal from fat, fed once daily) feeding. Conversely, Kim et al. (2007) noted increased body weight and a marked increase in trunk adiposity (76 %) following 6 wk of hypercaloric meal feeding (54 % of Kcal from fat). Interestingly, fasting glucose and FFA concentrations were unaltered with high fat feeding, whereas, insulin response to a meal was profoundly higher after 6 wk of high fat feeding. Together these findings suggest that high fat diets increase adiposity and impair insulin mediated glucose disposal.
1.1.2. Defining energy expenditure (EE)

Historically, obesity was considered to be the result of excessive energy intake. However, reduced physical activity and dysregulations in nutrient metabolism play an important role in the energy imbalance. In companion animals, the dietary energy available to the animal is considered to be the metabolizable energy, which represents the energy remaining after fecal, urinary and gaseous losses. The metabolizable energy that is expended (heat production) is expressed on a daily basis and referred to as energy expenditure (EE). EE encompasses the heat production associated with supporting basal metabolism (often referred to as resting energy expenditure, REE), physical activity (or work), and the digestion and assimilation of nutrients (referred to as dietary thermogenesis). REE comprises the largest component of EE (60 -70% of EE) and provides the basis of energy requirement recommendations. Physical activity can be quite variable and difficult to measure (25 - 35% of EE). Dietary thermogenesis describes the heat production associated with digestion and assimilation of nutrients, including bond breakage, nutrient absorption, and synthesis of digestive proteins and storage of nutrients (Baldwin, 1995). While dietary thermogenesis contributes the least to EE (less than 10%) it can be quite variable. When dietary thermogenesis is subtracted from metabolizable energy of the diet, net energy is determined. However, no studies in companion animals have sought to determine net energy.

Each component of EE is comprised of obligatory and facultative contributions. Obligatory thermogenesis includes the heat produced for basal metabolism of cells and organs (REE), the heat associated with involuntary physical activity, and the digestion and absorption of nutrients. In general, the obligatory contribution is relatively fixed, whereas, the facultative component is variable. Facultative thermogenesis encompasses voluntary physical activity (exercise), non-exercise physical activity (i.e. pacing), cold induced shivering and non-
shivering thermogenesis and nutrient metabolism (reviewed by van Marken Lichtenbelt and Schrauwen, 2011).

Quantifying energy expenditure (EE) and its individual components in dogs remains inherently difficult. In veterinary practice, resting energy expenditure (REE) is estimated using predictive equations and provides the basis of energy intake recommendations. REE is more accurately determined experimentally, using long term feeding trials, doubly labeled water or indirect calorimetry techniques. In the doubly labeled water technique, EE is derived from the exchange of isotopically labeled water ($^{2}$H$_{2}^{18}$O) given orally or intravenously with the body water and carbon pools. Labeled oxygen will be eliminated from the body as CO$_{2}$ and water, whereas, labeled hydrogen will be eliminated as water only. Therefore the difference between the rates of elimination represents CO$_{2}$ flux. The method assumes that the body water pool is constant, which may be accurate in adult maintenance, but not during periods of rapid growth, weight gain or weight loss (Wolfe and Chinkes, 2005). This technique is best suited for determining average EE over time (i.e. days or weeks), as it cannot distinguish individual components of EE. Indirect calorimetry estimates heat production based on O$_{2}$ consumption and CO$_{2}$ production. A detailed description of indirect calorimetry is presented in the Chapters 2 – 5. The method is advantageous in that dietary thermogenesis can also be determined by subtracting post-prandial heat production from REE. The relative amounts of carbohydrate, fat, and protein (if urine is collected) oxidized can also be estimated. However, the use of calorimetry in dogs has been limited.

Overweight and obese animals have an increased ratio of adipose to lean tissue. Adipose tissue being less metabolically active than muscle has led to the assumption that lowered REE associated with increased adiposity contributes to obesity. However, REE is higher in obese animals compared to lean animals due to the additional energy required to support basal metabolism of the excess body mass. Therefore, it is unclear whether changes in
REE contribute to the development of obesity. Similarly, the contribution of dietary thermogenesis to total heat production in obese animals is poorly understood. As obesity is associated with dysregulations in nutrient metabolism, it is likely that dietary thermogenesis would be altered in the obese state. However, no studies in dogs have examined dietary thermogenesis as it relates to obesity. In fact, only one study has examined dietary induced thermogenesis in healthy Beagles at weight maintenance (Pouteau et al. 2002). As high fat feeding has been shown to increase adiposity and induce metabolic abnormalities, the influence of dietary macronutrient concentrations on dietary thermogenesis would be of particular interest.

The final component of energy expenditure, physical activity, encompasses complex behaviours (i.e. spontaneous activity, exploration and exercise) that are elicited by a wide range of internal and external stimuli, making it difficult to measure. In normal weight laboratory Beagles, physical activity has been shown to decline with age and be affected by housing area (size), access to outdoors, lighting, and human interaction (Siwak et al. 2005). It is important to acknowledge that not all physical exercise is beneficial. For example, physical activity includes stereotypical behaviours such as pacing, and increased physical activity has been observed in cognitively impaired dogs (Siwak et al., 2005). Determining physical activity in human-owned animals has been attempted using owner surveys. In several surveys performed in the United States and Australia, 40 – 60 % of owners reported walking their dog 1 -2 days per week (reviewed by Cutt et al., 2007). However, the duration or intensity of exercise bouts was not reported. The use of pedometers to measure physical activity of dogs in their home environment is currently being validated (Michel et al., 2008; Wrigglesworth et al., 2011). There is evidence to suggest that overweight and obese dogs receive less exercise than lean animals. In comparison to owners of normal weight dogs, owners of overweight and obese dogs prioritized food over exercise, as they reportedly would trade their dogs’ food
over a walk (Kienzle et al., 1998). In support, Bland et al. (2010) found that overweight dogs received less exercise than normal weight dogs. Specifically, 19% of overweight dogs did not receive any walks vs. 4% of normal weight dogs.

In conclusion, obesity is the most prevalent nutritional disorder affecting companion animals. The pathophysiology of obesity is complex. The key features of obesity include systemic inflammation, dysregulations in nutrient metabolism, and reduced health related quality of life. The aetiology of obesity is not fully understood. There are certain animal risk factors, including age, genetics and spay and neuter status. The human-animal relationship, specifically the feeding and activity practices of owners is an important contributor to obesity.

1.2. Nutritional management of canine obesity

Nutritional management remains the front line treatment for canine obesity. Traditional management strategies are centered on total energy restriction (ER) and/or therapeutic (weight loss) diets. However, it is increasingly appreciated that a multidimensional approach to weight loss is needed. Owner education, physical activity, and adjunct therapies, including nutraceuticals, all play a role in obesity management.

1.2.1. Energy restriction (ER)

Energy restriction (ER), without malnutrition, is the most robust and repeatable strategy for weight management across species (reviewed by Fontana and Klein, 2007). Indeed, in a lifelong study of Labrador Retrievers, 25% ER decreased body weight and attenuated loss of lean body mass (Kealy et al. 2002). Furthermore, ER increased median and maximal lifespan and provided protection from age related disease development (reviewed by Lawler et al. 2008). The mechanisms by which ER exerts these beneficial effects are not completely understood. One theory is that ER alters nutrient sensing pathways leading to increased mitochondrial biogenesis and efficiency and enhanced insulin sensitivity (reviewed
by Cantó and Auwerx, 2011). These effects are thought to be mediated, in part, by the activation of the key energy sensing protein, 5' adenosine monophosphate-activated protein kinase (AMPK). Once activated, AMPK promotes ATP-generating pathways (i.e. glucose and fatty acid oxidation), and reduces anabolic processes (i.e. synthesis of fat, glucose and protein) creating a direct link between cellular energy status and whole body EE. Despite the noted benefits of ER in dogs, the rate of weight loss observed in client-owned animals undergoing ER is much slower than that observed experimentally (German et al. 2009). The lack of success clinically with ER strategies is thought to be attributed to significant behavioural changes in pets, including begging, aggression and scavenging. Such behaviours are often perceived as negative by the owner, thereby straining the owner-animal bond and presumably resulting in poor owner compliance with ER strategies.

1.2.2. Therapeutic diets

Therapeutic diets are commonly used for weight management in dogs. These diets aim to reduce energy intake and stimulate satiety by altering the macronutrient profile of the diet. In general, therapeutic diets provide low dietary fat, moderate to high dietary protein concentrations and include a source of soluble fibre. The purpose of lowering dietary fat content is to reduce the energy density of the diet; fat is more energy dense (9 kcal/g) than protein (4 kcal/g) or carbohydrate (4 kcal/g). Dietary fiber can also be used to lower the energy density of the diet. Cole et al. (1999) observed a linear decrease in dry matter and apparent gross energy digestibility with increasing inclusion of total dietary fiber. In support Burkhalter et al. (2001) found reduced dry matter digestibility with increasing ratios of insoluble to soluble fiber intakes. The addition of insoluble fiber increases fecal volume, an undesirable outcome for consumers. Soluble fibers, on the other hand, have been shown to be beneficial for gut health and lower post prandial glucose and insulin response (Respondek et al. 2008; Carciofi et al., 2008; Knapp et al., 2010). Dietary protein aids in weight loss through
several mechanisms, including promoting satiety, increasing dietary induced thermogenesis, and preserving lean body mass (reviewed by Westerterp-Plantenga et al., 2012). Indeed, dietary protein has been shown to promote satiety (Vester Boler et al., 2011) and induce weight loss (Hannah and Laflamme, 1998; Diez et al., 2002; Bierer and Bui, 2004) in dogs. However, providing high dietary protein in combination with high dietary fiber has a greater effect on satiety (Weber et al., 2007) and weight loss (German et al., 2010) than high dietary protein or fiber alone.

1.2.3. Nutraceuticals

In canines (and humans) there is a growing interest in the use of nutraceuticals for weight management. While a universal definition does not exist, a nutraceutical can broadly be considered a food or a part of a food that provides a health benefit. There is a paucity of research concerning the efficacy of nutraceuticals with respect to weight management strategies for dogs. To my knowledge only two compounds, diacylglycerol and epigallocatechin gallate (EGCG), have been investigated for weight loss in dogs. Dietary supplementation of an oil enriched with diacylglycerol decreased body weight in obese Beagles (Umeda et al. 2006), and lowered post-prandial serum triglycerides in normal weight (Bauer et al. 2006) and obese dogs (Umeda et al. 2006). These findings are in agreement with humans studies (reviewed by Hibi et al., 2009). Triacylglycerols comprise a substantial proportion (up to 90 %) of the fat found in most vegetable oils. However, through enzymatic processes, oils enriched in diacylglycerol are commercially available. In the intestinal lumen, dietary triacylglycerols are emulsified by bile salts and hydrolyzed to monoglycerides and FFAs by pancreatic lipases. Without further metabolism, these products aggregate to form mixed micelles which are taken up into the epithelium, synthesized into triacylclycerols, and packaged into chylomicrons. It is believed that the 1,3-diacylglycerol isomer (predominant form of diacylglycerol in enriched oils) is hydrolyzed to glycerol and FFA in the intestinal
lumen, thereby limiting their incorporation into chylomicrons. The glycerol and FFA derived from 1,3-diacylgerol is thought to be directly transported to the liver (Tada and Hoshida, 2003). The observed increase in whole body fat oxidation associated with diacylglycerol supplementation in human and rodent studies is thought to be attributed to the increased availability of FFA.

Numerous human and rodent studies have demonstrated the beneficial health effects of green tea consumption. Specifically, the polyphenol, EGCG found in green tea, has positive effects on weight loss and glucose tolerance (reviewed by Sudathip et al. 2011). In agreement green tea extract improved insulin sensitivity and decreased circulating triglycerides in obese insulin resistant Beagles (Serisier et al., 2008). Furthermore, the authors observed increase expression of peroxisome proliferator-activated receptors alpha (PPARα) in skeletal muscle and increased expression of PPARγ, glucose transporter 4 (GLUT4) and adiponectin in adipose. PPAR are a group of nuclear transcription factors that regulate the expression of many genes related to whole body energy homeostasis. PPARα is predominantly expressed in the liver, where it activates fat oxidation and PPARγ regulates adipogenesis and lipid metabolism within white adipose tissue. More specifically, PPARγ regulates the expression of GLUT4 and several adipokines, including adiponectin, resistin and leptin (reviewed by Ahmadian, 2013). Leptin and adiponectin activate AMPK in muscle, thereby increasing fatty acid oxidation (Kubota et al., 2006). In agreement, EGCG has been shown to activate AMPK in vitro (Hwang et al., 2005).

The ability of plant compounds to modulate pathways of fatty acid metabolism could be of particular benefit to overweight and obese animals and humans. Specifically, promotion of fat oxidation associated with prolonged daily feeding of nutraceuticals would be expected to reduce fat mass and ultimately body weight. Whether nutraceuticals alter daily EE remains unclear. In humans, short term supplementation with EGCG has not had any effect on REE or
diet induced thermogenesis. However, when EGCG was given in combination with caffeine (Dulloo et al., 1999; Berube-Parent et al., 2005) or resveratrol (polyphenol found in grapes) (Most et al., 2014) both REE and diet induced thermogenesis were greatly increased.

1.3. 5' adenosine monophosphate-activated protein kinase (AMPK), a critical regulator of cellular and whole body energy metabolism.

It is well-documented that AMPK acts as a master regulator of cellular and whole body energy homeostasis. As such, many studies have investigated AMPK activation as the mechanism by which energy restriction and nutraceutical compounds exert their beneficial effects. Indeed, several nutraceuticals (i.e. resveratrol, capsaicin) and pharmacological agents (i.e. metformin) have been shown to activate AMPK (Hardie, 2011).

AMPK is a heterotrimeric serine/threonine kinase consisting of a catalytic alpha (α1/α2) subunit and regulatory beta (β1/ β2) and gamma subunits (γ1-γ3). Conditions which lower the ATP to ADP (AMP) ratio, including exercise and nutrient deprivation, lead to AMPK activation by reversible phosphorylation of the alpha subunit. There is evidence to support that hypothalamic AMPK is under hormonal control (reviewed by Stark et al., 2013). Specifically, ghrelin and adiponectin are believed to activate AMPK, whereas, leptin and insulin reduce AMPK phosphorylation. However, the mechanisms by which these hormones modulate hypothalamic AMPK are not fully understood. Current evidence is based exclusively on in vitro reports.

The downstream effects of AMPK activation are widespread. Acutely, AMPK increases glucose uptake by stimulating GLUT4 translocation and activation of GLUT1. AMPK promotes fatty acid oxidation and suppresses fat synthesis by inhibiting acetyl CoA carboxylase (ACC). Inhibition of ACC leads to a decrease in its product malonyl CoA, an allosteric inhibitor of carnitine palmitoyltransferase I, thereby increasing fatty acid uptake into
mitochondria. Protein synthesis is also suppressed by AMPK, via inhibition of the mammalian target of rapamycin complex 1 signaling pathway. Transcription of gluconeogenic and lipogenic genes is down regulated by AMPK. Furthermore, AMPK directly phosphorylates the transcriptional co-activator, peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α). PGC-1α is a transcriptional coactivator that interacts with several transcription factors to regulate genes involved in thermogenesis, mitochondrial biogenesis, fatty acid uptake and oxidation, glucose utilization, skeletal muscle fibre switching and heart development (Benton et al., 2008).

1.4. Mannoheptulose (MH)

The efficacy of mannoheptulose (MH) as a novel nutraceutical for canines is under preliminary investigation. MH is a seven carbon sugar that was first discovered and isolated in avocado (Persea americana, Lauraceae) by La Forge in 1917. Although MH is found in other plants, such as alfalfa, fig and primrose, high contents of MH (up to 10% of tissue dry weight) have only been reported in avocados (Kappler-Tanudiyaya et al., 2007). The metabolic effects of MH were first examined by Roe and Hudson (1936). The authors found that intraperitoneal (i. p.) administration of MH (2 – 5 g/kg) to rabbits induced hyperglycemia within 1 h and persisting for 4 h. Indeed, MH has been shown to induce transient hyperglycemia in several species, including dogs (Table 1.1).
<table>
<thead>
<tr>
<th>Reference</th>
<th>Species, BW, (N)</th>
<th>MH Dose</th>
<th>Route</th>
<th>Metabolic Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoshi and Shreeve, 1969</td>
<td>Bar Harbor strain (C57BL/65) of hyperglycemic-obese mice (5)</td>
<td>20 mg s.c.</td>
<td>No change in blood glucose</td>
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<td>Argiles et al., 1992</td>
<td>Female Wistar rats, 150-180 g</td>
<td>1 g s.c.</td>
<td>Decreased glucose oxidation (in response to oral glucose load, $^{14}$C glucose given enterally) Increased blood glucose Decreased insulin:glucose</td>
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<td>Simon and Kraicer 1965</td>
<td>Wistar Rats, 200 g (12)</td>
<td>2 g/kg s.c.</td>
<td>Hyperglycemia</td>
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<td></td>
<td>Wistar Rats, 200 g (9) plus 0.3 U insulin</td>
<td>2 g/kg s.c.</td>
<td>No change in blood glucose</td>
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<tr>
<td>Frenkle 1972</td>
<td>Rats, 184 g</td>
<td>2 g/kg s.c.</td>
<td>Hyperglycemia Decreased RQ</td>
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<tr>
<td>Muller et al. 1971</td>
<td>Mongrel dogs, 11 - 25 kg (4)</td>
<td>1.2 g/kg i.v. (1 h constant infusion)</td>
<td>Insulin completely blocked (i.e. 0 values) 5 fold increase in glucagon</td>
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<td></td>
<td>Mongrel dogs, 11 - 25 kg (4)</td>
<td>1.2 g/kg i.v. (1 h constant infusion) plus 0.2 U/kg h insulin</td>
<td>Hyperglycemia 3 fold increase in glucagon</td>
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<tr>
<td>Coore and Randle, 1964</td>
<td>Rabbits, 1.6 ± 0.2 kg (6)</td>
<td>2 g/kg i.v.</td>
<td>Hyperglycemia persisting 4.5 h after administration</td>
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<tr>
<td>Study</td>
<td>Species</td>
<td>Treatment</td>
<td>Dose</td>
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<tr>
<td>Issekutz et al., 1974</td>
<td>Mongrel dogs, 11-16 kg</td>
<td>1.5 g/kg</td>
<td>i.a.</td>
<td>Mongrel dogs, 11-16 kg (5) treated dogs (6)</td>
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<tr>
<td>Langhans 1983</td>
<td>Mongrel dogs, 11-16 kg</td>
<td>1.5 g/kg</td>
<td>i.a.</td>
<td>Mongrel dogs, 11-16 kg (6) treated with methyprednisolone</td>
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<td></td>
<td>Rats</td>
<td>400 mg/kg</td>
<td>i.p.</td>
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<td>High fat vs. high CHO</td>
<td>i.p.</td>
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<tr>
<td>Klain 1976</td>
<td>Holtzman rats, 350 g</td>
<td>2 g/kg</td>
<td>i.p. (msmts)</td>
<td>Increased liver F6Pase &amp; PEPase activities</td>
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<td>Sprague-Dawley albino</td>
<td>4 g/kg vs.</td>
<td>oral (force fed) vs. i.p.</td>
<td>Both routes produced similar magnitude of hyperglycemia, but maximal glycemia reach at 1 h with i.p. vs. 2 h with oral.</td>
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<td>Viktora et al. 1969</td>
<td>Sprague-Dawley albino</td>
<td>0.5 g/kg</td>
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<td>rats, 250 g (5/group)</td>
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<td>Sprague-Dawley albino</td>
<td>0.25 g/kg</td>
<td>i.p.</td>
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<td>rats, 250 g (10/group)</td>
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<td>Davenport et al., 2010 a</td>
<td>Labrador Retrievers and</td>
<td>2, 10, 20</td>
<td>Oral</td>
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<td></td>
<td>Fox Terriers</td>
<td>mg/kg</td>
<td>(capsule)</td>
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<tr>
<td>Viktora et al. 1969</td>
<td>Humans (8)</td>
<td>33 – 200</td>
<td>Oral</td>
<td></td>
</tr>
</tbody>
</table>
Viktora et al. 1969  
**Rhesus monkeys, 4.4 - 5.3 kg (3/group)**  
1 g/kg vs. 2 g/kg vs. 4 g/kg  
Oral (gelatin solution)  
All doses resulted in hyperglycemia and decreased plasma insulin

Viktora et al. 1969  
**Mongrel dogs, 5.8 – 8.9 kg (8)**  
4 g/kg  
Oral (mixed with beef)  
Hyperglycemia persisting 5 h after administration

Johnson and Wolff, 1970  
**Human (6)**  
5 g vs. 10 g vs. 20 g  
Oral (in water)  
All doses resulted in hyperglycemia (~15% above fasting) that disappeared by 6 h. Nausea and diarrhea noted in all subjects who received the 20 g dose.

**Human (12)**  
10 g  
Oral (in water)  
Hyperglycemia  
No difference in plasma insulin.  

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1 Only available information is presented, many studies did not report body weight or sample size  
2 s.c., subcutaneous; i.p., intraperitoneal; i.v. intravenous; i.a., intra-arterial  
3 All studies were conducted in fasted animals

The metabolic effects of MH are ascribed to its ability to competitively inhibit hexokinase IV (HK IV) in brain, liver and pancreas (Crane and Sols, 1954; Coore and Randle, 1964). In comparison to hexokinases 1-3, HK IV has a much higher Km for glucose (5 mmol/L vs. 0.02 mmol/L) and is not allosterically inhibited by its product, glucose-6-phosphate. When given at doses greater than 1 g/kg BW (i.v. or i.a.), MH profoundly reduces the insulin to glucagon ratio, via glucagon hyper-secretion. Indeed, combined administration of MH and insulin does not prevent glucagon hyper-secretion, yet simultaneous administration of MH and tolbutamide stimulates insulin secretion (Simon and Kraicer, 1965). While circulating glucose concentrations are the primary regulator of insulin and glucagon release, pancreatic alpha cells may be more sensitive to glucose than beta cells. Specifically, alpha cells have a narrow threshold for suppression of glucagon release (2 – 3 mmol/L) in comparison to the beta cell threshold for stimulation of insulin release (3 – 6 mmol/L). Furthermore, glucose uptake does not appear to be limiting for glucose utilization.
within alpha cells, as rates of glucose utilization are similar between alpha and beta cells. Interestingly, alpha cells express substantially higher levels of plasma membrane monocarboxylase transport proteins, lactate dehydrogenase, and pyruvate carboxylase than beta cells (Sekine et al., 1994; Schuit et al., 1997; Zhoa et al., 2001). Together, these findings suggest that pancreatic alpha cells rely on anaerobic metabolism. Therefore, it is not surprising that inhibition of glycolysis, as imposed by MH, would stimulate glucagon hyper-secretion.

High circulating glucagon relative to insulin is a fundamental characteristic of fasting metabolism. In fasting, hepatic glycogenolysis and gluconeogenesis provide glucose for tissues such as the brain. In comparison to the role of insulin in glucose homeostasis, less is known about the regulation of glucose homeostasis in periods of low glucose availability. Many of the responses to low glucose are believed to be mediated by a lack of insulin. However, glucagon has been shown to stimulate hepatic glycogenolysis and gluconeogenesis through its second messenger, cAMP. cAMP-dependent protein kinase A (PKA) activates the bZIP family of transcription factors, increasing the expression of gluconeogenic enzymes. In agreement, MH (> 1 g/kg) has been shown to increase circulating cAMP and alanine concentrations and result in uncontrolled hepatic glucose output due to considerable increases in the activity of gluconeogenic enzymes fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase (Muller et al., 1971; Issekutz et al. 1974; Klain et al., 1976). Fasting stimulates lipolysis in adipose providing FFA to skeletal muscle for oxidation. These responses are coordinated largely by PPAR activation. As discussed previously, PPAR regulate lipid metabolism through several mechanisms, including adiponectin and leptin release and activation of AMPK.

It is important to note that the metabolic responses observed in previous studies were well outside normal physiological ranges suggesting that doses greater than 1 g/kg BW are
supra-physiological. Specifically, up to five fold increases in blood glucose (22 – 28 mmol/L) coupled with a near complete blockage of insulin secretion (0 – 2 U/mL) have been noted. Moreover, the metabolic changes elicited by MH doses over 1 g/kg, were likely further exaggerated due to the fact animals were fasted (insulin to glucagon already reduced). It is also possible that intravenous (i.v.) or intra-arterial (i.a.) administration of MH led to inflated responses, as MH was not subject to intestinal and first pass splanchnic metabolism.

Whether low doses of MH administered orally could provide a health benefit is largely unknown. Current evidence is limited to two published abstracts (Davenport et al., 2010\textsuperscript{a,b}). The authors demonstrated that ingested MH appears into the plasma of Labrador Retrievers fed a MH containing diet (2 mg/kg BW), peaking between 2-4 h after ingestion and disappearing within 24 h (Davenport et al., 2010\textsuperscript{b}). These findings demonstrate that ingested MH is available to the animal. Davenport et al. found that fasting insulin was lower in adult Labrador Retrievers fed a gelatin capsule containing 2, 10 and 20 mg MH/kg BW compared to dogs given a placebo capsule.

1.5. Thesis objectives and hypotheses

Based on the preliminary studies of Davenport et al. (2010\textsuperscript{a,b}), dietary doses ranging 2 - 8 mg/kg BW were used to evaluate the metabolic effects of MH. As the end goal of this work was to create a commercial dog food containing MH, MH was incorporated into the diet (Chapters 3 – 5) or fed as a supplement with the meal (Chapter 2). Our hypothesis was that MH induced inhibition of glucokinase in the pancreas would reduce glycolytic flux thereby inhibiting (or delaying) insulin secretion. MH inhibition hexokinases was expected to limit glucose as a substrate of energy production and consequently increase fat utilization. If glycolysis was inhibited substantially, there may be a decrease in intracellular ATP. Lowering the ATP to ADP ratio would activate AMPK. Once activated, AMPK directly decreases EE
by inhibiting anabolic pathways, and while at the same time increases ATP production via glucose and fat oxidation. However, as glucose availability would be limited, fat utilization would be further increased.

To test this hypothesis, three experiments were performed each with increasing doses of MH (2, 4 and 8 mg/kg BW; Chapters 3, 4, and 2 respectively). In all studies EE, REE and dietary thermogenesis, and respiratory quotient (RQ) were determined by indirect calorimetry. RQ was used to estimate the relative amount of carbohydrate to fat being oxidized. Because MH directly inhibits glycolysis, MH effects on glucose metabolism was assessed using several approaches, including measurement of fasting and post-prandial serum glucose concentrations (Chapters 2 – 5) and plasma glucose kinetics using stable isotope tracers in combination with indirect calorimetry (Chapters 3 and 5). The consequence of reduced glucose availability was assumed to be increased fat utilization. To test this hypothesis plasma glycerol and free fatty acid kinetics were assessed (Chapter 5) and the phosphorylation of AMPK in skeletal muscle was quantified (Chapters 3 and 4). Interpretation of the kinetic data generated from isotope tracer studies requires the use of mathematical modeling. Chapter 6 describes a novel two-pool model for assessing glucose kinetics. Appendix A and B review the existing models for the study of whole body glucose and insulin kinetics respectively.

The benefits of daily MH feeding were expected to be transient increases fat utilization (due to decreased glucose availability) without altering dietary intake. In the short term, transient increases in fat utilization may not impact body composition. However, with prolonged daily feeding of MH, increased fat oxidation was expected to lead to a reduction of body fat mass while preserving lean body mass. Reducing body fat would be of particular benefit to overweight and obese animals. Furthermore, altering body composition without limiting the animal’s energy intake would be desirable for pet owners.
CHAPTER 2

Mannoheptulose has differential effects on fasting and post-prandial energy expenditure and respiratory quotient in adult Beagle dogs fed diets of different macronutrient contents

2.1. Abstract

This study aimed to determine the effects of mannoheptulose (MH) (8 mg/kg BW) on energy expenditure (EE), respiratory quotient (RQ) and glycaemic response in healthy adult Beagle dogs (N = 8, 9.62 ± 0.31 kg, BCS 3.0). The study was designed as replicated 4 × 4 Latin squares with a 2 × 2 factorial treatment structure. The dietary treatments were low carbohydrate (CHO) relative to fat diet (LC; 31 % CHO, 28 % fat as fed) with placebo (0 mg/kg) or MH supplement and high CHO relative to fat diet (HC; 54 % CHO, 11 % fat as fed) with placebo (0 mg/kg) or MH supplement. Dogs were fed to maintain body weight (HC and HC+MH 867 ± 71 Kcal and LC and LC+MH 847 ± 68 Kcal). Resting and post-prandial (0 - 4 h; 5 – 10 h; 11-17 h; 18-23 h) EE and RQ were determined by indirect calorimetry (d 12 or 14). Glycaemic response to a meal (24 h) and plasma MH concentrations were determined on d 12 or 14. Plasma MH followed first order kinetics, confirming that MH is absorbed and available to the animal. In the presence of high dietary CHO, MH increased post-prandial EE (5 – 10 h only), suggesting MH increased dietary induced thermogenesis. In contrast to earlier reports, MH did not affect serum glucose or insulin in this study. Irrespective of MH, dogs adapted RQ to diet composition and dogs consuming the LC diet had a greater incremental AUC for glucose, but not insulin, than dogs consuming the HC diet.

Key Words: Mannoheptulose, Beagle, energy expenditure, respiratory quotient

2.2. Introduction

Obesity is a major health concern for companion animals, with 55% of dogs in the United States reported to be overweight or obese (Ward et al., 2011). As in humans, canine obesity is associated with metabolic diseases (i.e. insulin resistance and diabetes), and the aetiology is multi-factorial (i.e. genetic disposition, advancing age and sedentary lifestyle). Overweight dogs are commonly treated with nutritional management strategies that include specially formulated therapeutic (weight loss) diets and/or total calorie restriction (Laflamme, 2006). Weight loss diets achieve calorie dilution by altering the macronutrient content of the diet. Generally these diets contain low fat and high carbohydrate (CHO) concentrations. As dogs preferentially utilize fat over CHO for energy (Hill, 1998), it is unclear whether increased inclusion of dietary CHO relative to fat infers any metabolic benefit beyond calorie dilution for managing obesity. Studies in humans have been controversial with some reporting greater weight loss in people consuming high fat, low CHO diets (Brehm et al., 2005; Due et al., 2008). It is well-established that energy restriction (ER), without malnutrition, is the most robust and repeatable strategy for weight management. In addition, ER has been shown to delay the ageing process by increasing median lifespan in yeast, flies, nematodes, rodents and dogs (Kealy et al., 2002; Piper and Bartke, 2008). Studies in humans and non-human primates have not found ER to increase lifespan, but have shown that ER provides protection from age related disease development (Roth et al., 2007; Fontana and Klein, 2007) that would result in increased quality of life. How ER exerts these beneficial effects is incompletely understood. However, increased metabolic efficiency, enhanced insulin signaling pathways, and reduced oxidative damage are all hallmarks of ER (Fontana and Klein, 2007). Unfortunately, ER generally results in significant behavioural changes in pets that are often perceived as negative by the owner (i.e. begging and aggression) thereby straining the human-animal bond (Roudebush et al., 2008).
Energy restriction mimetics provide an alternative to ER. As the name implies, compounds with calorie restriction mimetic activity mimic the metabolic, hormonal and physiological effects associated with ER without altering dietary caloric intake (Ingram et al., 2006). Mannoheptulose (MH) is a seven carbon sugar found in avocados that has been proposed as a calorie restriction mimetic (Roth et al., 2009). Early research demonstrated MH functions as a glycolytic inhibitor by its ability to competitively inhibit hexokinases (EC 2.7.1.1) (Viktora et al., 1969). Dogs given MH doses ranging 1 – 2 g/kg BW (intravenous or intra-arterial) exhibit a transient diabetic state, characterized by marked hyperglycemia, dramatically decreased insulin to glucagon ratio and increased hepatic glucose output (Muller et al., 1971; Issekutz et al., 1974; Klain et al., 1976; Cryer, 2006). These responses were well outside normal physiological ranges suggesting that the dosage was supra-physiological. However, the effects of low doses of MH are largely unknown. Furthermore, early studies only examined MH effects on fasting metabolism. As MH inhibits glucokinase in intestine, liver and pancreas, it is of interest to examine MH effects on post-prandial metabolism.

The objective of this study was to determine the effects of avocado-derived MH on glycaemic response, macronutrient oxidation, and energy expenditure (EE) in adult Beagle dogs. Given MH’s known role as a glycolytic inhibitor, we expected MH to differentially affect these outcomes when fed in the presence of low or high dietary CHO concentrations.

2.3. Methods and Materials

2.3.1. Animals and housing

All procedures were approved by the Institutional Animal Care and Use Committee of P&G Pet Care (Mason, OH). A total of eight adult Beagle dogs (seven spayed females and one neutered male, 9.62 ± 0.31 kg body weight; 7.55 ± 0.39 y of age) were used in this study. All dogs were at optimal body condition score 3.0 (5-point scoring scale, adapted from
Laflamme, 1997) at the initiation of the study. All dogs resided at the P&G Pet Health and Nutrition Center (Lewisburg, OH) and were considered healthy based on a general health evaluation by a licensed veterinarian prior to study. Dogs were pair housed with free access to water and indoor and outdoor runs. Indoor runs were maintained on a 12 h light and dark cycle, in addition to natural light, and were equipped with raised canvas beds, toys and heated flooring. Outdoor runs were equipped with toys and play yard equipment. All dogs received 40 min of supervised group exercise and socialization in a separate fenced yard daily.

2.3.2. Study Design

The study was designed as replicated 4 × 4 Latin squares with a 2 × 2 factorial treatment (diet) structure. Each dog (N = 8) was randomly allocated to a Latin Square sequence defining the order to receive the four dietary treatment combinations. The total duration of the study was fifty-six days with each treatment leg (or period) lasting fourteen days. Indirect calorimetry and a meal challenge were performed during each fourteen day treatment leg. The indirect calorimetry method only allowed for four dogs to be measured per day (four calorimetry chambers total). Dogs were divided into two groups of four balanced by treatment sequence (each diet × MH combination represented on each day). Indirect calorimetry was performed on day twelve for group one followed by the meal challenge on day fourteen. In contrast, dogs in group two underwent the meal challenge on day twelve followed by indirect calorimetry on day fourteen.

2.3.3. Diets and Feeding

The dietary treatments were a low CHO relative to fat diet with placebo supplement (LC) or MH containing supplement (LC+MH) and a high CHO relative to fat diet with placebo supplement (HC) or MH containing supplement (HC+MH). The diets were nutritionally balanced and complete and made of identical ingredients and formulated to deliver an equivalent ratio of protein to energy (Table 2.1). The supplements were made of cocoa butter.
with a baker’s white coating (94 % dry matter, 25 % fat, 5.7 % protein, 5.9 % ash, 0.2 % crude fibre). MH was incorporated into the supplement by mixing whole-fruit avocado extract with the cocoa butter and baker’s white coating. The MH enriched avocado extract was produced using commercially available avocados (Hass variety). Frozen, whole avocados comprised of the flesh, peel and pit were initially ground before suspension in water (1:3 w/w). The resultant slurry was centrifuged to remove non-aqueous solids. A series of microfiltration (de-oiling), ultrafiltration (10 kDa) and nanofiltration (100 kDa) was used to produce the MH-enriched fraction. Lyophilization was used to form the final crystalline powder yielding 18 % MH (Massimino et al., 2005).

Dogs were fed to maintain body weight, based on their historical caloric intake records (HC and HC+MH 867 ± 71 Kcal and LC and LC+MH 847 ± 68 Kcal). For one week prior to study initiation dogs were fed their initial test diet (“wash in” period). Dogs were individually fed their daily ration of test diet in two meals (08:00 h and 13:00 h). At each meal, dogs received a supplement containing either 0 mg MH (placebo) or 40 mg MH. The daily dose of MH was ~ 8 mg/kg for each dog (two supplements per dog per d).
Table 2.1. Ingredient and chemical composition of the test diets, formulated with low (LC) and high (HC) concentrations of dietary carbohydrate.

<table>
<thead>
<tr>
<th>Ingredient (%)</th>
<th>Composition</th>
<th>LC</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn meal</td>
<td></td>
<td>18.5</td>
<td>32.0</td>
</tr>
<tr>
<td>Sorghum grain, ground whole</td>
<td></td>
<td>18.5</td>
<td>31.9</td>
</tr>
<tr>
<td>Chicken meal</td>
<td></td>
<td>18.5</td>
<td>12.8</td>
</tr>
<tr>
<td>Chicken byproduct meal</td>
<td></td>
<td>18.4</td>
<td>12.8</td>
</tr>
<tr>
<td>Chicken fat</td>
<td></td>
<td>15.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Beet pulp, dried</td>
<td></td>
<td>3.6</td>
<td>2.9</td>
</tr>
<tr>
<td>Chicken broth</td>
<td></td>
<td>2.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Chicken liver</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Fish oil</td>
<td></td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Mineral mix</td>
<td></td>
<td>2.1</td>
<td>1.7</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td></td>
<td>0.4</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Analyzed Chemical Profile (DM basis)

<table>
<thead>
<tr>
<th></th>
<th>Composition</th>
<th>LC</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (%)</td>
<td></td>
<td>92.8</td>
<td>90.8</td>
</tr>
<tr>
<td>Crude protein (%)</td>
<td></td>
<td>32.2</td>
<td>27.0</td>
</tr>
<tr>
<td>Fat (%)</td>
<td></td>
<td>27.9</td>
<td>11.2</td>
</tr>
<tr>
<td>Crude fiber (%)</td>
<td></td>
<td>2.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Ash (%)</td>
<td></td>
<td>7.1</td>
<td>5.6</td>
</tr>
<tr>
<td>Nitrogen Free Extract (%)</td>
<td></td>
<td>30.6</td>
<td>53.6</td>
</tr>
<tr>
<td>Gross energy (Kcal/g)</td>
<td></td>
<td>5.9</td>
<td>5.0</td>
</tr>
<tr>
<td>Metabolizable energy (Kcal/kg)</td>
<td></td>
<td>4574</td>
<td>3773</td>
</tr>
<tr>
<td>Protein to gross energy ratio</td>
<td></td>
<td>5.4</td>
<td>5.4</td>
</tr>
</tbody>
</table>
2.3.4. Indirect Calorimetry

Respiratory gas exchange measurements were conducted via whole-body indirect calorimetry on day twelve or fourteen of each treatment leg. The calorimetry chambers (76 cm × 53 cm × 61 cm, L × W × H) were made of clear plexiglass and fitted with a hinged access top door for providing food to the dog. Chambers were designed as open-circuits with room air pulled into the chambers at a rate of 9 – 18 L/min to maintain CO2 concentrations in the chamber between 0.4 – 0.8 %. Exiting chamber air was dried by passing it through columns of Drierite™ and magnesium perchlorate before reaching the O2 and CO2 analyzers (Qubit Systems Inc., Kingston, ON). Each chamber was sampled every 3 s over a 5 min period. O2 and CO2 exchange and respiratory quotient (RQ) data were logged using data acquisition software (Qubit C950-MCGES; Qubit Systems Inc., Kingston, ON). Prior to study initiation, dogs were acclimated over an eight week period (minimum exposure of one h per week) to rest comfortably in the chamber with no excessive activity or movement and were discouraged from urinating or defecating in the chamber.

Gas exchange measurements consisted of two fasting collections occurring 18 h after the dog’s last meal (time –50 min and –25 min). Dogs were then fed their full daily ration of test diet and supplements as a single meal (considered time zero). After feeding, gas exchange measurements were collected every 25 min for 23 h. Dogs were provided a brief 10
min break at 4, 10, and 17 h post-feeding in which they were removed from the chamber, taken outdoors into a fenced area and provided an opportunity to urinate and defecate. During these breaks the O₂ and CO₂ analyzers were re-calibrated. After each break, dogs rested in the calorimetry chambers for a minimum of 25 min to ensure adequate CO₂ accumulation prior to the resumption of gas exchange measurements. Energy expenditure (EE) was calculated from O₂ consumption and CO₂ production using the abbreviated Weir equation (de Weir, 1949) and expressed on a per kg metabolic BW basis (BW⁰.⁷⁵).

**2.3.5. Glycemic Meal Challenge**

An eighteen gauge catheter was inserted into the jugular vein for blood sampling. One fasting blood sample (~18 h since last meal) (time -5 min) was collected, after which dogs were fed their full daily ration of test diet and supplements as a single meal (considered time zero). Blood samples were collected at 15, 30, 45 min, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h post-feeding. Blood was centrifuged for 8 min at 3000 × g at 7 ºC and the serum and plasma were stored separately at –80 ºC for subsequent analysis of serum glucose and insulin, and plasma MH. The ratio of glucose to insulin was calculated at each time point. MH followed first-order elimination kinetics, and the elimination rate constant (K) was calculated as the slope of the line from the semi-log plot of MH plasma concentration versus time. The half life (tₜ/₂) of MH was calculated as ln(2)/K while MH turnover time was calculated as 1.44 × (tₜ/₂).

**2.3.6. Biochemical Analyses**

Serum glucose was measured by a clinical chemistry analyzer (Beckman Coulter AU480). Insulin was assayed using a paramagnetic particle, chemiluminescent immunoassay (Beckman, Fullerton, CA). The assay was not canine specific but was validated using canine serum (lower limit of detection is 0.03 μIU/mL, upper limit is 300 μIU/mL). HPLC tandem mass spectrometry was utilized to determine plasma MH concentrations. Briefly, 200 μL of
plasma was diluted with 800 µL MeCN and 10 µL of internal standard solution (\textsuperscript{13}C\textsubscript{7} D-MH, Toronto Research Chemicals, Inc.) and sonicated for 5 min, followed by centrifugation 5 min at 3750 rcf. The supernatant (10 µL) was injected onto a Shodex NH2P-40 3E column (Showa Denko America Inc., Lexington, NY), with guard on a Shimadzu LC10AVP HPLC (flow rate 300 µL/min, column temperature 50°C). Mobile phase A was 61 % acetonitrile, 39 % water with 10 mmol/L formic acid. Mobile phase B was 50 % acetonitrile, 50 % water with 10 mmol/L formic acid. The gradient was 100 % A isocratic for 43 min followed by a 6 min gradient to 100 % B. After a 6 min hold at 100 % B, the system was equilibrated at 100 % A for 12 min. MS/MS was performed in negative ionization mode on a Sciex API 4000 MS/MS. For MH, the formic acid loss was monitored at m/z 255 to 209. For C\textsubscript{7} MH, the formic acid loss was monitored at m/z 262 to 216.

2.3.7. Statistical Methods

All data were analyzed using Statistical Analysis Systems statistical software package version 9.2 (SAS Institute, Cary, NC) and are expressed as means and pooled SEM. Mixed effects models were fitted assuming fixed treatment, period and time effects and dogs as random variable. Repeated measures within period on dog were analyzed using the autoregressive order 1 covariance structure. Multiple comparisons were performed using the Tukey-Kramer method. Results were considered statistically significant if p < 0.05. For calorimetry measurements data were analyzed in time blocks of 0 – 4 h, 5 – 10 h, 11 – 17 h and 18 – 23 h post-prandial. This analytical approach was taken because the data set in its entirety cannot be considered continuous, despite the care taken to ensure all dogs treated the same during the breaks in collections.
2.4. Results

2.4.1. Energy Expenditure

There were no differences in body weight between dietary treatments or study periods (p = 0.99). Resting EE was not affected by diet (Table 2.2). Post-prandial EE was not affected by diet, with the exception of the 5 – 10 h post-prandial period (Table 2.2, Figure 2.1(a)). During this time, dogs who received HC^{MH} had higher EE than dogs fed HC (p = 0.06) and LC (p = 0.04).

2.4.2. Macronutrient Oxidation

While fasting RQ was not statistically significantly affected by diet, it was highest in dogs fed HC^{MH} (Table 2.2). Diet was significant for the entire post-prandial period (Table 2.2, Figure 2.1(b)); with dogs being fed HC and HC^{MH} having significantly higher RQ than dogs fed LC and LC^{MH}.
Table 2.2. Resting \(^1\) and post-prandial (PP) \(^2\) energy expenditure (EE, Kcal/kg\(^{0.75}\)·d) and respiratory quotient (RQ) as measured by indirect calorimetry in adult Beagle dogs (N = 8 in a cross-over design).

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>HC+MH</th>
<th>LC</th>
<th>LC+MH</th>
<th>SEM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting</td>
<td>81.8</td>
<td>87.7</td>
<td>92.8</td>
<td>86.3</td>
<td>8.5</td>
<td>0.70</td>
</tr>
<tr>
<td>PP, 0 – 3.8 h</td>
<td>102.0</td>
<td>104.7</td>
<td>109.4</td>
<td>111.2</td>
<td>5.6</td>
<td>0.25</td>
</tr>
<tr>
<td>PP, 5 – 9.6 h</td>
<td>106.9 (^a)</td>
<td>115.0 (^b)</td>
<td>106.1 (^a)</td>
<td>111.0 (^a,b)</td>
<td>4.1</td>
<td>0.02</td>
</tr>
<tr>
<td>PP, 10.9 – 16.7 h</td>
<td>84.1</td>
<td>87.6</td>
<td>88.4</td>
<td>88.3</td>
<td>3.4</td>
<td>0.71</td>
</tr>
<tr>
<td>PP, 18 – 22.5 h</td>
<td>80.0</td>
<td>80.4</td>
<td>84.5</td>
<td>81.1</td>
<td>3.2</td>
<td>0.28</td>
</tr>
<tr>
<td>RQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting</td>
<td>0.780</td>
<td>0.803</td>
<td>0.764</td>
<td>0.759</td>
<td>0.022</td>
<td>0.10</td>
</tr>
<tr>
<td>PP, 0 – 3.8 h</td>
<td>0.838 (^a,b)</td>
<td>0.851 (^a)</td>
<td>0.819 (^c)</td>
<td>0.824 (^b,c)</td>
<td>0.007</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PP, 5 – 9.6 h</td>
<td>0.914 (^a)</td>
<td>0.915 (^a)</td>
<td>0.834 (^b)</td>
<td>0.828 (^b)</td>
<td>0.010</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PP, 10.9 – 16.7 h</td>
<td>0.918 (^a)</td>
<td>0.920 (^a)</td>
<td>0.829 (^b)</td>
<td>0.825 (^b)</td>
<td>0.014</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PP, 18 – 22.5 h</td>
<td>0.869 (^a)</td>
<td>0.880 (^a)</td>
<td>0.797 (^b)</td>
<td>0.793 (^b)</td>
<td>0.016</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

\(^{a,b,c}\) Mean values within a row with unlike superscript letters were significantly different (p < 0.05)

\(^1\) Resting measures were taken after an overnight fast (~18 h since last meal).

\(^2\) Post-prandial measures were taken for 22.5 h after ingestion of a single test meal and supplements (low carbohydrate (high fat) diet with placebo supplement (LC) or mannoheptulose (8 mg/kg) containing supplement (LC+MH) and high carbohydrate (low fat) diet with placebo supplement (HC) or mannoheptulose (8 mg/kg) containing supplement (HC+MH)) at time zero.
Figure 2.1. Post-prandial\(^1\) energy expenditure (a) and respiratory quotient (b) as measured by indirect calorimetry in adult Beagle dogs (N = 8 in a cross-over design).

\(^1\) Post-prandial measures were taken for 22.5 h after ingestion of a single test meal and supplements (low carbohydrate diet with placebo supplement (LC); mannoheptulose (8 mg/kg) containing supplement (LC\(^{MH}\)); high carbohydrate diet with placebo supplement (HC); mannoheptulose (8 mg/kg) containing supplement (HC\(^{MH}\)) at time zero. Data were analyzed in time blocks of 0 to 4 h, 5 – 10 h, 11 – 17 h and 18 – 23 h. The main effect of diet was significant for the 5 – 10 h time block only (p = 0.02).
2.4.3. Glycaemic Response

The blood sampling catheters failed during collections in two dogs (one HC+MH and one HC dog). These dogs were not included in the final analysis. Neither treatment nor period significantly affected serum glucose (Figure 2.2(a), insulin (Figure 2.2(b)) or the glucose to insulin ratio (data not shown). However, the incremental AUC for glucose was significantly affected by diet (Table 2.3). Dogs who received the LC diet had higher incremental glucose AUC than dogs fed the HC.

Table 2.3. Incremental AUC for glucose and insulin after ingestion of a single test meal and supplements ¹ in adult Beagle dogs (N = 8 in a cross-over design).

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>HC+MH</th>
<th>LC</th>
<th>LC+MH</th>
<th>SEM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>iAUC glucose</td>
<td>25.6</td>
<td>19.9</td>
<td>36.4</td>
<td>32.0</td>
<td>4.52</td>
<td>0.03</td>
</tr>
<tr>
<td>iAUC insulin</td>
<td>218</td>
<td>238</td>
<td>202</td>
<td>225</td>
<td>21.5</td>
<td>0.66</td>
</tr>
</tbody>
</table>

¹ Mean values within a row with unlike superscript letters were significantly different (p < 0.05)

¹ Low carbohydrate (high fat) diet with placebo supplement (LC) or mannoheptulose (8 mg/kg) containing supplement (LC+MH) and high carbohydrate (low fat) diet with placebo supplement (HC) or mannoheptulose (8 mg/kg) containing supplement (HC+MH)
Figure 2.2. Fasting (time – 5 min) and 24 h post-prandial serum glucose (mmol/L)\(^a\) (a), serum insulin (µU/mL) (b) and plasma MH (µg/mL) (c) in adult Beagle dogs fed their full daily ration of test diet and supplements\(^1\) at time zero. Data are presented as means with pooled standard error and N = 8 in a complete cross-over design.

\(^1\)Low carbohydrate diet with placebo supplement (LC) or mannoheptulose containing supplement (LC\(^{+MH}\)) and high carbohydrate diet with placebo supplement (HC) or mannoheptulose containing supplement (HC\(^{+MH}\))

2.4.4. Mannoheptulose Kinetics

Analysis revealed no detectable MH in the placebo supplement. Plasma MH followed first-order elimination kinetics in all dogs but one (Figure 2.2(c)). Results showed peak MH (2.7 µg/mL) occurred 3.0 h post-feeding, while MH half-life and turnover time averaged 3.7 and 5.3 h\(^{-1}\), respectively. MH had returned to non-detectable levels by 24 h.
2.5. Discussion

This study found that in the presence of high dietary carbohydrate relative to fat, MH increased fasting RQ and post-prandial EE (5 – 10 h). In contrast to earlier reports, MH did not affect serum glucose or insulin in this study. These findings suggest MH elicits changes in energy sensing pathways and macronutrient fuel selection. Interestingly, this study found that acute consumption (14 d) of a diet low in CHO relative fat content increased the incremental AUC for glucose (irrespective of MH). This finding persisted despite the dogs’ ability to adapt macronutrient oxidation to diet composition.

MH appeared in plasma within 1 h of administration, peaked at 3 h and returned to non-detectable levels by 24 h. Similarly, Davenport et al. (2010b) observed peak MH concentrations within 2 – 4 h after oral administration of MH (2 mg/kg) to Labrador Retrievers. These findings confirm that MH is absorbed and available to the animal.

While resting EE was not different between diets, it was similar to values reported in dogs in the literature (74 – 94 Kcal/kg·d, Pouteau et al., 2002; 86 – 89 Kcal/kg·d, Yoo et al., 2006). In the present study, dogs were fed equivalent grams of dietary protein to energy, and the daily food allowance was set to maintain a dog’s body weight over the course of the study. As such, dietary macronutrient differences were not expected to significantly alter whole body EE. This finding agrees with other studies conducted using dogs (Yoo et al., 2006) and humans (Brehm et al., 2005) that were fed different concentrations of CHO and/or fat. These observations suggest energy metabolism is highly controlled in mammals and is not influenced by acute changes in dietary macronutrient concentrations. In contrast, MH increased post-prandial EE in the 5 – 10 h post-feeding period indicating its ability to affect energy metabolism. It is possible that MH feeding may have increased the thermic effect of food (TEF) based on increased EE. In dogs (Lablanc and Diamond, 1986) and humans (Kunz et al., 2005), increased TEF has been observed for as long as 6 h after a meal. TEF represents
the acute increase in EE in response to feeding and accounts for approximately 10 % of daily energy expenditure (Westerterp-Plantenga et al., 2012). It can be further divided into obligatory and facultative EE. Obligatory EE is relatively fixed and encompasses energy needs for digestion and absorption. Facultative EE is mediated by sympathetic nervous system activation and β-A responses and can be highly variable (Lablanc and Diamond, 1986). Direct stimulation of the β-A system has been shown to increase post-prandial TEF in humans (Stob et al., 2007). MH can potently stimulate glucagon secretion and inhibit insulin secretion, even in the presence of exogenous insulin infusion (Muller et al., 1971). In addition, MH significantly increases hepatic glucose output and circulating cAMP concentrations (Issekutz et al., 1974). All of these metabolic responses are characteristic of a β-A response and may provide a possible mechanism by which MH affects whole-body EE. It should be noted, however, that these previous MH induced responses resulted from supra-physiological doses (~1.2 g/kg) of MH administered intra-arterially. In contrast, the observed effects of MH on EE in the present study were associated with a significantly lower level of avocado-derived MH (~8 mg/kg) administered orally with the diet. Increasing TEF contributes little to daily energy expenditure (3 – 5 %). However, even this small increase in daily energy expenditure would be beneficial to overweight and obese animals. The fact that MH increased TEF within the high CHO relative to fat (HC) diet is especially important given that overweight and obese animals are often prescribed therapeutic diets of a similar macronutrient profile. Additional research is warranted to more fully delineate the metabolic mechanism(s) by which MH is exerting its effects on whole body energy metabolism.

Not surprisingly, dogs in this study demonstrated their ability to adapt macronutrient oxidation to diet composition, as evidenced by changes in the post-prandial RQ based on the inclusion of dietary CHO relative to fat. Increased RQ was observed in the HC fed dogs, suggesting an increased proportion of CHO to fat oxidation. Conversely, a lower RQ
indicates a greater proportion of fat relative to CHO oxidized, as seen in dogs consuming the low CHO relative to fat diet (LC). It is important to note that RQ does not indicate net carbohydrate and fat oxidation, nor does it account for protein oxidation. Only one other study to our knowledge has published post-prandial RQ values in dogs (Pouteau et al., 2002). Pouteau et al. (2002) also fed adult Beagle dogs a diet of similar composition to the HC diet. They reported comparable changes in post-prandial RQ (0.91 ± 0.01, 24 h average).

However, this study is the first to demonstrate changes in post-prandial RQ in response to different dietary composition. These data further validate the use of indirect calorimetry as a research tool to measure fasting and meal-induced responses in whole-body energy metabolism in dogs. Fasting RQ was greatest in dogs fed HC+MH, suggesting increased carbohydrate oxidation relative to fat oxidation in fasting. The ability of MH to affect fasting RQ was unexpected as previous reports demonstrate that MH has transient effects on metabolism that are reversed within 1 – 6 h of administration (Viktora et al., 1969; Muller et al., 1971; Issekutz et al., 1974). The effect may be a result of chronic MH feeding. However, only one other study to our knowledge has examined the effects of chronic MH supplementation (Davenport et al., 2010a), but those authors did not measure RQ. It is unclear whether changing resting RQ would have any biological significance. These results are somewhat surprising. As MH is purported to function as a competitive inhibitor of glucokinase, one may have expected MH to slow the rate of glucose utilization, especially when faced with a high carbohydrate (glucose) load. These results further demonstrate the need for additional research to evaluate the role of MH on glucose and fat oxidation in fasting and the post-prandial period to see if they are repeatable.

In contrast to previous studies, MH did not affect serum glucose or insulin. Previous studies showed high doses (> 1.2 g/kg) of MH induced transient hyperglycaemia and hypoinsulinemia in rats, dogs and humans (Klain et al., 1974; Issekutz et al., 1974; Johnson et
However, these doses elicited responses well outside the range of normal metabolism suggesting the dose was supra-physiological. Low dietary MH concentrations (2 mg/kg) have been reported to lower post-prandial serum insulin concentrations, but not glucose, when fed to senior Labrador Retrievers (average age 11.8 years) for 30 d (Davenport et al., 2010a). The lack of a post-prandial insulin response in the current study may be attributed to the fact that dogs were young and lean which may have precluded any noticeable metabolic changes. It is worth noting that aside from Davenport et al. (2010a), this is the only study to examine MH effects in the post-prandial state and a small sample size was used.

Owing to its ability to inhibit glucokinase in small intestine, liver and pancreas, MH is quite likely to have differential effects on glycaemia in fasting and fed conditions.

Dogs fed the LC diet exhibited higher serum glucose than dogs consuming the HC diet. However, serum glucose values were within the normal range for healthy dogs. Serum insulin concentrations were not different between diets suggesting that the LC diet induced slight insulin resistance. These findings may be attributed to the high fat relative to CHO content of the LC diet. High fat diets have been shown to induce impairments in insulin mediated glucose disposal in as quickly as 3 – 5 d in humans (Bachmann et al., 2001; Stettler et al., 2005; Numao et al., 2012). High fat, high fructose feeding for six to twelve weeks induced glucose intolerance in dogs (Moore et al., 2011; Coate et al., 2011). However, to our knowledge no acute studies examining the effects of exclusively high fat (relative to CHO) have been performed in dogs. In order to fully understand the effects of feeding a diet high in fat relative to CHO on insulin sensitivity and glucose tolerance, more sensitive measures are needed partnered with a longer dietary adaptation period. In addition, measuring serum lipids, including triglycerides and free fatty acids, would provide insight into the metabolic adaptations that occur with high fat feeding. The minimal glucose response observed in dogs consuming HC diet is consistent with other studies using dogs fed similar concentrations of
dietary CHO (Coate et al., 2011; Strack et al., 1994). In comparison to humans, dogs tend to have a blunted glucose response to a mixed meal which has been attributed to the prolonged absorptive phase (12 – 24 h) in dogs (Hill et al., 2006). Furthermore, the HC diet had high inclusion of sorghum grain, which is considered to have a low glycemic index in dogs due to its high dietary fibre content (Carciofi et al., 2008).

In conclusion, MH has acute effects on post-prandial EE and RQ. Specifically, MH increased fasting RQ indicating an increase in CHO relative to fat oxidation. MH increased dietary induced thermogenesis in the high CHO relative to fat diet, supporting its use as an energy restriction mimetic. Increasing dietary thermogenesis would be of particular benefit to overweight and obese animals, especially those consuming a high CHO low fat diet. Future research is warranted and needs to involve more sensitive measures of macronutrient oxidation to fully elucidate the mechanism of action of MH on whole-body metabolism in fasting and post-prandial conditions. Irrespective of MH, this study demonstrated that dogs adapt relative carbohydrate to fat oxidation to diet composition. Despite this finding, dogs consuming a low CHO high fat diet had increased serum glucose.
CHAPTER 3

Dietary mannoheptulose has differential effects on fasting and post-prandial glucose oxidation in Labrador Retrievers (Pilot Study) ²

3.1. Abstract

This study aimed to determine the effects of dietary mannoheptulose (2 mg/kg BW), a glycolytic inhibitor, on glucose oxidation and biomarkers of energy metabolism in neutered, adult, male Labrador Retriever dogs (N = 6). Fasting and post-prandial respiratory quotient and energy expenditure were determined by indirect calorimetry (d 16). Glucose turnover and oxidation were assessed during fasting and repeated meal feeding using indirect calorimetry and a constant intravenous infusion of U-13C-glucose (d 18). A sample from the biceps femoris was obtained to determine the muscle protein content of AMPK and acetyl CoA carboxylase (ACC) (total and phosphorylated forms) (d 21). MH did not affect energy expenditure, serum glucose, insulin or free fatty acids or ACC protein content. In fasting, MH significantly increased fasting respiratory quotient (p = 0.01) and glucose oxidation (p = 0.02) and tended to decrease the ratio of phosphorylated to total AMPK protein content (p = 0.16). In contrast, post prandial glucose oxidation tended to be lower (p=0.14) in dogs fed MH. These results suggest that MH has differential effects on fasting and post-prandial whole body glucose and fat oxidation. However, further research that uses a greater number of animals and/or higher dose of MH is necessary.

Key Words: energy expenditure, glucose oxidation, Labrador Retriever, mannoheptulose, respiratory quotient

² McKnight et al. Journal of Applied Animal Research (JAAR-2013-0346), In Review
3.2. Introduction

Energy restriction (ER) without malnutrition (30-60% of *ad libitum*) has been shown to effectively delay the ageing process by increasing median and maximal lifespan and/or providing protection from age-related disease development in several species, including dogs and humans (Kealy et al. 2002; Roth et al. 2004; Fontana and Klein 2007). The mechanism by which ER exerts its beneficial effects is not completely understood. Current evidence suggests ER impacts intracellular energy metabolism by altering key energy-sensing pathways leading to improved metabolic efficiency, enhanced insulin signaling, and reduced oxidative damage (Fontana and Klein 2007).

Despite the reported benefits of ER, maintaining a low-energy diet remains a challenge for people and companion animals. The obesity epidemic plaguing the human population also impacts companion animals with 55% of dogs in the United States reported to be overweight or obese (Ward et al. 2011). Addressing this problem in pets has been challenging due to difficulties in defining the ideal weight for dogs, coupled with poor owner adherence to recommended feeding regimes. In addition, the perceived negative behavioral changes associated with ER (begging and aggression), has prompted researchers to seek alternative approaches to ER, such as calorie restriction mimetics.

Energy restriction mimetics are therapeutic agents or food ingredients that mimic the metabolic, hormonal and physiological effects of ER without altering caloric intake (Ingram et al. 2006). Mannoheptulose (MH), a seven carbon sugar found in high concentrations in avocados has been proposed as a calorie restriction mimetic (Roth et al. 2009). Early research demonstrated MH functions as a glycolytic inhibitor through its ability to competitively inhibit hexokinases (HK, EC 2.7.1.1) (Viktora et al. 1969). Dogs given MH doses ranging 1-2 g/kg (intravenous or intra-arterial) exhibit a transient diabetic state, characterized by marked hyperglycemia, dramatically decreased insulin to glucagon ratio and increased hepatic glucose
output (Viktora et al. 1969; Muller et al. 1971; Issekutz et al. 1974; Klain et al. 1976). These responses were well outside normal physiological ranges suggesting that the dosage was supra-physiological. A recent study of Fox Terriers and Labrador Retrievers found that MH doses of 2, 10 and 20 mg/kg BW delivered in a gelatin capsule lowered fasting insulin concentrations with no change in circulating glucose (Davenport et al. 2010a). However the effects of low oral doses of MH on other aspects of glucose metabolism are largely unknown. Furthermore, previous studies have only examined MH effects on fasting metabolism. As MH inhibits glucokinase in intestine, liver and pancreas (Crane and Sols, 1954; Coore and Randle, 1964), it is of interest to examine MH effects on post-prandial metabolism.

The objective of this pilot study was to determine the effects of low dietary doses of MH (2 mg/kg BW) on glucose metabolism, specifically glucose oxidation. Given that MH inhibits glucokinase, we expected MH to decrease glucose utilization. In addition, we investigated the effects of MH on biomarkers of energy metabolism known to be associated with ER, including energy expenditure (EE) and 5’ adenosine monophosphate-activated protein kinase (AMPK) and skeletal muscle acetyl-CoA carboxylase (ACC) protein content.

3.3 Materials and Methods

3.3.1. Animals and Housing

All procedures were approved by the Institutional Animal Care and Use Committee of P&G Pet Care (Mason, OH, USA). A total of 6 neutered male Labrador Retrievers (3 black-coated 3.9 y; 27.3 kg and 3 chocolate-coated 6.8 y; 35.0 kg) were used in this study. All dogs were housed at the P&G Pet Health and Nutrition Center (Lewisburg, OH) and were considered healthy at the initiation of the study based on a general health evaluation by a licensed veterinarian. All dogs were at an optimal body condition score of 3.0 (five point scale) at the initiation of the study. Dogs were pair-housed with free access to water and
indoor and outdoor runs. Indoor runs were maintained on a 12 h light and dark cycle, in addition to natural light, and were equipped with raised canvas beds, toys and heated flooring. Outdoor runs were equipped with toys and play yard equipment. All dogs received 40 min of supervised group exercise and socialization in a separate fenced yard daily.

3.3.2. Study Design

This study was designed as a cross-over with each dog receiving both dietary treatments, control (CON) and mannoheptulose (MH), in random order. The duration of this study was 44 d with each period lasting 22 d. For each period, blood parameters, indirect calorimetry, and glucose kinetics were measured and muscle biopsies were collected. The indirect calorimetry method only allowed three dogs to be measured per day. Therefore, dogs were blocked by coat colour (i.e. chocolate and black) for all collections and were staggered one day apart irrespective of their dietary treatment.

3.3.3. Diets and Feeding

Test diets were formulated to represent Eukanuba Large-Breed Senior Maintenance Formula, which is a commercially available diet that is nutritionally complete and balanced. Test diets were similar in overall nutrient content (92% dry matter; 24% protein; 15% fat; 43% carbohydrate; 4600 cal/g). The MH diet was made by incorporate avocado whole-fruit extract into the CON diet to deliver 200 mg/kg MH. The MH enriched avocado extract was produced using commercially available avocados (Hass variety). Frozen, whole avocados comprised of the flesh, peel and pit were initially ground before suspension in water (1:3 w/w). The resultant slurry was centrifuged to remove non-aqueous solids. A series of microfiltration (de-oiling), ultrafiltration (10 kDa) and nanofiltration (100 kDa) was used to produce the MH-enriched fraction. Lyophilization was used to form the final crystalline powder yielding 18% MH (Massimino et al., 2005).
Dogs were fed to maintain body weight, based on their historical caloric intake records (2066 ± 191 kcal/d CON; 2111 ± 196 kcal/d MH). For one week prior to study initiation dogs were fed the CON diet (“wash in” period). Dogs were individually fed their daily ration in two meals (0800 and 1300) to deliver a daily dose of 2 mg/kg MH. As mentioned previously, a dose of 2 mg/kg was shown to decrease circulating insulin, but not glucose, in Labrador Retrievers (Davenport et al. 2010a). The dose therefore, appears to be bioavailable and non-pharmacological.

3.3.4. Measurements

Food intake was measured daily and body weight weekly. On days 1 (baseline), 7 and 14 of each treatment leg, blood samples (each 3 mL) were obtained via jugular venipuncture prior to the dog’s morning meal (fasting) and again precisely 4 h after the morning meal. All dogs were acclimated to the venipuncture procedure prior to study. Fasting blood samples were analyzed for glucose, insulin, and free fatty acids. Plasma MH concentrations were measured in the fed samples only to confirm dogs received the correct dietary treatment.

3.3.5. Indirect Calorimetry

Respiratory gas exchange measurements were conducted via whole body indirect calorimetry on day 16 of each period. The calorimetry chambers (76 cm × 53 cm × 61 cm, L × W × H) were made of clear plexiglass and fitted with a hinged access top door or feeding drawer for providing food to the dog. Chambers were designed as open-flow circuits with room air pulled into the chambers at a rate of 22 to 26 L/min to maintain CO₂ concentrations in the chamber between 0.4 and 0.8 %. Exiting chamber air was dried by passing it through columns of Drierite™ and magnesium perchlorate before reaching the O₂ and CO₂ analyzers (Qubit Systems Inc., Kingston, ON, Canada). Each chamber was sampled every three seconds over a five minute period. O₂ and CO₂ exchange and respiratory quotient (RQ) data were logged using data acquisition software (Qubit Systems Inc., Kingston, ON, Canada). Prior to
study initiation, dogs were acclimated over an eight week period (minimum exposure of one h per week) to rest comfortably in the chamber with no excessive activity or movement and were discouraged from urinating or defecating in the chamber.

Prior to any measurements, the O2 and CO2 analyzers were calibrated and dogs rested in the chamber for a minimum of 25 min to ensure adequate CO2 accumulation. Gas exchange measurements consisted of two fasting collections occurring 18 h after the dog’s last meal (time -50 min and -25 min). Dogs were then fed their full daily ration of test diet as a single meal (considered time zero). After feeding, gas exchange measurements were collected every 25 min for 12 h. Dogs were provided a brief 10 min break at 6 h post-feeding in which they were removed from the chamber, taken outdoors into a fenced area and provided an opportunity to urinate and defecate. During this break the O2 and CO2 analyzers were re-calibrated. After the break, dogs rested in the calorimetry chambers for a minimum of 25 min to ensure adequate CO2 accumulation prior to the resumption of gas exchange measurements. Energy expenditure (EE) was calculated from O2 consumption and CO2 production using the abbreviated Weir equation (de Wier 1949) and expressed per kg metabolic BW (BW^{0.75}).

3.3.6. Glucose kinetics

Glucose kinetics were assessed during fasting and repeated meal feeding (fed state) on d 19 of both periods using indirect calorimetry to measure CO2 exchange and a primed, continuous intravenous infusion of U-^{13}C-Glucose (99 % atom, Sigma Aldrich, Isotec, Miamisburg, OH, USA, 45342) to quantify glucose flux. Both front legs were aseptically prepared and an 18 gauge catheter was inserted into the cephalic vein of each leg. One catheter was used for isotope infusion, while the other catheter was only used if that catheter failed. All blood samples were taken by jugular venipuncture.

Animals began measurements in the fasted state (18 h since last meal). Baseline breath and blood samples were collected prior to isotope infusion to determine background ^{13}C
enrichment. At time 0, a bolus dose (14 mg/kg) of isotope was administered, followed immediately by a continuous intravenous infusion (0.025 mg/kg·min) delivered via the cephalic catheter using a NE-1000 Syringe Pump (New Era Pump Systems Inc., Farmingdale, NY, USA). Six fasting blood samples (each 3 ml) were taken at 60, 85, 110, 135, 160, and 185 min followed by four breath samples collected at 220, 245, 270, and 295 min post-infusion. After the completion of fasting measurements, fed-state kinetics were examined. Dogs were fed their test diet divided into 15 equal meals (14 g/kg glucose per meal). Meals were initially fed three times, 10 min apart, and then every 25 min thereafter. This feeding regimen was based on reported gastric emptying times of ~20 min for dogs fed small meals (Gooding et al. 2012). Therefore, it was assumed that feeding small meals in 25 min intervals would mimic a continuous fed state and provide a constant dietary infusion of MH. Blood and breath collections were taken at the same time points as fasting measurements, with an additional two breath collections at 320 and 345 min.

Blood samples were centrifuged for 8 min at 3000 x \( g \) at 7 °C and the serum and plasma were stored separately at −80 °C for subsequent analysis of glucose enrichment. Expired CO\(_2\) was trapped in 1 mol/L NaOH and stored at room temperature for later analysis of \(^{13}\)C enrichment.

A single pool model was used to calculate plasma glucose turnover (Eq. 1) and oxidation (Eq. 2). Plasma glucose rate of appearance (Ra) was assumed to equal the rate of disappearance at isotopic steady state (S/S) and represent whole body plasma glucose turnover (Eq. 1). If a plateau in isotopic enrichment did not occur, S/S was extrapolated using simple negative exponential curve-fitting over the period of post-peak decline (Eq. 3) or using Eq. 4.

\[
Ra (\text{mg/kg·min}) = F \times [(e_F / e_P) - 1] \quad \text{[1]}
\]

\[
\text{Oxidation (mg/kg·min)} = [VCO_2 \times e_{CO2}] / [e_F \times 6] \quad \text{[2]}
\]
where $F$ is the isotope infusion rate, $e_F$, $e_P$ and $e_{CO2}$ represent the isotopic enrichments of the infusate, plasma and expired CO$_2$, respectively, and $6$ represents the number of carbon atoms per glucose molecule oxidized.

\[ Y = (Y_o - \text{plateau}) e^{(-kt)} + \text{plateau} \]  

\[ e_B = \frac{\text{breath}_{\text{max}} / \text{plasma}_{\text{max}}}{e_P} \]

where “max” refers to the maximum observed enrichment and $e_P$ the projected steady state plasma enrichment (Mol, %)

3.3.7. Muscle Biopsy

All six dogs underwent muscle biopsies on the same day (day 20 or 21 of both period). Dogs were fasted overnight and sedated using Dexmedetomidine (0.02 mg/kg, Dexdomitor®, Pfizer) and Carprofen (4 mg/kg, Rimadyl®, Pfizer). Propofol (5-7 mg/kg, Propoflo®, Abbott) was administered i.v. for induction in the first study period. As Propofol was unavailable for purchase due to a worldwide shortage in the second study period, Ketamine (5.5 mg/kg) and Diazepam (0.275 mg/kg) were used instead. The use of different induction chemicals was not believed to affect any outcome measures, as each dog received both agents as part of the crossover design. A 3 - 4 cm incision was made along the dorsal caudal thigh and a #10 scalpel blade was used to remove two muscle tissue samples (wet weight ~100 mg per sample) from the biceps femoris. The incision was closed using surgical skin staples (AutoSuture Appose ULC™, Tyco). At the end of the procedure, each dog was administered atipamezole (0.125 mg/kg, Antisedan®, Pfizer) as a sedative reversal and placed in a heated cage until recovered. Dogs were monitored one week for complications. Muscle samples were immediately frozen in liquid N$_2$ and stored at -80 °C for later analysis of phosphorylated (p) and total (t) ACC and AMPK protein abundance.

Muscle was homogenized in cold NP40 Cell Lysis Buffer with 1 mmol/L PMSF (Invitrogen Corp., Camarillo CA) and protease inhibitor cocktail (Sigma, Oakville ON) and
centrifuged at 4°C for 30 min. Resulting supernatants were removed and protein concentration was determined by Bradford protein assay. Protein (30 µg) from muscle lysates was diluted (1 v/v) in Laemmli sample buffer (Bio-Rad Laboratories, Mississauga ON) and loaded onto 7.5 % Tris HCL pre-cast resolving gels (Bio-Rad Laboratories, Mississauga ON) and separated by SDS-PAGE (1 h, 150 v). Gels were equilibrated in cold Transfer Buffer (25 mmol/L Tris, 192 mmol/L glycine and (20% v/v methanol, pAMPK and AMPK only)) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Mississauga ON). Membranes were incubated at 4 °C for a minimum of 8 h with either ACC, pACC, AMPK or pAMPK antibody (Cell Signaling Technology Inc, Boston MA) diluted (1:1000) in 7.5 % BSA. Membranes were washed in 1x TBST and then incubated with anti-rabbit immunoglobulin–horseradish-peroxidase–linked whole antibody (1:2000 dilution in TBST) for ~1 h. Washed membranes were submerged in enhanced chemiluminescence reagents (GE Health Care Biosciences Corp., Piscataway, NJ) and exposed using an Alpha Innotech imager. Bands were visualized and quantified using AlphaView Software for FluorChem Systems. Data are expressed as the ratio of phosphorylated (p) to total protein content.

3.3.8. Biochemical Analyses

Serum glucose was measured by a clinical chemistry analyzer (Beckman Coulter AU480). Serum insulin was assayed using a paramagnetic particle, chemiluminescent immunoassay (Beckman, Fullerton, California) and serum free fatty acids using an enzymatic calorimetric assay (Wako Diagnostics). HPLC tandem mass spectrometry was utilized to determine plasma MH. Briefly, 200 µL of serum was diluted with 800 µL MeCN and 10 µL of internal standard solution (13C7 D-MH, Toronto Research Chemicals, Inc.) and sonicated for 5 minutes, followed by centrifugation for 5 min at 3750 rcf. The supernatant (10 µL) was injected onto a Shodex NH2P-40 3E column (Showa Denko America Inc.), with guard on a Shimadzu LC10AVP HPLC (flow rate 300 µL/min, column temperature 50 C). Mobile phase
A was 61% acetonitrile, 39% water with 10 mmol/L formic acid. Mobile phase B was 50%
acetonitrile, 50% water with 10 mmol/L formic acid. The gradient was 100% A isocratic for
43 min followed by a 6 min gradient to 100% B. After a 6 min hold at 100% B, the system
was equilibrated at 100% A for 12 min. MS/MS was performed in negative ionization mode
on a Sciex API 4000 MS/MS. For MH, the formic acid loss was monitored at m/z 255 to 209.
For C7 MH, the formic acid loss was monitored at m/z 262 to 216. Plasma isotope enrichment
was measured by Metabolic Solutions Inc. (Nashua, NH) using the method described by
Previs et al. (1994). Briefly, the isotopic enrichment of plasma samples was determined by
gas chromatography–mass spectrometric analysis of the glucose aldonitrile pentaacetate
derivative formed with acetic anhydride and hydroxylamine hydrochloride. $^{13}$C$_6$-glucose
enrichment was measured using an Agilent 5975C EI/CI MSD with an Agilent 7890 GC in
the electron ionization mode. A Phenomenex ZB-1MS capillary column was used to separate
the derivative of glucose. Selected ion chromatograms were obtained by monitoring ions m/z
328 and 334 for D-glucose and D-$^{[13}$C$_6$]-glucose, respectively. Isotope enrichment in mole %
excess was calculated from peak area ratios. The final value for all determinations was
corrected using an enrichment calibration curve. Breath samples were analyzed using an
automated $^{13}$CO$_2$ breath-analysis system, BreathMat Plus (Thermo Finnigan, San Jose, CA)
(Phillips et al. 2004). Briefly, 2 mL of the expired breath sample were introduced into the gas
chromatograph. Gases not of interest (N$_2$ and O$_2$) as well as water were removed online via a
continuous-flow diffusion pump. Pure CO$_2$ was introduced to the isotope ratio mass
spectrometer (IRMS) and was analyzed at a mass-to-charge ratio of 44/45.

3.3.9. Statistical Methods

All data were analyzed using SAS version 9.2 (SAS Institute, Cary, NC) and
expressed as mean with pooled standard error. Data were analyzed using the proc mixed
procedure with period and diet considered fixed effects and dogs random variables. Repeated
measures within period on dogs were subjected to the autoregressive order 1 covariance structure. For calorimetry data, time was considered a fixed effect and fasting, post-prandial (0 -6 h) and post-absorptive (7 – 12 h) data were analyzed separately. Statistical significance was considered p < 0.05. The interactions between fixed effects were also tested but only reported if significant.

3.4. Results

3.4.1. Macronutrient Oxidation

Fasting RQ was significantly (p = 0.01) greater in dogs fed MH (Table 3.1). However, there was no affect of diet on post-prandial RQ (Figure 3.1(a)). An isotopic S/S was not achieved in breath during fasting and glucose oxidation was calculated using extrapolated values (derived from Eq. 3). Fasting glucose oxidation was significantly (p = 0.02) higher in dogs fed MH than those who received control (Table 3.1). During repeated meal feeding, plateaus in isotope enrichment were achieved and considered S/S values. While there was not statistically significant difference in post-prandial glucose oxidation, it was numerically lower in dogs who consumed the MH containing diet (Table 3.1).
Table 3.1. Fasting and post-prandial\(^1\) respiratory quotient (RQ), energy expenditure (EE), plasma glucose turnover (Ra), and glucose oxidation in adult male neutered Labrador Retrievers receiving either a control diet or mannoheptulose (MH) containing diet (2 mg/kg BW)\(^2\).

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>MH</th>
<th>SEM</th>
<th>(P_{\text{period}})</th>
<th>(P_{\text{diet}})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fast</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting EE, kcal/kg(^{0.75})·d</td>
<td>66.6</td>
<td>68.8</td>
<td>3.3</td>
<td>0.64</td>
<td>0.64</td>
</tr>
<tr>
<td>RQ</td>
<td>0.74</td>
<td>0.76</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Ra, μmol/kg·min</td>
<td>19.4</td>
<td>14.6</td>
<td>4.5</td>
<td>0.80</td>
<td>0.47</td>
</tr>
<tr>
<td>Oxidation, mg/kg·min</td>
<td>2.1</td>
<td>2.4</td>
<td>0.1</td>
<td>0.14</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Fed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ra, mg/kg·min</td>
<td>76.1</td>
<td>47.8</td>
<td>25</td>
<td>0.74</td>
<td>0.47</td>
</tr>
<tr>
<td>Oxidation, mg/kg·min</td>
<td>34.9</td>
<td>16.3</td>
<td>6.9</td>
<td>0.48</td>
<td>0.14</td>
</tr>
</tbody>
</table>

\(^1\) Post-prandial plasma glucose turnover and glucose oxidation data are also presented.

\(^2\) Data are means with pooled standard error and \(N = 6\) in a complete cross-over design.
Figure 3.1. Post prandial respiratory quotient (RQ) (a) and energy expenditure (EE, kcal/kg^{0.75}·d) (b) in adult male neutered Labrador Retrievers fed their full daily ration of test diet, control (CON) or mannoheptulose (MH, 2 mg/kg) at time 0. Data are presented as means with pooled standard error and N = 6 in a complete cross-over design.
3.4.2. Glucose Metabolism

There were no significant changes in fasting glucose (Figure 3.2(a)), insulin (Figure 3.2(b)), or FFA (Figure 3.2(c)) over time and there was no affect of diet (or period). True isotopic steady state (S/S) was not achieved in plasma during fasting or repeated meal feeding likely due to an over prime of isotope. Therefore, plasma glucose turnover was calculated using extrapolated S/S values. Fasting and post-prandial plasma glucose turnover was not affected by diet (Table 3.1).
Figure 3.2. Serum glucose (mmol/L) \( (a) \), insulin (µU/mL) \( (b) \) and free fatty acids (mEq/L) \( (c) \) concentrations in adult male neutered Labrador Retrievers receiving control (CON) or mannoheptulose (MH 2 mg/kg) diets taken on day 1, 7 and 14 of study. Data are presented as means with pooled standard error and \( N = 6 \) in a complete cross-over design.
### 3.4.3. Energy Metabolism

Fasting (Table 3.1) and post-prandial EE (Figure 3.1(b)) was not affected by diet. Dogs receiving MH tended to have a lower ratio of phosphorylated to total AMPK protein content ($p = 0.16$) (Figure 3.3). MH did not affect the ratio of phosphorylated to total ACC (Figure 3).

![Figure 3.3](image.png)

**Figure 3.3.** The ratio of phosphorylated to total protein content of adenosine-monophosphate protein kinase (AMPK) and acetyl Co-A carboxylase (ACC) in Labrador Retrievers fed control (CON) or mannoheptulose (MH 2 mg kg$^{-1}$) diets. Muscle was sampled from the biceps femoris after an overnight fast. Data are presented as means with pooled standard error and $N = 6$ in a complete cross-over design.
3.5. Discussion

Overall, MH did not affect circulating glucose, insulin or FFA or energy expenditure. However, MH significantly increased RQ and glucose oxidation in fasting and tended to lower post-prandial glucose oxidation. In addition, MH tended to decrease the ratio of phosphorylated to total AMPK protein content in fasted skeletal muscle, suggesting that fat utilization was decreased (indirect evidence). Together these results suggest that MH shifts the time course of glucose utilization from after a meal to the fasting period. However, a study using a greater number of animals and/or higher dose of MH is needed to fully understand the effects of dietary MH on glucose and energy metabolism.

The ability of MH to induce transient metabolic changes has been demonstrated in rats, dogs and humans with effects lasting from one to four hours after MH administration (Johnson and Wolff 1970; Muller et al. 1971; Klain and Meikle, 1974; Klain et al. 1976). In contrast to the present study, these studies examined the effects of MH exclusively in the fasted state using very high doses of MH administered intravenously (or intra-arterially). These conditions elicited responses well outside normal physiological ranges suggesting that the dosage was supraphysiological. In addition, injecting MH does not allow one to examine the potential effects of MH on gut and/or liver metabolism (i.e. first pass responses). Nevertheless, the patterns of change in glucose oxidation (fast vs. fed) induced by dietary exposure in our study are consistent with the temporary changes in metabolism observed in these previous reports.

This study was the first study to examine in vivo glucose oxidation in response to dietary MH and statistically significant differences between groups were achieved in fasting only. The lack of significance during repeated meal feeding was likely due to a lack of statistical power and future studies with larger sample sizes are warranted. Similarly, glucose oxidation and turnover rates were calculated using extrapolated S/S enrichment values. While
fasting plasma extrapolated S/S enrichments were comparable to reported literature values (Tounian et al. 1994; Coggan et al. 1990), $^{13}$CO$_2$ enrichment has been poorly reported in the literature, making it difficult to compare extrapolated S/S breath enrichment values. While fasting oxidation values in this study were lower than those previously reported in mongrel dogs (~0.89 mg/kg min Wolfe and Shaw 1986; ~1.19 mg/kg min Paul et al. 1966), they are still within the normal physiological range. As the repeated meal feeding technique has not been used to study glucose turnover or oxidation, we did not have comparable literature data. However, the repeated meal feeding technique was essential to this study as it provided a constant dietary supply of MH and mimicked a continuous fed state. The fed oxidation values were similar to those observed in humans under clamped hyperinsulinemic conditions (Bonadonna et al. 1993; Fery et al. 2004).

In contrast to earlier studies, MH did not affect circulating glucose or insulin. The lack of consensus may be due to the major methodological differences between these early reports and our study and have been already noted. The only other published report of oral MH administration observed lower fasting insulin but no change in glucose in Fox Terriers and Labrador Retrievers given 2 mg/kg MH for 30 days (Davenport et al. 2010a). These findings suggest that the dose of MH used in the present study should have been sufficient to elicit changes in circulating insulin levels. However, previous reports demonstrate that MH has a greater impact on circulating glucagon than insulin levels. Indeed, a fivefold increase in glucagon has been reported in dogs during constant MH infusion (Muller et al. 1971). As such, it would be beneficial for future studies to examine MH effects on both insulin and glucagon.

MH tended to decrease the ratio of phosphorylated to total AMPK protein content. AMPK is considered a master regulator of intracellular energy metabolism (Ruderman and Prentki 2004). When activated (via phosphorylation), AMPK increases cellular energy
generation mainly by promoting lipid oxidation but also by increasing glucose uptake (Ruderman and Prentki 2004). The observed decrease in active AMPK indirectly suggests that fat utilization was decreased in fasting. This notion compliments our findings that RQ and glucose oxidation were increased in fasting.

In conclusion, MH at a dose of 2 mg/kg increased fasting glucose oxidation and RQ but did not appear to significantly affect post-prandial glucose or energy metabolism. Future studies using larger sample sizes and/or higher doses of MH, coupled with direct measurements of fat oxidation are needed. Furthermore, the effects of MH on other proteins known to be associated with calorie restriction and cellular fuel selection are needed to better characterize the effects of low doses of dietary MH.
CHAPTER 4

Dietary mannoheptulose decreases diet induced thermogenesis and physical activity in adult Labrador Retrievers

4.1. Abstract

This study aimed to determine the effects of dietary mannoheptulose (MH), delivered as an extract of un-ripened avocado, on energy expenditure (EE) in healthy adult Labrador Retriever dogs (total of 12 dogs, 26.99 ± 0.634 kg, 4.9 ± 0.2 yr). The study was a double-blind, cross-over with each dog receiving both dietary treatments, control (CON) and MH (4 mg/kg BW), in random order. Resting and post-prandial (10 h) EE and respiratory quotient (RQ) were determined by indirect calorimetry (d 42). The following day, body composition was assessed using dual X-ray absorptiometry. Continuous activity monitoring was conducting using an Atical® accelerometer (d 43 – 47). A vastus lateralis muscle biopsy was obtained prior to the morning meal (d 49) and 4 h after consumption of their meal (d 56) to determine the protein content and phosphorylation of 5′ adenosine monophosphate-activated protein kinase (AMPK). Diet did not affect body composition, resting EE and RQ, or skeletal muscle AMPK. Dogs fed MH tended to have lower post-prandial EE (P = 0.08) and significantly lower RQ (P = 0.02) and ratio of fat to lean body mass (p = 0.02). Activity during light time periods (but not dark) was lower in dogs fed MH (P < 0.05). In conclusion, dietary MH decreased dietary thermogenesis and physical activity. However, changes in EE were not mediated by AMPK.

**Keywords:** 5′ adenosine monophosphate-activated protein kinase, energy expenditure, indirect calorimetry, Labrador Retriever, Mannoheptulose, physical activity

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4.2. Introduction

Obesity is the most common nutritional disorder affecting companion animals (German, 2006). Energy restriction is the most robust strategy for weight management, but results in negative behavioural changes in dogs thereby straining the human-animal bond (Roudebush et al., 2008). Energy restriction mimetics (ERM) may provide an alternative to ER. In humans, several plant-derived ERM are currently under investigation, including resveratrol and epigallocatechin gallate. These compounds have been shown to activate 5' adenosine monophosphate-activated protein kinase (AMPK) (Hardie, 2011). Once activated, AMPK promotes ATP-generating pathways and reduces anabolic processes, creating a direct link between cellular energy status and whole body energy expenditure (EE).

Mannoheptulose (MH), a seven-carbon sugar found in avocados, has been preliminarily investigated as an ERM in dogs. Early research demonstrated that MH competitively inhibits hexokinases (Crane and Sols, 1954; Coore and Randle, 1964). As glucose deprivation has been shown to activate AMPK (Salt et al., 1998), it is plausible that MH-induced inhibition of glycolysis would decrease ATP production thereby activating AMPK and in turn decreasing diet induced thermogenesis and increasing lipid oxidation and glucose uptake. However, this hypothesis has not been tested.

The primary objective of this study was to examine the effects of dietary MH (4 mg/kg BW) on EE in adult Labrador Retrievers. In addition, we examined the effects of MH on skeletal muscle AMPKα protein content and phosphorylation. We hypothesized that MH will decrease diet induced thermogenesis and this decrease will be associated with increases in the activation of AMPK in skeletal muscle. Our secondary aim was to examine the effect of dietary MH on respiratory quotient (RQ) and serum glucose and insulin.
4.3. Materials and Methods

4.3.1. Animals and Housing

All procedures were approved by the Institutional Animal Care and Use Committee of The Iams Company (Lewisburg, OH). A total of 12 black Labrador Retrievers (5 spayed females and 7 neutered males, 26.99 ± 0.634 kg; 4.9 ± 0.2 yr of age) were used in this study. All dogs resided at The Iams Company (Lewisburg, OH) and were considered healthy based on a general health evaluation by a licensed veterinarian prior to study. Dogs were pair-housed in indoor runs (in the same building) with free access to water and indoor and outdoor runs. The indoor kennel was maintained on a 12 h light (0600 h to 1800 h) and dark (1800 h to 0600 h) cycle, in addition to natural light. Indoor temperature was set at 22°C (range 18°C to 24°C) and humidity at 50% (range 40% to 70%) with 10 to 15 fresh air exchanges per hour. All indoor runs were equipped with raised canvas beds, toys and heated flooring. Outdoor runs were equipped with toys and play yard equipment. All dogs received 40 min of supervised group exercise and socialization in a separate fenced yard daily.

4.3.2. Study Design

This study was designed as a parallel, double-blind, cross-over with each dog receiving both dietary treatments, control (CON) and mannoheptulose (MH), in random order. The study took place January 2013 to July 2013 (total duration 154 d) and included two dietary washout periods (each 14 d) and two periods (each 63 d). In each period indirect calorimetry and physical activity were measured and muscle biopsies were collected after an overnight fast (49 d) and 4 h post feeding (56 d). The indirect calorimetry method only allowed for 4 dogs to be measured per day. Therefore, the 12 dogs were divided into 3 groups of 4 dogs with each diet represented on each day and staggered 1 d apart. As this experiment was part of a larger study, glucose and lipid kinetics were assessed on study d 35 and 63, respectively (data presented in Chapter 5).
Both diets, CON and MH, were made from identical ingredients and were similar in terms of nutrient content (Table 4.1). The MH diet was made by incorporating a water-soluble extract of flesh-only un-ripened fruit avocado (MH source) (Kemin Industries, Des Moines, IA) into the CON diet to deliver a MH dose of approximately 400 mg/kg diet. Only two studies have administered MH orally to dogs. Labrador Retrievers received 2 mg/kg BW dietary MH and plasma MH peaked 2 to 4 h after ingestion (Davenport et al., 2010b). MH has also been given as an oral supplement (8 mg/kg BW) to adult Beagles and plasma MH peaked 3 to 4 h after ingestion and an increase in dietary thermogenesis was observed (Chapter 2). A pilot study performed in our laboratory did not find any changes in EE in Labrador Retrievers fed a dietary dose of 2 mg/kg BW MH, suggesting a higher dose of MH may be necessary to elicit changes in EE. As such, a dose of 4 mg/kg BW dietary MH was used in this study.

The MH enriched avocado extract was produced using commercially available avocados (Hass variety). Frozen, avocados comprising the flesh were initially ground before suspension in water (1:3 w/w). The resultant slurry was centrifuged to remove non-aqueous solids. A series of microfiltration (de-oiling), ultrafiltration (10 kDa) and nanofiltration (100 kDa) was used to produce the MH-enriched fraction. Samples of the diets were taken at four separate time points (twice per period) to confirm the MH content. Animals were individually fed their daily ration in two meals (0700 h and 1300 h) and food intake was measured daily. Beginning 10 wk prior to study initiation dogs were fed the CON diet and caloric intake and body weights were monitored weekly. During this dietary wash-in period caloric intakes were adjusted to maintain dogs at a body condition score of ~3.0 (5-point scoring scale with half points (adapted from Laflamme, 1997). The day before study initiation, dogs were randomized to dietary treatment and their most recent caloric intakes were used throughout
the entire experimental period, resulting in a MH dose of 4 mg/kg BW. In between treatment periods, dogs received the CON diet for 3 wk (wash-out).

**Table 4.1.** Ingredient composition and proximate analysis of the control (CON) and mannoheptulose (MH) containing diets

<table>
<thead>
<tr>
<th>Ingredient inclusion, %</th>
<th>CON</th>
<th>MH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>17.7</td>
<td>17.7</td>
</tr>
<tr>
<td>Corn Meal</td>
<td>14.6</td>
<td>14.6</td>
</tr>
<tr>
<td>Chicken-By-Product Meal</td>
<td>14.4</td>
<td>14.4</td>
</tr>
<tr>
<td>Ground Whole Grain Sorghum</td>
<td>13.6</td>
<td>13.6</td>
</tr>
<tr>
<td>Corn Grits</td>
<td>11.1</td>
<td>11.1</td>
</tr>
<tr>
<td>Ground Whole Grain Barley</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Fish Meal</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Chicken Fat 1</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Chicken Flavor</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Beet Pulp</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Mineral mix 2</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Egg Product</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Brewers Dried Yeast</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Vitamin mix 3</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Other 4</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Avocado Extract</td>
<td>0.04</td>
<td>0.04</td>
</tr>
</tbody>
</table>

**Analyzed chemical profile (DM basis)**

<table>
<thead>
<tr>
<th>Dry matter, %</th>
<th>CON</th>
<th>MH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>92.1</td>
<td>92.1</td>
</tr>
<tr>
<td>Nutrient</td>
<td>Percentage</td>
<td>ME, kcal/g</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>Crude protein, %</td>
<td>26.3</td>
<td>26.5</td>
</tr>
<tr>
<td>Crude fat, %</td>
<td>16.6</td>
<td>16.8</td>
</tr>
<tr>
<td>Crude fiber, %</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Ash, %</td>
<td>6.5</td>
<td>6.6</td>
</tr>
<tr>
<td>ME, kcal/g</td>
<td>3709</td>
<td>3712</td>
</tr>
</tbody>
</table>

1. Preserved with mixed tocopherols  
2. Potassium chloride, calcium carbonate, sodium chloride, dicalcium phosphate, ferrous sulfate, zinc oxide, manganese sulfate, copper sulfate, manganous oxide, potassium iodide, cobalt carbonate  
3. Vitamin E, choline chloride, ascorbic acid, vitamin A acetate, cross-linked beta carotene calcium pantothenate, biotin, thiamine mononitrate, vitamin B12, niacin, riboflavin, inositol, pyridoxine hydrochloride, vitamin D3, folic acid  
4. Sodium hexametaphosphate, fructooligosaccharides, flax meal, dried chicken cartilage, DL-methionine, L-carnitine, rosemary extract  
5. Metabolizable energy content (ME) was determined using the modified Atwater factors where fat, protein and carbohydrate provide 8.5, 3.5, and 3.5 Kcal/g respectively.

4.3.4. Serum Biochemistry

At baseline, d 14 and d 28 of each period, a blood sample (3 mL) was taken by jugular venipuncture prior to the dog’s morning meal (fasting) and again precisely 4 h after the initial morning meal (fed). Blood was centrifuged for 8 min at 3000 × g at 7ºC and the serum was stored at −80ºC for later analysis of mannoheptulose, glucose and insulin.

4.3.5. Biochemical Analyses

Serum glucose was measured by a clinical chemistry analyzer (Beckman Coulter AU480). Insulin was assayed using a paramagnetic particle, chemiluminescent immunoassay (Beckman, Fullerton, CA). The assay was not canine-specific but was validated using canine serum (lower limit of detection is 0.03 μIU/mL and upper limit is 300 μIU/mL). The LC/MS/MS approach was used to determine serum MH. Briefly, 200 μL of plasma was diluted with 800 μL MeCN and 10 μL of internal standard solution (13C7 D-MH, Toronto Research Chemicals, Inc.) and sonicated for 5 min, followed by centrifugation 5 min at 3750 rcf. The supernatant (10 μL) was injected into a polymer-based HILIC Shodex NH2P-40 3E
column (3.0 × 250 mm, 4-µm particles) for an isocratic separation using 76% Mobile Phase A and 24% Mobile Phase B (84.2% organic) at a flow rate of 0.3 mL/min followed by a gradient column wash in 100% Mobile Phase B. The column temperature was maintained at 50°C. The detector was a Sciex API4000 mass spectrometer operated in negative ion electrospray mode and employing multiple reaction monitoring (MRM). The mass transitions used for mannoheptulose and its internal standard were (m/z 255.1 → 209.1) and (m/z 262.1 → 216.1) respectively. The total run time per sample was approximately 60 min.

4.3.6. Indirect Calorimetry

Respiratory gas exchange measurements were conducted via whole-body indirect calorimetry. The calorimetry chambers (76 cm × 53 cm × 61 cm, L × W × H) were made of clear plexiglass and fitted with a hinged access top door for providing food to the dog. Chambers were designed as open-flow circuits with room air pulled into the chambers at a rate of 22 to 26 L/min to maintain CO₂ levels in the chamber between 0.4 and 0.8%. Exiting chamber air was dried by passing it through columns of Drierite™ and magnesium perchlorate before reaching the O₂ and CO₂ analyzers (Qubit Systems Inc., Kingston, ON).

Prior to study initiation, dogs were acclimated over an 8-wk period (1 to 8 h per week) to rest comfortably and calmly in the chamber with no excessive activity or movement. Dogs that did not acclimate were ineligible to participate in the study. Gas exchange measurements were conducted on d 42 of each period. Prior to any measurements, the O₂ and CO₂ analyzers were calibrated with standard gases and dogs rested in the chamber for a minimum of 25 min to ensure adequate CO₂ equilibration. Two fasting measurements were taken, after which dogs were fed their full daily ration of test diet as a single meal (time 0) and gas exchange measurements continued for 10 h. Each chamber was sampled every 3 s over a 5 min period every 25 min. O₂ and CO₂ exchange and respiratory quotient data were logged in real time using data acquisition software (Qubit Systems Inc., Kingston, ON). Energy expenditure was
calculated from O₂ consumption and CO₂ production (VO₂ and VCO₂) using the abbreviated Weir equation (de Weir, 1949) and expressed on a per kg lean mass basis.

4.3.7. Body Composition Analysis

On d 43 of each treatment leg body composition analysis was completed using an X-Ray Bone Densitometer (QDR4500, Hologic Inc., Bedford MA). Dogs were fasted overnight (18 h since last meal) and sedated using Dexmedetomidine (Dexdomitor, Pfizer) at a dose of 0.02 mg/kg and Carprofen (Rimadyl, Pfizer) at a dose of 2 to 4 mg/kg administered i.m. Propofol (Propoflo, Abbott) at a dose of 5 to 7 mg/kg was administered i.v. for induction. Dogs were positioned on their sternum with the cranial aspect of ante brachium placed on the table to ensure the phalanges faced caudally. The hind limbs were extended with the tail placed straight and in between the hind limbs. A whole body scan was performed of the following regions: left arm, right arm, trunk, left leg, right leg and head. Scans were done in triplicate for each dog and the median value of the three scans was recorded. Following the scan, atipamezole (Antisedan®, Pfizer) was administered to each dog at a dose of 0.2 mg/kg. Dogs were placed in a heated cage until fully recovered and monitored for 1 wk for complications.

4.3.8. Physical Activity Monitoring

Continuous physical activity measurements were made using the Actical accelerometer (Philips Respironics, Bend, OR). The Actical device (28mm × 27 mm × 10mm, ~17.5 g) was attached to the dog’s collar in the ventral position and all dogs were acclimated to wearing the device prior to study initiation. The device includes an accelerometer that is sensitive to movement in all directions and a piezoelectric sensor that generates a voltage when the device is subjected to a change in velocity per unit time. The voltage generated by the sensor is converted to a digital value that is used to adjust a running baseline value. The difference between the current digital value and baseline is used to create a raw activity value.
for the measurement period (1 min). Raw activity values were converted to activity counts using ActiReader software (Philips Respironics, Bend, OR).

Baseline activity measurements were taken the week prior to study initiation and again the third week of the dietary washout period. Treatment measurements were taken on d 43 to 47 of each period (March 2013, period 1 and June 2013, period 2). The average activity per minute was calculated during dark (1800 h to 0600 h) and light (0600 h to 1800 h) time periods. The percent of active time was calculated by dividing the time when dogs were active (activities greater than or equal to 250 per minute) by the total time. Weekday (0600 h Wednesday through 0600 Saturday) and weekend (0600 h Saturday through 0600 h Monday) activity were analyzed separately, as there is less human interaction with the animals on weekends.

4.3.9. Muscle Biopsy

Muscle biopsies were taken after an overnight fast (18 h since last meal) on d 49 of each treatment leg. On d 56 of both treatment legs, dogs were fed their full daily ration of test diet at their morning meal and muscle biopsies were taken 4 h post-feeding (contralateral leg to that of the fasted sample). This sampling time point coincides with peak MH concentrations in the plasma (Davenport et al., 2010b; Chapter 2). Dogs were sedated using Dexmedetomidine (Dexdomitor, Pfizer) at a dose of 0.02 mg/kg and Carprofen (Rimadyl, Pfizer) at a dose of 2 to 4 mg/kg i.m. The hair over the incision point was clipped and the skin was prepared aseptically. A 1 to 2 cm incision was made with a #11 scalpel blade in the lateral aspect of the proximal thigh, and a Bergstrom needle (Surgipro, Inc. Shawnee, KS) was used to obtain 20 – 30 mg of muscle tissue from the vastus lateralis. The incision was closed using surgical skin staples (AutoSuture Appose ULC TM, Tyco) and atipamezole (Antisedan, Pfizer) was administered (0.2 mg/kg). Muscle samples were immediately frozen in liquid N2 and stored at –80°C for later immunoblot analysis.
4.3.10. Western Blot

Muscle was homogenized in ice cold NP40 Cell Lysis Buffer (15:1 v:w) with 1 mmol/L PMSF (Invitrogen Corp., Camarillo CA) and protease inhibitor cocktail (Sigma, Oakville ON) and spun at 4°C for 5 min at 1500 x g. Supernatants were removed and protein concentration was determined by Bradford protein assay. Protein (25 µg) from muscle lysates was diluted in Laemmli sample buffer (Bio-Rad Laboratories, Mississauga ON) and loaded onto 7.5% Tris HCL pre-cast resolving gels (Bio-Rad Laboratories, Mississauga ON) and separated by SDS-PAGE. Gels were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Mississauga ON) (200 mA/tank). Membranes were blocked in 5% milk for 1 h at room temperature and washed in TBST for 10 min before being incubated in primary antibody overnight at 4°C. Primary antibodies (AMPK and pAMPK, Cell Signaling Technology Inc., Danvers, MA) were diluted (1:1000) in 5% BSA/TBST. Membranes were washed twice in TBST for 15 min and then incubated with anti-rabbit immunoglobulin–horseradish-peroxidase–linked secondary antibodies (1:2000 dilution in TBST) for ~1 h. The proteins of interest were visualized using enhanced chemiluminescence (GE Health Care Biosciences Corp., Piscataway, NJ) and captured and quantified using AlphaView Software for FluorChem Systems. All samples from individual dogs were loaded on to the same gel, in addition to a control sample. Data were normalized to the control sample and expressed as the ratio of phosphorylated to total protein content.

4.3.11. Statistical Methods

All data were analyzed using SAS version 9.2 (SAS Institute, Cary, NC) and are expressed as means and pooled SEM. Mixed effects models were fitted using the PROC MIXED procedure of SAS assuming fixed period and diet effects and dogs as random variables. Denominator degrees of freedom were calculated using the Kenward-Rogers approximation. Repeated measures within period on dog were analyzed using the
autoregressive order 1 covariance structure. Multiple comparisons were made using the Tukey-Kramer method. Interactions between fixed effects were tested but only discussed if significant. For calorimetry data, fasting, 0 to 10 h post-prandial and 3 to 5 h post-prandial data were analyzed separately and time was considered a fixed effect. MH concentrations have been shown to peak in plasma between 3 to 5 h after feeding and the effects of MH on metabolism are transient. Therefore, the post-prandial time period of 3 to 5 h was selected to examine maximal MH effects, in addition, to provide a basis of comparison for muscle protein analysis. Results were considered statistically significant if $P < 0.05$ and a statistical trend was defined as $P$ value between 0.05 and 0.1.

4.4. Results

4.4.1. Serum Biochemistry

There were no detectable MH concentrations (< 50 ng/mL) in serum of dogs fed CON on d 14 or 28 (post-prandial sample). Serum from dogs fed MH had significantly greater MH concentrations than from dogs fed CON ($P < 0.05$), and MH concentrations were not different between d 14 (1206 ± 132 ng/mL) and d 28 (1155 ± 88 ng/mL).

MH did not affect fasting or post-prandial serum glucose (Table 4.2). Both fasting and post-prandial glucose were higher in the first study period than the second; and highest on d 14 of study. For fasting glucose only, the interaction between day and period was significant ($P = 0.01$) (Fig. 4.1). Specifically, within period 1, serum glucose on d 14 was higher than that at baseline and d 28 (no difference between baseline and d 28). Serum glucose was not different between days in period 2. Diet, day and period did not significantly affect fasting or post-prandial serum insulin (Table 4.2). However, post-prandial serum insulin tended to be lower in the first study period than the second period.
Table 4.2. Fasting and post-prandial (4 h post-feeding) serum glucose and insulin in adult Labrador Retrievers fed a control (CON, no mannoheptulose) or mannoheptulose containing diet (MH, 4 mg/kg BW) (a total of 12 dogs in a complete cross-over design).

<table>
<thead>
<tr>
<th></th>
<th>Diet</th>
<th>Period</th>
<th>SEM</th>
<th>$P$-value</th>
<th>Diet</th>
<th>Period</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MH</td>
<td>CON</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fast</td>
<td>5.94</td>
<td>5.98</td>
<td>6.33</td>
<td>5.59</td>
<td>0.09</td>
<td>0.48</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Post-prandial</td>
<td>6.28</td>
<td>6.28</td>
<td>6.44</td>
<td>6.12</td>
<td>0.17</td>
<td>0.99</td>
<td>0.03</td>
</tr>
<tr>
<td>Insulin, μU/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fast</td>
<td>6.44</td>
<td>6.70</td>
<td>6.69</td>
<td>6.44</td>
<td>0.70</td>
<td>0.70</td>
<td>0.95</td>
</tr>
<tr>
<td>Post-prandial</td>
<td>15.47</td>
<td>13.66</td>
<td>12.65</td>
<td>16.47</td>
<td>1.43</td>
<td>0.35</td>
<td>0.06</td>
</tr>
</tbody>
</table>

1 baseline, 5.96 mmol/L; d 14, 6.06 mmol/L; d 28, 5.86 mmol/L
2 baseline, 6.13 mmol/L; d 14, 6.58 mmol/L; d 28, 6.14 mmol/L
Figure 4.1. Fasting and post-prandial (4 h post-feeding) serum glucose in the first (●) and second (○) study periods in adult Labrador Retrievers fed either a control diet or mannoheptulose containing diet in a cross-over design (total of 12 dogs)\(^1\).

\(^1\) Fasting serum glucose was significantly affected by period \((P < 0.01)\) and day \((P = 0.04)\), but not diet \((P = 0.48)\). The interaction between period and day was also significant \((P = 0.01)\). Post-prandial serum glucose was not affected by diet \((P = 0.99)\). However, period and diet were significant, \(P = 0.03\) and \(P < 0.01\), respectively (no significant interactions).

4.4.2. Body Weight and Composition

Food intake was fixed in this study based on the dogs historical energy intake records and was not different between diets (CON, 718 ± 65; MH, 765 ± 78 kcal/d; \(P = 0.65\)). Body weight significantly increased throughout the study \((P_{\text{time}} < 0.01)\) irrespective of diet \((P_{\text{diet}} = 0.27)\) (Fig. 4.2) and there was no interaction between diet and time \((P = 0.35)\).
Figure 4.2. Body weights (kg) were measured weekly over the entire study. Dogs were randomized to dietary treatment order, a MH containing diet (MH, 4 mg/kg BW) followed by control diet (CON) (■) (n = 6) or CON followed by MH (□) (n = 6), with a 3 wk dietary washout between periods.

Body composition is presented in Table 4.3. Diet did not significantly affect body weight, fat or lean mass. However, dogs fed MH had a significantly lower ratio of fat to lean mass. There was a significant affect of period for body weight, fat mass, and percent fat mass, which were all higher in the second period. Percent lean mass was lower in the second period (Table 4.3), but absolute lean mass did not differ between treatments or periods. These findings are not surprising given that dogs gained weight throughout the study.
Table 4.3. Body composition measured by dual-energy X-ray absorptiometry of adult Labrador Retrievers fed either a control diet (CON, no mannoheptulose) or a mannoheptulose containing diet (MH, 4 mg/kg BW) (a total of 12 dogs in a complete cross-over design).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Period</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH</td>
<td>CON</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>27.5</td>
<td>28.2</td>
<td>27.1</td>
</tr>
<tr>
<td>Lean mass, kg</td>
<td>21.3</td>
<td>21.4</td>
<td>21.2</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>5.23</td>
<td>5.45</td>
<td>4.87</td>
</tr>
<tr>
<td>Lean mass, %</td>
<td>77.4</td>
<td>76.8</td>
<td>78.3</td>
</tr>
<tr>
<td>Fat mass, %</td>
<td>17.0</td>
<td>19.0</td>
<td>18.0</td>
</tr>
<tr>
<td>Fat : lean, %</td>
<td>24.7</td>
<td>27.7</td>
<td>23.1</td>
</tr>
</tbody>
</table>

4.4.3. Energy Expenditure and Respiratory Quotient

Resting energy expenditure (REE) was not affected by diet, but was higher ($P = 0.03$) in the first period than the second period (42.9 vs. 38.3 kcal/(kg lean mass · d), respectively) (Table 4.4). Post-prandial (0 to 10 h) EE (Supplemental Fig. 1a) was not significantly different between diets. From 3 to 5 h post-prandium, EE tended ($P = 0.08$) to be lower in dogs fed MH than those fed CON. Diet did not affect fasting or post-prandial (0 to 10 h) RQ (Supplemental Fig. 1b); however, during 3 to 5 h post-prandium, RQ was significantly lower in dogs fed MH compared to dogs fed CON.
Table 4.4. Resting and post-prandial energy expenditure (EE) and respiratory quotient (RQ) in adult Labrador Retrievers fed either a control (CON, no mannoheptulose) or mannoheptulose containing diet (MH, 4 mg/kg) (a total of 12 dogs in a complete cross-over design).

<table>
<thead>
<tr>
<th>Diet</th>
<th>MH</th>
<th>CON</th>
<th>SEM</th>
<th>P value</th>
<th>Diet</th>
<th>Period</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE, kcal/(kg lean mass · d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting</td>
<td>39.6</td>
<td>41.5</td>
<td>1.90</td>
<td>0.31</td>
<td>0.03</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Post-prandial (0 – 10 h)</td>
<td>62.9</td>
<td>64.9</td>
<td>1.69</td>
<td>0.16</td>
<td>0.31</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Post-prandial (3 – 5 h)</td>
<td>64.6</td>
<td>67.9</td>
<td>1.65</td>
<td>0.08</td>
<td>0.55</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>RQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>0.77</td>
<td>0.76</td>
<td>0.01</td>
<td>0.44</td>
<td>0.47</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Post-prandial (0 – 10 h)</td>
<td>0.87</td>
<td>0.87</td>
<td>0.01</td>
<td>0.31</td>
<td>0.20</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Post-prandial (3 – 5 h)</td>
<td>0.87</td>
<td>0.88</td>
<td>0.01</td>
<td>0.02</td>
<td>0.15</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

1 Period 1, 42.9 Kcal/(kg lean mass · d); Period 2, 38.3 Kcal/(kg lean mass · d)
Figure 4.3. Post-prandial (0 – 10 h) resting energy expenditure (a) and respiratory quotient (b) in adult Labrador Retrievers fed either a control diet (□) (no mannoheptulose) or a mannoheptulose containing diet (■) (4 mg/kg). Data are means with pooled standard errors and a total of 12 dogs in a complete crossover design.
4.4.4 Physical Activity

For all baseline and period activity measurements, dogs were more active in light than dark time periods.

Weekday and weekend baseline activity per minute was not different between diets prior to study initiation or during the dietary washout period. Similarly, there was also no effect of diet on percent of time activity during baseline measurements (data not shown).

There was no affect of diet on percent of time active in dark or light periods (Table 4.5). Weekday activity per minute tended to be lower in dogs fed MH compared to CON during the light time period only. On the weekend, daytime activity per minute was significantly lower in dogs fed MH than those fed control. Similarly, dogs fed MH tended to have lower percent of active time than dogs fed CON.

Period was significant for weekday and weekend activity per minute and percent active time in the dark time period only (Table 4.5). For both weekday and weekend, activity measures were higher in the first period, compared to the second period.
Table 4.5. Spontaneous physical activity counts as measured using an accelerometer in adult Labrador Retrievers fed either a control (CON, no mannoheptulose) or mannoheptulose containing diet (MH, 4 mg/kg) (a total of 12 dogs in a complete cross-over design).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Period</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH</td>
<td>CON</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weekday&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average activity per minute</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark&lt;sup&gt;2&lt;/sup&gt;</td>
<td>70.9</td>
<td>69.5</td>
<td>100</td>
</tr>
<tr>
<td>Light&lt;sup&gt;3&lt;/sup&gt;</td>
<td>251</td>
<td>272</td>
<td>258</td>
</tr>
<tr>
<td>Percent of active time&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark, %</td>
<td>6.0</td>
<td>6.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Light, %</td>
<td>19</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Weekend&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average activity per minute</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>65.6</td>
<td>62.9</td>
<td>86.8</td>
</tr>
<tr>
<td>Light</td>
<td>223</td>
<td>258</td>
<td>233</td>
</tr>
<tr>
<td>Percent of active time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark, %</td>
<td>6.0</td>
<td>6.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Light, %</td>
<td>17</td>
<td>19</td>
<td>16</td>
</tr>
</tbody>
</table>

<sup>1</sup> Weekday = measurements taken 0600 h Wednesday through 0600 h Saturday
<sup>2</sup> Dark = measurements taken from 1800 h – 0600 h
<sup>3</sup> Light = measurement taken from 0600 h – 1800 h
<sup>4</sup> Percent of active time = activities greater than or equal to 250 per minute divided by the total time
<sup>5</sup> Weekend = measurements taken 0600 h Saturday through 0600 h Monday
4.4.5. Skeletal Muscle Protein Abundance

An inadequate amount of muscle tissue (< 20 mg) was harvested to complete the necessary analyses in some animals and as such, statistical power was reduced. AMPK was expressed as the ratio of phosphorylated to total AMPK protein content (Fig. 4.4). There was no significant effect of diet or period on fasting ($P = 0.72; N = 9$ MH; $N = 9$ CON) or post-prandial ($P = 0.24; N = 7$ MH; $N = 10$ CON) AMPK.

Figure 4.4. The ratio of phosphorylated to total AMPK (pAMPK/total) protein abundance in vastus lateralis muscle of adult Labrador Retrievers. Fasting muscle samples were taken prior to the dog’s morning meal (18 h since last meal). Fed muscle samples were taken 4 h after the dogs consumed their full daily ration of test diet, either control or mannoheptulose (4 mg/kg). Data are means with pooled standard errors.

1 $N = 9$
2 $N = 9$
3 $N = 7$
4 $N = 10$
4.5. Discussion

Dietary MH (4 mg/kg), delivered as an extract of un-ripened avocados, resulted in lower dietary induced thermogenesis and physical activity. These MH induced changes in EE were not associated with alterations in the content and phosphorylation of AMPK in skeletal muscle. Interestingly, decreased EE associated with MH feeding, led to a reduction in body fat to lean mass, without altering body weight (despite equivalent energy intakes). As expected, MH decreased post-prandial RQ, indicating higher fat relative to carbohydrate oxidation. However, MH did not affect fasting or post-prandial serum glucose or insulin.

Energy expenditure as measured by indirect calorimetry encompasses REE, adaptive thermogenesis and a small amount of voluntary activity. It does not include the energy expended during physical activity. REE represents the major component of total EE (~70%) and is largely predicted by lean mass (Johnstone et al., 2005). As lean mass was not affected by diet in this study, it is not surprising that diet did not affect REE. The REE values observed in this study are similar to those reported in Beagles (Pouteau et al., 2002; Chapter 2) and Labrador Retrievers (Yoo et al., 2006).

Adaptive thermogenesis includes the energy produced in response to environmental temperature and diet and accounts for a relatively small proportion of total EE (~10%). Unexpectedly, MH transiently decreased diet-induced thermogenesis. This finding is in contrast to that observed previously in Beagles fed a MH supplement (8 mg/kg) (Chapter 2) and to our original hypothesis. It is worth noting that this study utilized Labrador Retrievers of higher body condition score than the Beagles in the previous study. In agreement with reduced dietary thermogenesis associated with MH feeding, RQ was reduced, suggesting a higher proportion of fat relative to carbohydrate oxidation. Dietary fat, in comparison to protein and carbohydrate, contributes the least to the thermic effect of food. However, RQ
does not indicate net carbohydrate or fat oxidation, nor does it account for protein oxidation. More sensitive measures of substrate oxidation are needed.

Changes in RQ were not accompanied by changes in circulating glucose or insulin concentrations. Previous studies using higher doses of MH (1 – 2 g/kg) administered intravenously or intra-arterially observed profound, transient hyperglycaemia and hypoinsulinemia in fasted rats, dogs and humans (Johnson and Wolff, 1970, Klain and Meikle, 1974, Muller et al., 1971, Klain et al., 1976). Specifically, up to five-fold increases in blood glucose (22 – 28 mmol/L) coupled with a near complete blockage of insulin secretion (0 – 2 U/mL in plasma) were noted, suggesting that doses greater than 1 g/kg were supra-physiological. In the current study, there were no detectable serum MH concentrations in fasted dogs. Therefore, it is not surprising that MH did not affect fasting glucose or insulin concentrations. Studies administering low oral doses of MH are limited. Davenport et al. (2010a) reported lower post-prandial serum insulin concentrations, but not glucose, in senior Labrador Retrievers (average age 11.8 yr) fed 2 mg/kg dietary MH, whereas no changes in fasting or post-prandial (24 h) serum glucose or insulin were observed in healthy adult Beagles fed an oral supplement containing 8 mg/kg MH (Chapter 2). The lack of post-prandial glucose and insulin responses in the current study may be attributed to the inclusion of dietary fibres, especially sorghum grain. In comparison to brewer’s rice, corn and cassava, sorghum elicited a minimal change in incremental blood glucose and insulin when provided as the primary carbohydrate source (Carciofi et al., 2008).

As MH has been shown to inhibit hexokinases, the reduced flux through glycolysis was expected to limit glucose as a substrate of energy production and lead to a decrease in intracellular ATP. Lowering the ATP to ADP ratio would activate AMPK. Once activated, AMPK directly decreases EE by inhibiting anabolic pathways, and concomitantly increases ATP production via glucose and fat oxidation. Indeed, glucose deprivation, as imposed by
another glycolytic inhibitor, 2-deoxyglucose, has been shown to activate AMPK (Jaing et al., 2008; Hawley et al., 2010). In this study, MH had no effect on the ratio of phosphorylated to total AMPK protein content (used as a marker of AMPK activation). Glucose utilization in skeletal muscle is mediated by hexokinase II isoform, which has a low $K_m$ for glucose compared to that of hexokinase IV found in liver, pancreas and brain. As glucose phosphorylation in skeletal muscle is saturated at low glucose concentrations, AMPK may not be as sensitive to changes in glucose supply as it would be in tissues that express glucokinase. In addition, MH was delivered in a mixed meal. Therefore, it is also likely that effectiveness of MH as competitive inhibitor of hexokinase II was overcome by increased substrate concentrations. A dose response study is necessary to better understand the role of dietary MH as a glycolytic inhibitor in skeletal muscle (and other tissues). Results from the immunoblot were highly variable and sample size was reduced as an inadequate amount of muscle tissue was harvested to complete the necessary analyses. As this is the first study to examine changes in AMPK protein content between fasting and post-prandial conditions in dogs, further research is warranted using a larger sample size.

The final component of EE, physical activity, can account for 20 – 30% of EE. Dogs in this study exhibited diurnal patterns of activity; specifically dogs were more active during the light than dark periods (irrespective of diet). This finding agrees with the activity patterns displayed by laboratory Beagles (Siwak et al., 2003). In contrast, feral dogs have been shown to be more active in dark periods than light periods (Scott and Causley, 1973). Differences in physical activity patterns between laboratory-housed dogs and feral dogs suggest that dogs are able to adapt activity patterns to their environmental conditions. In the dark time period, dogs were more active in the first study period (March 2013) than the second period (June 2013). This finding may be attributed to differences in light exposure and intensity between study periods. Specifically, exposure to natural light was reduced in the first period compared to the
second period due to the time of year. The reduced exposure to natural light would have imposed an abrupt transition between light and dark periods, as artificial lighting was maintained on a 12 h cycle. Previous research in Beagles has demonstrated that dogs exhibit anticipatory activity prior to the lights coming on in an indoor housing arrangement (Siwak et al., 2003). When these animals were transitioned to an indoor/outdoor housing facility this anticipatory activity was no longer present. This finding is thought to be attributed to the more gradual light-dark transition provided by exposure to natural sunlight (similar to that observed in our second study period). As all dogs had access to outdoor runs, it is possible that differences in climate between study periods may have also influenced activity. Feral dogs were noted to be move greater distances during cooler seasons than warmer seasons in the southern United States (Scott and Causley, 1973). However, no research has investigated the interaction between physical activity patterns and climate in laboratory dogs, likely due to the standardization of housing conditions in research settings. Study period did not affect daytime activity patterns. This finding is in agreement with Siwak et al. (2003) who found daytime activity patterns in laboratory Beagles to be largely dictated by standardized daily routine that dogs experienced in their housing facility. In contrast, MH decreased daytime physical activity, particularly on the weekend when there is reduced human-animal contact. It is important to note that activity, as measured in this study, represents the mean activity count (locomotion) over the entire light (06:00 – 18:00) and dark periods (18:00 – 06:00). It is unclear how MH affects discrete activity bouts and conversely rest periods, but this finding is interesting. Furthermore, locomotion encompasses complex behaviours (i.e., spontaneous activity, exploration and exercise) that are elicited by a wide range of internal and external stimuli. A more critical analysis of physical activity and related behaviours is necessary to fully understand the effects of MH.
Together, diet induced thermogenesis and physical activity comprise up to 30% of total EE. With prolonged reductions in these components, as imposed by daily MH feeding, one may expect weight gain. However, MH decreased the ratio of body fat to lean mass, despite all dogs having equivalent energy intakes. These findings may be partly attributed to increased fat oxidation with daily MH feeding. To better understand the impact of MH on EE, other measurement techniques, including the doubly labelled water technique, should be considered. The doubly labelled water technique would enable average estimates of EE over variable time periods in the dog’s regular environment.

In conclusion, daily MH feeding (4 mg/kg) decreased dietary thermogenesis and physical activity, particularly on the weekend when there is reduced human animal contact. As physical activity encompasses a broad range of complex behaviours, a more critical analysis of physical activity (and related behaviours) is warranted. Transient reductions in dietary thermogenesis may be reflective of increased fat oxidation, as evidenced by a lower RQ and a reduction in fat to lean body mass with MH feeding (despite equivalent energy intakes).
5.1. Abstract

This study aimed to determine the effects of dietary mannoheptulose (MH), delivered as an extract of un-ripened avocados, on glycerol, palmitate and glucose kinetics in healthy adult Labrador Retriever dogs (total of 12 dogs, 26.99 ± 0.634 kg, 4.9 ± 0.2 yr). The study was a double-blind, cross-over with each dog receiving both dietary treatments, control (CON) and MH (4 mg/kg BW), in random order. Plasma glucose turnover and oxidation in fasting and in response to repeated meal feeding were assessed on d 35 using a primed constant intravenous infusion of U-\textsuperscript{13}C-glucose and indirect calorimetry. On d 63, fasting plasma glycerol was measured using a primed constant intravenous infusion of 1,1,2,3,3-D\textsubscript{5}-glycerol, after which plasma glycerol turnover and plasma palmitate turnover and oxidation were examined during repeated meal feedings. Meals were fed 3 times 10 min apart and every 25 min thereafter. The fifth meal (time 70 min) contained U-\textsuperscript{13}C-K\textsubscript{2}-Palmitate (4 mg/kg). MH had no effect on fasting or post-prandial plasma glucose turnover and oxidation. Furthermore, MH did not affect fasting or post-prandial lipolytic rate or post-prandial plasma palmitate turnover or oxidation. Future work should consider measuring fasting palmitate kinetics and additionally, seek to understand the effects of different doses of MH on macronutrient metabolism.

**Keywords**: free fatty acids, glucose, indirect calorimetry, Labrador Retriever, mannoheptulose
5.2. Introduction

In companion animals there is a growing interest in the use of nutraceuticals for weight management. A nutraceutical can broadly be considered a food or a part of a food that provides a health benefit. Mannoheptulose (MH), a seven carbon sugar found in avocados, is being evaluated as a novel nutraceutical for canines.

The metabolic effects of MH are ascribed to its ability to competitively inhibit glucokinase (Crane and Sols, 1954; Coore and Randle, 1964). When given at doses greater than 1 g/kg intravenously or intra-arterially, MH transiently elicits profound hyperglycaemia and hypoinsulinemia in fasted rats, dogs and humans (Johnson and Wolff, 1970, Klain and Meikle, 1974, Muller et al., 1971, Klain et al., 1976). Furthermore, MH (> 1 g/kg) has been shown to increase circulating glucagon, cAMP and alanine concentrations and result in uncontrolled hepatic glucose output (Muller et al., 1971; Issekutz et al. 1974) due to considerable increases in the activity of gluconeogenic enzymes (Klain et al., 1976).

The ability of MH to transiently lower the insulin to glucagon ratio could be of benefit to overweight animals as it would promote free fatty acid (FFA) oxidation. However, the metabolic responses observed in previous studies were well outside normal physiological ranges suggesting that doses greater than 1 g/kg are supra-physiological. Few studies have examined the metabolic effects of low oral doses of MH and no studies have investigated the effects of MH on lipid metabolism.

The purpose of this study was to determine the effects of avocado-derived MH extract (delivered in the diet, 4 mg/kg) on fasted and post-prandial lipolysis and FFA oxidation in adult Labrador Retrievers. Additionally, we examined MH effects on fasted and post-prandial plasma glucose turnover and oxidation. Dietary MH was not expected to significantly impact fasting metabolism, but was expected to increase FFA turnover and oxidation and decrease glucose oxidation post-prandially.
5.3. Materials and Methods

5.3.1. Animals

All procedures were approved by the Institutional Animal Care and Use Committee of The Iams Company (Lewisburg, OH). A description of the animals and housing is given in Chapter 4.

5.3.2. Study Design

The study was designed as a parallel, double-blind, cross-over with each dog receiving both dietary treatments, control (CON) and mannoheptulose (MH), in random order. Both diets, CON and MH, were made from identical ingredients and were similar in nutrient content. Full details of the experimental diets and enriched avocado-derived MH extract are provided in Chapter 4.

The study took place from January 2013 to July 2013 (total duration 154 d) and included two dietary washout periods and two treatment period. In each period (63 d) glucose and lipid kinetics were assessed on study d 35 and 63, respectively. This methodology required the use of indirect calorimetry, which only allowed 4 dogs to be measured per day. Therefore, the 12 dogs were divided into 3 groups of 4 dogs with each diet represented on each day and staggered 1 d apart.

5.3.3. Experimental Protocols

Two experimental protocols were performed, I: Assessment of glucose kinetics (d 35) and II: Assessment of lipid kinetics (d 63). Prior to the initiation of each experimental protocol, dogs were fasted overnight (18 h since last meal) and both front legs were aseptically prepared and an 18 gauge catheter was inserted into the cephalic vein of each leg. One catheter was used for stable isotope infusion, and the other catheter was only used if the first catheter failed.
Both protocols required repeated blood sampling and expired CO₂ collection. All blood samples (each 3 mL) were collected by jugular venipuncture. Dogs were well acclimated to jugular venipuncture prior to study initiation. Blood samples were centrifuged for 8 min at 3000 \times g at 7°C and the plasma was stored at –80°C for later biochemical analysis. Indirect calorimetry was used to measure the exchange of respiratory gases (see Chapter 4) and for the collection of expired CO₂. Expired CO₂ was trapped in 1 mol/L NaOH over a 20 min period within each calorimetry measurement cycle and stored at room temperature for later analysis of isotopic enrichment.

**Protocol I: Assessment of glucose kinetics**

Glucose kinetics were assessed in fasting and during repeated meal feeding on d 35 of each period (Figure 5.1). Two baseline breath and blood samples were collected prior to isotope infusion to determine background ¹³C enrichment.

At time 0, a primed (8.1 μmol/kg) constant (0.136 μmol/kg·min) intravenous infusion of U-¹³C-Glucose (99% atom, Sigma Aldrich, Isotec, Miamisburg, OH) was administered via the cephalic catheter. Six fasting blood samples (each 3 ml) were taken at 90, 105, 120, 135, 150, and 165 min post infusion followed by 6 breath samples taken at 180, 205, 230, 255, 280 and 305 min post infusion.

After the completion of fasting measurements, post-prandial kinetics were examined. To ensure that all dogs consumed equivalent grams of dietary glucose dogs were fed a total ration of 23 g/kg (as fed) divided into 14 equal sized meals. The ration was based on the dog with highest energy intake. Meals were fed 3 times, 10 min apart and every 25 min thereafter. Six blood samples (each 3 ml) were taken at 90, 105, 120, 135, 150 and 165 min post feeding followed by 6 breath samples taken at 180, 205, 230, 255, 280 and 305 min post feeding.
Figure 5.1. Experimental protocol for the determination of glucose kinetics.

1 Breath was collected by indirect calorimetry
2 Blood was sampled via jugular venipuncture
3 Animals received their total ration of test diet (23 g/kg, control or mannoheptulose containing diet) in equal sized meals
4 Primed (8.1 μmol/kg) constant (0.136 μmol/kg·min) intravenous infusion of U-13C-Glucose (99% atom, Sigma Aldrich, Isotec, Miamisburg, OH)

Protocol II: Assessment of lipid kinetics

Two baseline breath and blood samples were taken prior to isotope infusion to determine background D5 and 13C enrichment. Fasting plasma glycerol turnover was assessed (A), after which, post-prandial glycerol and palmitate kinetics were examined (B). Due to difficulties in obtaining canine serum albumin for experimental use, required for palmitate intravenous infusion, we were unable to use the constant intravenous infusion technique to assess palmitate kinetics.

A) Fasting plasma glycerol turnover. A primed (1.0 μmol/kg) constant (0.1 μmol/kg·min) intravenous infusion of 1,1,2,3,3-D5-Glycerol (98% Atom, Sigma Aldrich, Isotec, Miamisburg, OH, USA, 453420) was administered via the cephalic catheter. Six fasting blood samples (each 3 ml) were taken at 35, 45, 55, 65, 75 and 85 min post infusion.

B) Post-prandial glycerol and palmitate kinetics. After the completion of fasting measurements, post-prandial kinetics were examined (Figure 5.2). As in Protocol I, dogs were fed a total ration of 23 g/kg divided into 14 equal sized meals. Meals were fed 3 times 10 min apart and every 25 min thereafter. The fifth meal (time 70 min) contained a bolus dose of U-
$^{13}$C-K$_2$-Palmitate (4 mg/kg, 99% Atom, Sigma Aldrich, Isotec, Miamisburg, OH). Six blood samples were collected at times 90, 115, 145, 165, 190, 215 and 440 min post-feeding. Breath was collected at 85, 110, 135, 160, 185, 210, 235, 260, 285, 310, 335, 360, 385, 410 and 435 min post-feeding.

**Figure 5.2.** Experimental protocol for the determination of glycerol and palmitate kinetics.

1 Breath was collected by indirect calorimetry
2 Blood was sampled via jugular venipuncture
3 Animals received their total ration of test diet (23 g/kg, control or mannoheptulose containing diet) in equal sized meals. The fifth meal contained U-$^{13}$C-K$_2$-Palmitate (4 mg/kg, 99% Atom, Sigma Aldrich, Isotec, Miamisburg, OH)
4 Constant (0.1 µmol/kg·min) intravenous infusion of 1,1,2,3,3-D$_5$-Glycerol (98% Atom, Sigma Aldrich, Isotec, Miamisburg, OH, USA, 453420)

### 5.3.4. Acetate Recovery

To quantify FFA oxidation using stable isotope tracers, the complete recovery of carbon label in expired CO$_2$ is assumed. However, carbon label becomes fixed into intermediary metabolites, leading to an underestimation of fatty acid oxidation. To account for this temporary fixation of carbon label, an acetate recovery factor is applied. Acetate enters the tricarboxylic acid cycle directly and from that point on is treated the same as fatty acid-derived acetyl-CoA. Therefore, the recovery of carbon label from acetate should reflect the fixation of carbon label experienced by carbon labeled fatty acids within the TCA cycle.
To our knowledge, acetate recovery has not been reported previously in dogs. Therefore, acetate recovery was determined during the third week of the dietary washout period using \( 1,2^{13}\text{C}\)-Na-Acetate (0.5 mg/kg BW) (99% Atom, Sigma Aldrich, Isotec, Miamisburg, OH) and indirect calorimetry (as described earlier). The experimental protocol matched that of Protocol IIB above with respect to meal feeding and breath collection (blood was not collected). To ensure that an equal amount \(^{13}\text{C}\) label was ingested as in Protocol IIB, dogs received an acetate dose equivalent to one eighth (by weight) of the palmitate dose (0.5 mg/kg).

### 5.3.5. Biochemical Analyses

In Protocol I, plasma glucose enrichment \( (m + 6/m) \) and expired \( \text{CO}_2 \) enrichment were determined. Analytes for Protocol II included plasma palmitate and FFA concentrations, plasma glycerol \( (m + 3/m) \) and palmitate \( (m + 16/m) \) enrichment, and expired \( \text{CO}_2 \) enrichment.

All plasma isotope enrichments were determined by MTI MetaTech (Edmonton, AB). For glucose, 300 µL of plasma was added to 1.0 mL of cold acetone, incubated on ice for 10 min and then centrifuged at 10 000 rpm for 10 min. A 10 µL aliquot of the supernatant was removed and added to 490 µL of acetonitrile/water (75/25) and subjected to liquid chromatography–mass spectrometry (LC-MS) analysis. Extracted sample (5 µL) was injected onto a zorbax RX-Sil normal phase column (3.0 × 100 mm, 1.8 µm) prior to MS detection. The LC flow rate was 0.5 µL/min and the separation was achieved using an isocratic elution profile consisting of 25% water and 75% acetonitrile (run time 10 min). The LC eluent was interfaced to an electrospray ionization chamber, where glucose was ionized prior to MS detection. The mass spectrometer was an Agilent 6430 triple-quad MS operated in selected ion monitoring mode. Glucose was detected in positive ion mode with an \( m/z = 203.1 \) and enriched glucose at \( m/z = 209.1 \).
For glycerol, 300 µL of plasma were added to 1.3 mL of cold acetone/chloroform (9:4) and centrifuged at 10 000 rpm for 10 min. The supernatant was removed and dried under nitrogen. Samples were then dissolved in acetonitrile/water (80/20) with 50 µM cesium acetate and subjected to LCMS analysis. A 100 µL aliquot of extracted sample was injected onto a zorbax carbohydrate analysis column (4.6 × 250 mm, 5 µm) prior to MS detection. The LC flow rate was 0.7 µL/min and the separation was achieved using an isocratic elution profile consisting of 20% water and 80% acetonitrile with 50 µM cesium acetate (run time 20 min). The LC eluent was interfaced to an electrospray ionization chamber, where glycerol was ionized prior to MS detection. The mass spectrometer was an Agilent 6430 triple-quad MS operated in multiple reaction monitoring mode (MRM). The ion corresponding to a glycerol-cesium adduct was monitored in positive ion mode and detected at m/z = 225 and enriched glycerol at m/z = 230.

FFA were extracted from plasma using a dole reagent (isopropyl alcohol/heptanes/hydrochloric acid, 79/19/2). Tubes were pre-chilled on ice and 25.8 µL of C17:0 internal standard (0.194 µg/µL) was added to each tube, followed by 1.0 mL of the dole reagent and a 200 µL aliquot of plasma. 300 µL of heptanes and 400 µL of milli-Q water were added and the samples were centrifuged at 8500 rpm for 10 min to allow the aqueous and organic phases to separate. A 300 µL aliquot of the upper heptane phase was removed and placed in LCMS vials. The samples were dried under nitrogen and then dissolved in methanol/isopropyl alcohol/water (70/15/15) containing 1.5 mmol/L ammonium acetate and 0.015% acetic acid. The samples where then subjected to LCMS analysis. A 25 µL aliquot of the extracted plasma sample was injected onto a poroshell 120 EC-C18 column (3.0 × 50 mm, 2.7 µm) and the FFA were separated using reverse phase chromatography prior to MS detection. The LC flow rate was 0.5 µL/min and the separation was achieved using a gradient elution. The initial LC solvent profile consisted of 70% solvent A (methanol) and 30%
solvent B (isopropyl alcohol/water, 50/50, with 5 mmol/L ammonium acetate and 0.05% acetic acid). The final LC solvent composition was 100% solvent A and the run time was 7 min. The LC eluent was interfaced to an electrospray ionization chamber, where the FFA were ionized prior to MS detection. The mass spectrometer was an Agilent 6430 triple-quad MS operated in selected ion monitoring mode. Palmitic acid was detected as a deprotonated species with m/z = 255.4 and enriched palmitic acid at m/z = 271.4. Ions corresponding to other common fatty acids were also monitored, and by comparing the intensity of the peaks to the C17:0 internal standard, the concentration of FFA in plasma was obtained. Breath enrichment was analyzed using an automated $^{13}$CO$_2$ breath-analysis system, BreathMat Plus (Thermo Finnigan, San Jose, CA) (Phillips et al. 2004). Briefly, 2 mL of the expired breath sample were introduced into the gas chromatograph. Gases not of interest (N$_2$ and O$_2$) as well as water were removed online via a continuous-flow diffusion pump. Pure CO$_2$ was introduced to the isotope ratio mass spectrometer (IRMS) and was analyzed at a mass-to-charge ratio of 44/45.

5.3.6. Calculations

On d 43 of each period lean body mass was measured using a X-Ray Bone Densitometer (QDR4500, Hologic Inc., Bedford MA) (see Chapter 4). All kinetic data are expressed on a per kg lean body mass basis (CON, 21.4 ± 0.6; MH, 21.3 ± 0.5 kg).

Protocol I: Assessment of glucose kinetics

Isotopic determination of plasma glucose turnover and oxidation were resolved using the a one-pool model (Bergman et al., 1979), where the rate of plasma appearance ($Ra$) was assumed equal to the rate of plasma disappearance ($Rd$) (Eq. 1) and plasma glucose oxidation was representative of whole-body oxidation (Eq. 2) in steady state. In the post-prandial state, repeatedly feeding small meals in timed intervals similar to the reported gastric emptying time in dogs (Gooding et al. 2012) was assumed to reflect a physiological steady state, where
circulating substrate concentrations were not changing over time. Furthermore, CO₂ production has been found to be significantly correlated with labelled carbon recovery in animals fed repeated small meals (Moehn et al., 2004). The fractional rate of recovery of tracer in expired CO₂ was calculated using Eq. 3, and the percent of VCO₂ from glucose was calculated using Eq. 4.

\[
Ra, \, \mu\text{mol/min·kg} = F\times[(e_F/e_P) - 1] \quad (1)
\]

Fractional recovery of label, \% = \left[\left(\frac{e_{CO2} \times VCO2}{F \times C}\right)\right] \times 100 \quad (2)

Oxidation, \, \mu\text{mol/min·kg} = \left[\frac{VCO2 \times e_{CO2}}{e_P \times C \times n}\right] \quad (3)

VCO₂ from glucose, \%, = \left[\frac{e_{CO2}}{(e_P \times C \times n)}\right] \times 100 \quad (4)

where \( F \) is the isotope infusion rate (\( \mu\text{mol/min·kg} \)), \( e_F \) the isotopic enrichment of the infusate and \( e_P \) the background corrected plasma isotopic enrichment, \( e_{CO2} \) the background corrected isotopic enrichment of expired CO₂, \( VCO2 \) is the volume of CO₂ production (\( \mu\text{mol/min·kg} \)), \( C \) is the correction factor to account for carbon retention within the body, and \( n \) represents the number of carbon atoms per substrate molecule oxidized.

**Protocol II: Assessment of lipid kinetics**

To assess post-prandial glycerol and free fatty acid kinetics, the same experimental feeding paradigm was used as in the glucose kinetics and was assumed to be representative of a physiological steady state. Isotopic determination of plasma glycerol turnover was resolved using the minimal model (described above) and considered to be a direct index of lipolysis (Wolfe and Chinkes, 2005). As a bolus dose of palmitate isotope was given orally, plasma palmitate \( Ra \) (Eq. 5) and disappearance (\( Rd \)) (Eq. 6) were calculated using the non-steady model of Steele et al. (1959). Plasma palmitate oxidation was calculated by multiplying its \( Rd \) by the percentage of tracer recovered in expired CO₂ (Eq. 2). Cumulative recovery of label, an indirect measure of oxidation, was calculated as the area under the curve from time 0 to 435 min.
\[ Ra, \mu\text{mol/min} \cdot \text{kg} = \frac{F - V \times [(C_1 + C_2)/2] \times [(e_2 - e_1)/(t_2 - t_1)]]}{[(e_2 - e_1)/2]} \]  
\[ Rd, \mu\text{mol/min} \cdot \text{kg} = Ra - V \times (C_2 - C_1)/(t_2 - t_1) \]

where \( F \) is the isotope dose (\( \mu\text{mol/kg} \)); \( V \) is the volume of distribution, which was assumed to be 48 mL/kg (Wamberg et al., 2002); \( C_1 \) and \( C_2 \) are the palmitate concentrations at times \( t_1 \) and \( t_2 \), respectively.

5.3.7. Statistical Methods

All data were analyzed using SAS version 9.2 (SAS Institute, Cary, NC) and are expressed as means and pooled SEM. Mixed effects models were fitted using the PROC MIXED procedure assuming fixed period and diet effects and dogs as random variables. Multiple comparisons were made using the Tukey-Kramer method. Interactions between fixed effects were tested but only discussed if significant. Results were considered statistically significant if \( P < 0.05 \) and a statistical trend was considered as \( P \) ranging from 0.05 to 0.1.

5.4. Results

5.4.1. Protocol I: Assessment of glucose kinetics

MH did not affect fasting or post-prandial \( Ra \), whole-body glucose oxidation, fractional recovery of label or the percentage of CO\(_2\) from glucose (Table 5.1). As expected, post-prandial glucose kinetics values were higher than those observed in fasting, reflecting the increased availability of exogenous glucose.
Table 5.1. Fasting and fed glucose kinetic parameters in adult Labrador Retrievers fed a control (CON, no mannoheptulose) or mannoheptulose containing diet (MH, 4 mg/kg) (a total of 12 dogs in a complete cross-over design).

<table>
<thead>
<tr>
<th>Diet</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>Period</td>
<td></td>
</tr>
<tr>
<td>MH</td>
<td>CON</td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ra, μmol/min·kg</td>
<td>31.2</td>
<td>33.6</td>
</tr>
<tr>
<td>Oxidation, μmol/min·kg</td>
<td>4.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Fractional recovery of label, %/min</td>
<td>15.5</td>
<td>14.2</td>
</tr>
<tr>
<td>VCO₂ from glucose, %</td>
<td>14.7</td>
<td>15.6</td>
</tr>
<tr>
<td>Fed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ra, μmol/min·kg</td>
<td>148.4</td>
<td>143.1</td>
</tr>
<tr>
<td>Oxidation, μmol/min·kg</td>
<td>46.6</td>
<td>42.9</td>
</tr>
<tr>
<td>Fractional recovery of label, %/min</td>
<td>25.7</td>
<td>25.6</td>
</tr>
<tr>
<td>VCO₂ from glucose, %</td>
<td>69.4</td>
<td>70.8</td>
</tr>
</tbody>
</table>

1 Period 1, 34.4; Period 2, 30.4

5.4.2. Protocol II: Assessment of lipid kinetics

Diet had no effect on fasting or post-prandial glycerol Ra, an index of lipolysis (Table 5.2). There was no notable change in glycerol Ra between fasting and post-prandial states. Similarly, MH did not affect fasting or post-prandial plasma palmitate or FFA concentrations (expressed as the average of 6 samples). The main effect of period was significant but not particularly discernable for fasting glycerol turnover and palmitate concentrations. Similarly, period was significant for post-prandial FFA concentrations. In all instances, values were higher in the first study period than in the second (Table 5.2).
Recovery of carbon label from acetate is shown in Figure 5.3. Cumulative recovery of carbon label from acetate recovery was 31.5 ± 2.1%, which is similar to that reported in the literature (Sidossis et al., 1995). Plasma tracer enrichment was not detected at 20 min post-tracer ingestion. Detectable plasma tracer enrichment was observed in half of the samples at 45 min post-tracer ingestion and in all the samples taken at 70 min post-tracer ingestion (Figure 5.4). Similarly, enrichments in expired CO2 were not detected until after 70 min post-tracer ingestion (Figure 5.5). MH did not affect plasma palmitate Ra, Rd, oxidation or the cumulative recovery of carbon label (indirect measure of oxidation) (Table 5.4). Palmitate Ra closely matched its Rd.

96
Table 5.2. Fasting and fed glycerol rate of appearance (Ra) and lipid kinetics in adult Labrador Retrievers fed a control (CON, no mannoheptulose) or mannoheptulose containing diet (MH, 4 mg/kg) (a total of 12 dogs in a complete cross-over design).

<table>
<thead>
<tr>
<th></th>
<th>Diet</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MH</td>
<td>CON</td>
<td></td>
</tr>
<tr>
<td><strong>Fasting</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol Ra, μmol/min·kg</td>
<td>7.0</td>
<td>5.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Free fatty acids, μg/mL</td>
<td>0.274</td>
<td>0.278</td>
<td>0.016</td>
</tr>
<tr>
<td>Palmitate, μg/mL</td>
<td>0.058</td>
<td>0.063</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>Fed</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol Ra, μmol/min·kg</td>
<td>8.4</td>
<td>6.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Free fatty acids, μg/mL</td>
<td>0.112</td>
<td>0.123</td>
<td>0.009</td>
</tr>
<tr>
<td>Palmitate, μg/mL</td>
<td>0.027</td>
<td>0.031</td>
<td>0.002</td>
</tr>
<tr>
<td>Palmitate Ra, μmol/min·kg</td>
<td>132.5</td>
<td>101.4</td>
<td>42.3</td>
</tr>
<tr>
<td>Palmitate Oxidation, μmol/min·kg</td>
<td>81.9</td>
<td>92.5</td>
<td>26.0</td>
</tr>
<tr>
<td>Cumulative recovery of label ⁴</td>
<td>34.3</td>
<td>33.1</td>
<td>5.0</td>
</tr>
</tbody>
</table>

² Period 1, 0.135; Period 2, 0.099  
³ Period 1, 0.066, Period 2, 0.054  
⁴ Area under the curve
**Figure 5.3.** Recovery of carbon label from an oral bolus of 1,2-\(^{13}\)C-Na-Acetate (0.5 mg/kg BW) (99% Atom, Sigma Aldrich, Isotec, Miamisburg, OH) in adult Labrador Retrievers fed repeated small meals.

**Figure 5.4.** Plasma palmitate enrichment \((m+16/m)\) in dogs fed an oral bolus of U-\(^{13}\)C-K\(_2\)-Palmitate (4 mg/kg, time 0).
Figure 5.5. CO₂ enrichment in dogs fed an oral bolus of U-¹³C₂-Palmitate (4 mg/kg, time 0).

5.5. Discussion

Contrary to our original hypothesis MH did not affect fasting or post-prandial lipolytic rate or plasma palmitate turnover and oxidation. Furthermore, MH had no effect on fasting or post-prandial plasma glucose turnover and oxidation. To our knowledge, this study is the first to quantify post-prandial FFA in canines. Such information is pertinent and provides a basis for quantitative measures of macronutrient metabolism in dogs.

In the present study, MH did not affect fasting lipid or glucose metabolism. These findings were expected as MH metabolic effects are transient. Indeed, Chapter 4 demonstrated that plasma MH concentrations return to non-detectable levels within 24 h of ingestion (8 mg/kg). Fasting plasma glucose turnover and oxidation values observed in the present study are comparable to those reported in the canine literature (Paul et al., 1966, Wolfe and Shaw, 1986, Bailhache et al., 2003, Kim et al., 2003). To our knowledge, only one study has
assessed glycerol turnover in dogs. Fasting plasma glycerol turnover values in the present study were lower than those observed in mongrel dogs selected for athletic performance (Issekutz et al., 1975; 13.4 – 15.6 μmol/min·kg BW vs. ~ 4 μmol/min·kg BW present study).

Only three studies have examined the effects of low oral doses of MH on post-prandial metabolism. Davenport et al. (2010) found MH decreased post-prandial serum insulin, but not glucose concentrations, in senior Labrador Retrievers (average age 11.8 years) fed a MH-containing diet (2 mg/kg BW). No changes in post-prandial (24 h) serum glucose or insulin were observed in adult Beagles fed an oral supplement containing 8 mg/kg BW MH (Chapter 2). Similarly, McKnight et al. (Chapter 4) did not observe any changes in serum glucose or insulin with MH feeding (4 mg/kg BW), but did find MH decreased respiratory quotient (3 - 5 h post-prandial) after a single meal. This finding is in contrast to the present study where MH did not affect post-prandial glucose or FFA oxidation. However, respiratory quotient represents the relative proportion of dietary fat to carbohydrate oxidation and does not account for protein oxidation. The inability of dietary MH to elicit changes in post-prandial glucose and lipid metabolism in the present study may be related to diet composition. Like McKnight et al. (Chapter 2), the diet used in the present study had high inclusion of dietary sorghum which has been demonstrated to minimize post-prandial glucose and insulin responses in dogs (Carciofi et al. 2008), making it difficult to observe notable changes in glucose metabolism associated with MH feeding. In comparison to glucose, plasma FFA kinetics, are not under tight biological regulation. Therefore, it is not surprising that FFA kinetics values in the present study had large standard errors. Indeed, within subject variation in plasma FFA kinetics has also been noted in humans (Wolfe 2004). To minimize this variation, experimental procedures including daily exercise routines were standardized. However, voluntary physical activity was higher in dogs during the first study period (January – April 2013) (results presented in Chapter 4) and may have impacted lipid kinetics making it
difficult to observe notable MH effects. The lack of MH effect on post-prandial metabolism may also be related to its bioavailability. While dietary MH has been shown to subsequently appear in plasma, no study has assessed the cellular and tissue specific bioavailability of ingested MH. Furthermore, MH effectiveness as a competitive inhibitor of glucokinase in the presence of dietary carbohydrate (and other nutrients) is unclear. Future work should seek to understand the effects of different doses of oral MH on macronutrient metabolism.

Irrespective of MH, this study is the first to quantify post-prandial plasma glycerol and FFA kinetics in canines. To examine post-prandial kinetics we used a repeated meal feeding technique in an attempt to elicit a physiological steady state, where circulating substrate concentrations (pool size) were not changing over time, and provide a constant dietary infusion of MH. Such approach enabled steady state kinetic modeling. Normand-Lauziere et al. (2010) used a repeated feeding technique to assess post-prandial glycerol and palmitate Ra and oxidation in humans. Similar to Normand-Lauziere, no discernable differences between fasting and post-prandial glycerol turnover were observed. However, the authors reported considerably lower glycerol and FFA kinetic values in human subjects (glycerol Ra ~4 μmol/min·kg, palmitate ~40 g/ml, Ra ~1 μmol/min·kg and oxidation ~0.2 μmol/min·kg), than we observed in the present study using a canine model. The discrepancy may be attributed to differences in experimental methodology (i.e. differences in dietary composition) and species. Indeed, Acevedo et al. (2006) found canine muscle fibres (type II) express considerably higher oxidative capacity in comparison to other mammals, including humans, horses, goats, rats, pigs. In agreement, McClelland et al. (1994) found dogs to have significantly higher fatty acid transport capacity than goats. These findings support the notion that dogs are highly adapted to fat metabolism and conclusions from other species may not accurately reflect canine metabolism.
In conclusion, dietary MH when given at a dose of 4 mg/kg has no effect on lipolysis, or plasma FFA and glucose turnover and oxidation. Future work aimed at determining an optimal dose of dietary MH is necessary to better understand the role of MH as a glycolytic inhibitor and potential nutraceutical. This study is the first to quantify post-prandial glycerol, palmitate and glucose kinetics in healthy adult dogs, highlighting the need for a greater understanding of the control of macronutrient metabolism in dogs.
CHAPTER 6

A kinetic model of whole-body glucose metabolism with reference to the domestic dog

*(Canis lupus familiaris)*

6.1. Abstract

A new two-pool model to describe glucose kinetics in the steady state is presented. The pools are plasma glucose, Q1, and tissue glucose, Q2 (both µmol). The flows (all µmol/min) into the plasma pool (Pool 1) are absorbed glucose entry from dietary sources, labelled glucose infusion, and hepatic glucose production. There is one flow out of Pool 1, glucose uptake by the tissues. Inflows to the tissues pool (Pool 2) are from plasma and glycogenolysis. Outflows from Pool 2 are to plasma, glucose oxidation, and glycogenesis and other metabolism.

Application of the model was illustrated using experimental data derived from healthy adult Labrador Retrievers in the fasted and fed (repeated meal feeding) state. In general, model derived estimates of glucose kinetics were representative of normal glucose metabolism, where rates of glucose production and uptake are similar and act to maintain blood glucose concentrations. Furthermore, estimates of within tissue glucose cycling indicated glycogenolysis in fasting and fed glycogenesis. There was a significant variation in post-prandial kinetic values (Ra) which may be explained, in part, by differences in experimental methodology.

*Key words:* Glucose oxidation, glucose rate of appearance, Labrador Retriever, stable isotope tracer, steady state

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5 McKnight et al. Journal of Theoretical Biology (JTB-D-14-00584), *In Review*
6.2. Introduction

In canids, as in all mammals, glucose is a ubiquitous cellular fuel source for all tissues. As such, the regulation of glucose metabolism has been under extensive investigation for the past century. Blood glucose homeostasis is predominantly regulated by the ability of the pancreas to balance insulin and glucagon secretions in response to glucose availability. When circulating glucose is in excess, such as after a meal, insulin is released from the pancreatic beta cells. Insulin increases glucose uptake and utilization in peripheral tissues, whereas in the liver, insulin promotes glycogen and fatty acid synthesis and inhibits gluconeogenesis. When glucose concentrations fall below a certain threshold, glucagon is released from the pancreatic alpha cells. Glucagon acts primarily on the liver to stimulate glycogenolysis and gluconeogenesis to re-establish glucose homeostasis. Skeletal muscle has the ability to store a limited amount of glucose as glycogen, thereby providing an endogenous source of glucose in times of low glucose availability.

Experimentally, isotopic determination of plasma glucose turnover has served as a marker of cumulative tissue glucose uptake. Using a labeled carbon substrate (i.e., $^{13}$C or $^{14}$C glucose) in combination with indirect calorimetry, allows for the quantification of whole body glucose oxidation. Traditionally, isotopic determination of plasma glucose kinetics has been resolved using a one-pool model (Bergman et al., 1979). This minimal scheme contains one pool (plasma glucose), one inflow (rate of glucose appearance, $R_a$), and one outflow (rate of glucose disappearance, $R_d$) (reviewed in Appendix A). Stable or radioactive isotope is administered by constant infusion, steady state is assumed, and $R_a$ calculated as infusion rate divided by enrichment or specific activity. The model is limited by the assumption that glucose is uniformly distributed in the extracellular fluid space. As a result, the single pool model cannot describe the exchange of glucose between plasma and tissue compartments. In an attempt to overcome such simplification of glucose distribution, Radziuk et al. (1978)
proposed a two pool scheme to describe glucose kinetics in the non-steady state, and variants of this have been applied to canids by Cobelli et al. (1997) and Steil et al. (1998) and to felines by Hoenig et al. (2006). However, model solution is computationally complex and requires previous knowledge of parameters, either measured in a separate experiment or prior to initiation of experimental conditions.

In this paper, a new two-pool model is proposed which gives explicit representation to the bidirectional exchange of glucose between plasma and tissue compartments and the within tissue cycling of glucose through glycogenesis and subsequent glycogenolysis. The model describes glucose kinetics in the steady state and is solved algebraically, thus preserving the inherent simplicity of the one-pool model. Application of the new model is illustrated using experimental data derived from our previous published work. Specifically, we present data obtained from adult Labrador Retrievers in the fasted and repeated meal feeding (fed) state.

6.3. The Model

The kinetic scheme for total glucose (labelled plus unlabelled) comprises two pools and seven flows (Figure 6.1a, see Table 6.1 for mathematical notation).
Figure 6.1. Two-pool model for describing glucose kinetics (a) and the kinetic scheme for labeled glucose (b). The first pool represents total (labelled plus unlabelled) glucose in plasma, and second represents total glucose in tissues. Arrowed solid lines show flows, hollow arrow shows glucose application (infusion), solid dots indicate flows active only in the fasted state, hollow dots indicate flows active only in the fed state, and the broken line represents sampling.
Table 6.1. Mathematical notation

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_{ij}$</td>
<td>Flow of total glucose to pool $i$ from pool $j$; $F_{io}$ represents external flow of glucose into pool $i$ and $F_{io}$ flow of glucose from pool $i$ out of the system [µmol/min]</td>
</tr>
<tr>
<td>$G, G^*$</td>
<td>Total and labelled glucose concentration, respectively, in primary pool [µmol/mL]</td>
</tr>
<tr>
<td>$I$</td>
<td>Constant rate of labelled glucose infusion into primary pool [µmol/min]</td>
</tr>
<tr>
<td>$Q_i$</td>
<td>Quantity of total glucose in pool $i$ [µmol]</td>
</tr>
<tr>
<td>$q_i$</td>
<td>Quantity of labelled glucose in pool $i$ [µmol]</td>
</tr>
<tr>
<td>$e_i$</td>
<td>Enrichment of pool $i$ [µmol labelled glucose/µmol total glucose]</td>
</tr>
<tr>
<td>$t$</td>
<td>Time since isotope first administered [min]</td>
</tr>
<tr>
<td>$V_i$</td>
<td>Volume of pool $i$; $V_1$ represents primary (plasma) pool volume [mL]</td>
</tr>
</tbody>
</table>

The pools are plasma glucose, $Q_1$, and tissue glucose, $Q_2$ (both µmol). The flows (all µmol min$^{-1}$) into the plasma pool (Pool 1) are absorbed glucose entry from dietary sources, $F_{10}$, labelled glucose infusion, $I$, and hepatic glucose production, $F_{12}$. There is one flow out of Pool 1, glucose uptake by the tissues, $F_{21}$. Inflows to the tissues pool (Pool 2) are $F_{21}$ and glycogenolysis, $F_{2R}$. Outflows from Pool 2 are $F_{12}$, and glucose oxidation, $F_{02}$, and glycogenesis and non-oxidative glucose disposal, $F_{R2}$. The flow $F_{10}$ is assumed to occur only in the fed state, and the flow $F_{12}$ only in the fasted state. The scheme for labelled glucose assumes no re-entry of label into Pool 1 during the infusion period and is shown in Figure 1b. It contains one pool, labelled plasma glucose ($q_1$, µmol), one inflow viz. the rate of infusion $I$, and one outflow viz. labelled glucose uptake by the tissues $F_{21}$. Sampling, e.g., total glucose concentration, $G_1$ (µmol mL$^{-1}$), and enrichment, $e_1$ (µmol labelled per µmol total glucose), is from the plasma pool.

The fundamental equations are, for total glucose:

\[
\frac{dQ_1}{dt} = F_{10} + F_{12} + I - F_{21}
\]

\[
\frac{dQ_2}{dt} = F_{21} + F_{2R} - F_{02} - F_{12} - F_{R2}
\]
and for labelled glucose:

\[ \frac{dq_{1}}{dt} = I - f_{21} = I - e_{1}F_{21} \]

Assume that steady state is reached after a few hours of infusion such that \( \frac{dQ_{1}}{dt} = \frac{dq_{1}}{dt} = 0 \) and \( \frac{dQ_{2}}{dt} \approx 0 \). Therefore:

\[
F_{10} + F_{12} + I - F_{21} = 0 \\
F_{21} + F_{2R} - F_{02} - F_{12} - F_{R2} \approx 0 \\
I - e_{1}F_{21} = 0
\]

This set of 3 simultaneous linear equations can be solved algebraically to give:

\[
F_{21} = I / e_{1} \quad (1) \\
F_{10} + F_{12} = F_{21} - I \quad (2) \\
F_{12} + F_{R2} - F_{2R} = F_{21} - F_{02} \quad (3)
\]

When the net flow \( F_{R2} - F_{2R} \) is positive, glycogenesis (and other metabolism) predominates, and when negative, glycogenolysis predominates. In the fasted state, absorbed glucose entry to the plasma pool from dietary sources is zero and Eqs. (1)-(3) yield:

\[
F_{10} = 0 \quad (4) \\
F_{21} = I / e_{1} \quad (5) \\
F_{12} = F_{21} - I \quad (6) \\
F_{R2} - F_{2R} = F_{21} - F_{02} - F_{12} \quad (7)
\]

In the fed state, hepatic glucose production is negligible. Eqs. (1)-(3) now yield:

\[
F_{12} = 0 \quad (8) \\
F_{21} = I / e_{1} \quad (9) \\
F_{10} = F_{21} - I \quad (10) \\
F_{R2} - F_{2R} = F_{21} - F_{02} \quad (11)
\]

The fasted model is therefore given by Eqs. (4)-(7), and the fed model by Eqs. (8)-(11).
6.4. Application

Data were taken from two separate experiments (A and B) conducted in our laboratories. The general aim of both studies was to determine the effects of dietary mannoheptulose, a glycolytic inhibitor, on glucose oxidation. Key details of the experimental designs, animals, and methodologies are provided in Table 6.2. Briefly, glucose oxidation was assessed after an overnight fast and during repeated meal feeding (fed state) using indirect calorimetry and a primed constant intravenous infusion of U-\(^{13}\)C-glucose. For repeated meal feeding, dogs were fed their daily ration divided into equal sized small meals every 25 min. This feeding regimen was based on reported gastric emptying time of \(~20\) min for dogs fed small meals, thereby providing a constant dietary supply of mannoheptulose (Gooding et al., 2012). Furthermore, CO\(_2\) production has been found to be significantly correlated with labelled carbon recovery in animals fed repeated small meals (Moehn et al., 2004). Glucose oxidation \((F_{\text{O}_2})\) was calculated according to Eq. (12) (Wolfe and Chinkes, 2005).

\[
F_{\text{O}_2} = \frac{V_{\text{CO}_2} \times e_{\text{CO}_2}}{e_{1} \times C_{\text{corr}} \times 6} \quad \text{(12)}
\]

where \(V_{\text{CO}_2}\) is the volume of CO\(_2\) production, \(e_{\text{CO}_2}\) the isotopic enrichment of expired CO\(_2\), \(e_{1}\) the plasma isotopic enrichment, \(C_{\text{corr}}\) is the bicarbonate correction factor to account for carbon retention and 6 represents the number of carbon atoms per glucose molecule oxidized.
Table 6.2. Key experimental details.

<table>
<thead>
<tr>
<th>Study design</th>
<th>Experiment A</th>
<th>Experiment B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diets:</td>
<td>control (CON) and mannoheptulose (MH, 2 mg/kg)</td>
<td>control (CON) and mannoheptulose (MH, 4 mg/kg)</td>
</tr>
<tr>
<td>Duration:</td>
<td>22 d with glucose kinetics assessed on d 19 of study.</td>
<td>63 d with glucose kinetics assessed on d 35 of study.</td>
</tr>
<tr>
<td>Study population</td>
<td>A total of 6 neutered male Labrador Retrievers, 3-black-coated (3.9 yr; 27.3 kg) and 3-chocolate-coated (6.8 yr; 35.0 kg)</td>
<td>A total of 12 black Labrador Retrievers, 5 spayed females and 7 neutered males (27.0 ± 0.6 kg; 4.9 ± 0.2 yr of age).</td>
</tr>
<tr>
<td>Key details of glucose kinetics</td>
<td>Isotope: U-13C-glucose Prime: 77.8 μmol/kg Constant: 0.136 μmol/min·kg Fasting collections: 6 blood samples were taken at 60, 85, 110, 135, 160, and 185 min followed by 4 breath samples taken at 220, 245, 270, and 295 min post infusion. Post-prandial collections: total food ration of 14 g/kg divided into 15 equal sized meals fed in 25 min intervals; 6 blood samples were taken at 60, 85, 110, 135, 160, and 185 min followed by 4 breath samples taken at 220, 245, 270, 295, 320 and 345 min post-initial meal.</td>
<td>Isotope: U-13C-glucose Prime: 8.1 μmol/kg Constant: 0.136 μmol/min·kg Fasting collections: 6 fasting blood samples were taken at 90, 105, 120, 135, 150, and 165 min followed by 6 breath samples taken at 180, 205, 230, 255, 280 and 305 min post infusion. Post-prandial collections: total food ration of 23 g/kg divided into 14 equal sized meals fed in 25 min intervals; 6 fasting blood samples were taken at 90, 105, 120, 135, 150, and 165 min followed by 6 breath samples taken at 180, 205, 230, 255, 280 and 305 min post-initial meal.</td>
</tr>
</tbody>
</table>

Kinetic measurements and calculated flows derived from Experiment A and Experiment B are presented in Table 6.3 (fasting) and Table 6.4 (fed), respectively. In Experiment A, plateaus in plasma enrichment were not achieved (due to an over prime of isotope) and steady state enrichments were extrapolated using simple negative exponential
curve fitting over the period of post-peak decline. Both measured (last sampling time point) and extrapolated values are given. In fasting, the calculated rate of plasma glucose appearance (Rₐ) (F₁₂) was similar to its rate of disappearance (F₂₁). Within tissue glucose recycling (Fᵣ₂ – Fᵣ₂) values were negative, suggesting that tissue glycogenolysis was predominant. Calculated flows in Experiment A derived from extrapolated steady state values were higher than those similarly derived from measured values. Indeed, extrapolated steady state values in Experiment A were similar to those observed in Experiment B where isotopic steady state was achieved. During repeated meal feeding, measured glucose oxidation (F₀₂) and calculated flows (F₂₁, Fᵣ₂ – Fᵣ₂) were greater than those observed in fasting, reflecting the increased availability of exogenous glucose. In agreement, within tissue glucose recycling (Fᵣ₂ – Fᵣ₂) values were positive, indicating glycogenesis. Glucose oxidation and calculated flows varied considerably within and between experiments. Specifically, oxidation and calculated flows were greater in Experiment B than Experiment A. In Experiment A, oxidation values were lowest when derived from extrapolated steady state enrichments, whereas calculated flows were lowest when derived from measured enrichments.
Table 6.3. Glucose kinetics parameters in fasted adult Labrador Retrievers fed a control diet (CON, no mannoheptulose) or a mannoheptulose containing diet (MH, Experiment A, 2 mg/kg; Experiment B, 4 mg/kg)\(^1\). Data are expressed as mean and standard error (flows are expressed as \(\mu\text{mol/min/kg}\)).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>BW, kg</th>
<th>(I)</th>
<th>(e_1)</th>
<th>(e_{CO2})</th>
<th>(F_{20})</th>
<th>(F_{10})</th>
<th>(F_{12})</th>
<th>(F_{21})</th>
<th>(F_{R2} - F_{R1})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>30.9 ± 1.6</td>
<td>0.136</td>
<td>0.021 ± 0.001</td>
<td>0.003 ± 0.000</td>
<td>2.8 ± 0.2</td>
<td>0</td>
<td>6.5 ± 0.3</td>
<td>6.9 ± 0.3</td>
<td>-2.5 ± 0.2</td>
</tr>
<tr>
<td>MH</td>
<td>31.3 ± 1.8</td>
<td>0.136</td>
<td>0.019 ± 0.001</td>
<td>0.002 ± 0.000</td>
<td>2.8 ± 0.5</td>
<td>0</td>
<td>7.3 ± 0.6</td>
<td>7.7 ± 0.6</td>
<td>-2.6 ± 0.5</td>
</tr>
<tr>
<td>CON</td>
<td>30.9 ± 1.6</td>
<td>0.136</td>
<td>0.010 ± 0.002</td>
<td>0.005 ± 0.001</td>
<td>2.1 ± 0.2</td>
<td>0</td>
<td>19.2 ± 4.9</td>
<td>20.0 ± 5.1</td>
<td>-1.9 ± 0.2</td>
</tr>
<tr>
<td>MH</td>
<td>31.3 ± 1.8</td>
<td>0.136</td>
<td>0.011 ± 0.002</td>
<td>0.006 ± 0.001</td>
<td>2.4 ± 0.1</td>
<td>0</td>
<td>14.4 ± 3.4</td>
<td>15.0 ± 3.5</td>
<td>-2.2 ± 0.1</td>
</tr>
<tr>
<td><strong>Experiment B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>27.4 ± 0.7</td>
<td>0.136</td>
<td>0.007 ± 0.000</td>
<td>0.001 ± 0.000</td>
<td>3.9 ± 0.4</td>
<td>0</td>
<td>19.7 ± 1.0</td>
<td>19.9 ± 1.2</td>
<td>-3.7 ± 0.5</td>
</tr>
<tr>
<td>MH</td>
<td>27.6 ± 0.7</td>
<td>0.136</td>
<td>0.007 ± 0.000</td>
<td>0.001 ± 0.000</td>
<td>4.0 ± 0.3</td>
<td>0</td>
<td>19.8 ± 1.2</td>
<td>19.7 ± 0.9</td>
<td>-3.5 ± 0.4</td>
</tr>
</tbody>
</table>

\(^1\) Data are expressed as mean and standard error (flows are expressed as \(\mu\text{mol/min/kg}\))

\(^2\) Measured enrichment values (last sampling time point)

\(^3\) Extrapolated steady state enrichments
Table 6.4. Post-prandial glucose kinetics parameters in adult Labrador Retrievers fed a control diet (CON, no mannoheptulose) or a mannoheptulose containing diet (MH). Data are expressed as mean and standard error (flows are expressed as μmol/min/kg).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>BW, kg</th>
<th>I</th>
<th>e₁</th>
<th>e₂</th>
<th>F₀₂</th>
<th>F₁₀</th>
<th>F₁₂</th>
<th>F₂₁</th>
<th>Fₓ₁₀–Fₓ₁₂</th>
<th>Fᵢₒ/Dose, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON ²</td>
<td>30.9 ± 1.6</td>
<td>0.136</td>
<td>0.004 ± 0.000</td>
<td>0.001 ± 0.000</td>
<td>12.5 ± 0.3</td>
<td>34 ± 5</td>
<td>0</td>
<td>35.0 ± 4.7</td>
<td>22 ± 4</td>
<td>16.3 ± 2.2</td>
</tr>
<tr>
<td>MH ²</td>
<td>31.3 ± 1.8</td>
<td>0.136</td>
<td>0.005 ± 0.000</td>
<td>0.001 ± 0.000</td>
<td>10.1 ± 1.1</td>
<td>29 ± 2</td>
<td>0</td>
<td>30.5 ± 2.0</td>
<td>20 ± 1</td>
<td>14.1 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.003 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>6.4 ± 0.6</td>
<td>78 ± 34</td>
<td>80.6 ±</td>
<td>72 ± 33</td>
<td>37.5 ± 16.3</td>
<td></td>
</tr>
<tr>
<td>CON ³</td>
<td>30.9 ± 1.6</td>
<td>0.136</td>
<td></td>
<td>0</td>
<td>34.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MH ³</td>
<td>31.3 ± 1.8</td>
<td>0.136</td>
<td>0.003 ± 0.001</td>
<td>0.003 ± 0.000</td>
<td>5.7 ± 0.4</td>
<td>48 ± 10</td>
<td>0</td>
<td>10.6 ±</td>
<td>42 ± 10</td>
<td></td>
</tr>
<tr>
<td>B⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON ²</td>
<td>27.4 ± 0.7</td>
<td>0.136</td>
<td>0.001 ± 0.000</td>
<td>0.001 ± 0.000</td>
<td>61 ± 18</td>
<td>56</td>
<td>0</td>
<td>225 ± 56</td>
<td>163 ± 40</td>
<td></td>
</tr>
<tr>
<td>MH ²</td>
<td>27.6 ± 0.7</td>
<td>0.136</td>
<td>0.001 ± 0.000</td>
<td>0.001 ± 0.000</td>
<td>37 ± 7</td>
<td>30</td>
<td>0</td>
<td>153 ± 30</td>
<td>116 ± 23</td>
<td></td>
</tr>
</tbody>
</table>

¹ Dogs were fed 2 mg/kg MH and 14 g/meal glucose
² Experimental enrichment values (last sampling time point)
³ Extrapolated steady state enrichments
⁴ Dogs were fed 4 mg/kg MH and 23 g/meal glucose
⁵ Crude estimate of glucose absorption, based on diets providing 760 g glucose/kg fed in 25 min intervals

A brief analysis was conducted into the effects of measurement errors in infusion rate and plasma enrichment on model solutions using the overall mean of experimentally derived values. The model was solved by perturbing each input (i.e., I and e₁) in turn by 0, ± 10 and ± 20%. Each calculated flow (y, μmol/min·kg) was then plotted against the perturbation (x, %), and a five-point linear regression of y on x performed to determine the slope of the line produced. Each average slope was subsequently scaled by its corresponding unperturbed average flow value, and multiplied by a hundred to give the scaled slopes dimensions of % change in y per % change in x (Table 6.5). In general, measurement error in either input did not substantially impact calculated flows. Overall, fasting plasma flows were most affected by
measurement errors in plasma enrichment. It is important to note that measurement errors in plasma enrichment affect not only calculated flows, but glucose oxidation calculated from plasma enrichment.

**Table 6.5.** Analysis of measurement errors in infusion rate ($I$) and plasma enrichment ($e_1$) on model solutions.  

<table>
<thead>
<tr>
<th></th>
<th>$F_{02}$</th>
<th>$F_{10}$</th>
<th>$F_{12}$</th>
<th>$F_{21}$</th>
<th>$F_{R2-R2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope, $(\Delta F)/(\Delta I)$</td>
<td>-</td>
<td>-</td>
<td>0.157 ± 0.036</td>
<td>0.160 ± 0.035</td>
<td>0.001 ± 0.006</td>
</tr>
<tr>
<td>Error, %</td>
<td>-</td>
<td>-</td>
<td>1.2</td>
<td>1.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Slope $(\Delta F)/(\Delta e_1)$</td>
<td>-0.062 ± 0.022</td>
<td>-</td>
<td>-0.164 ± 0.037</td>
<td>-0.165 ± 0.036</td>
<td>0.058 ± 0.027</td>
</tr>
<tr>
<td>Error, %</td>
<td>3.3</td>
<td>1.2</td>
<td>1.2</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Post-prandial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope $(\Delta F)/(\Delta I)$</td>
<td>-</td>
<td>0.873 ± 0.350</td>
<td>-</td>
<td>0.874 ± 0.350</td>
<td>0.874 ± 0.277</td>
</tr>
<tr>
<td>Error, %</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Slope $(\Delta F)/(\Delta e_1)$</td>
<td>-0.303 ± 0.090</td>
<td>-0.905 ± 0.357</td>
<td>-</td>
<td>-0.905 ± 0.357</td>
<td>-0.602 ± 0.295</td>
</tr>
<tr>
<td>Error, %</td>
<td>1.0</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^1 I$ and $e_1$ were perturbed in turn by 0, ±10 and ±20%.

### 6.5. Discussion

A two-pool model was presented to describe glucose kinetics in fasted and fed states. The model gave specific consideration to the bidirectional exchange of glucose between plasma and tissue compartments and to the within tissue cycling of glucose through glycogenesis and subsequent glycogenolysis. In general, model derived estimates of glucose kinetics were representative of normal glucose metabolism, where rates of glucose production and uptake are similar and act to maintain blood glucose concentrations. Furthermore,
estimates of within tissue glucose cycling indicated glycogenolysis in fasting and fed glycogenesis.

In the canine literature, plasma $R_a$ has generally been determined using a single pool model under a wide range of physiological conditions (Table 6.6). The underlying assumption of the one pool model is that blood glucose rapidly and uniformly distributes in all physiological pools (i.e., plasma, interstitial and intracellular). Provided the system is in steady state, such as after an overnight fast, the assumption is considered valid. Mathematically, the one-pool model calculates the rate of disappearance from plasma, which is assumed to equal the $R_a$. Therefore, literature values of $R_a$ presented in Table 6 were comparable to $F_2^1$, glucose uptake by the tissues, in our proposed fasted model application. Literature $R_a$ values were similar to those in Experiment A derived from extrapolated steady state values and Experiment B, whereas, $R_a$ derived from measured values in Experiment A were lower than experimental and literature values. These findings indicate $R_a$ is likely to be underestimated if steady state enrichment is not achieved experimentally. However, extrapolating steady state enrichments yield reasonable estimates of glucose kinetic parameters and can be employed if necessary.

The fasting application of the model provided negative within tissue glucose recycling values. Hepatic glycogenolysis and gluconeogenesis contribute to maintenance of blood glucose concentrations in fasting and as fasting progresses the relative contribution of glycogenolysis is reduced while gluconeogenesis is enhanced. Negative recycling values suggest hepatic glycogen depletion. However, at the completion of these experiments, dogs had been fasted for close to 24 h and Edgerton et al. (2001 and 2009) findings suggest that hepatic glycogenolysis is still occurring in dogs fasted for 60 h. As our model considers all tissues as a single pool, we cannot differentiate the relative tissue contributions to
glycogenolysis (and gluconeogenesis) without employing much more invasive multi-catheter models and looking at arterial venous balance kinetics.

Relatively few studies have assessed post-prandial plasma $R_a$ using isotope tracer methodology and no studies, to our knowledge, have been performed in canines. Existing methodologies (in humans) require the simultaneous use of at least 2 different isotope tracers and frequent arterial blood sampling. One isotope is delivered by constant intravenous infusion to quantify glucose volume distribution, and the second isotope is provided within a single meal to trace the rate of ingested glucose appearance. The kinetic data generated have been resolved using the non-steady state model of Steele (1959) and the two-compartmental model first proposed by Radziuk (1978) (further modified by Mari, 1992). Both models describe time-varying changes in glucose $R_a$. Steele’s equation assumes that $R_a$ and $R_d$ take place in the accessible (plasma) compartment, which has a volume equal to a fraction of the total distribution volume. The two-compartment model assumes time-varying $R_a$ and $R_d$ from the accessible (plasma) compartment and constant-rate parameters between the accessible and the remote (interstitial) compartments.

In a review of 30 dual radio-isotope tracer experiments performed in humans, Livesey et al. (1998) noted peak plasma $R_a$ from an oral glucose load ranged between 12 – 39 μmol/min·kg. Toffolo et al. (2006) reported peak plasma $R_a$ of 58.4 ± 5.5 μmol/min·kg and 82.5 ± 8.7 μmol/min·kg in humans using the dual tracer and triple stable isotope tracer techniques, respectively. The considerable variation in peak plasma $R_a$ observed values may be due to differences in study populations, methodology and data analysis between all published studies. The heterogeneity of the study populations with respect to age, ethnicity, gender, and basal glucose tolerance between studies make direct comparisons difficult. Differences in the quantity of oral glucose delivered, and the duration of study also would have affected $R_a$. Finally, the majority of studies (24/30) presented by Livesey et al. (1998)
utilized a one-compartment model to quantify $R_a$ and there was a wide variation in volume distribution values (95 – 230 ml/kg) applied.

In our model, the plasma $R_a$ from ingested glucose ranged from 39 – 78 $\mu$mol/min·kg in Experiment A and 122 – 224 $\mu$mol/min·kg in Experiment B. In general, these values are higher than those observed in the literature. However, direct comparisons are not appropriate, given the differences in species and methodology. Specifically, our experimental data reflect glucose $R_a$ in canines fed intermittent mixed meals, whereas the literature values are based on a single oral glucose load (in humans). Mixed meals delay gastric emptying, enhance insulin response and stimulate release of gastrointestinal hormones. Furthermore, the presence of other nutrients within the meal alters glucose absorption and utilization.

The present model assumed hepatic glucose production was completely suppressed and dietary glucose was the sole contributor to plasma $R_a$. However, a non-physiological dose of exogenous glucose would be required to fully suppress endogenous glucose production. Such a dose would not be achieved through intermittently feeding small mixed meals. Therefore, $R_a$ reflects glucose entry from exogenous and endogenous sources. Dietary glucose was assumed to enter the peripheral circulation directly. In actuality, ingested glucose is absorbed, released into portal circulation, and taken up by the liver prior to its release into peripheral circulation. Additionally, ingested glucose can serve as a substrate for volatile free fatty acid production in the colon. Moore et al. (1994) only accounted for 68 % of the labeled glucose administered within a single mixed meal fed to mongrel dogs. Of that, 82 % appeared within systemic circulation. These findings suggest that our model underestimates glucose $R_a$. Indeed, a crude calculation of glucose absorption was performed ($F_{10}/Dose$, %) (Table 2) and less than 40% of ingested glucose is accounted for by $F_{10}$.

In conclusion, a novel two-pool model to describe glucose kinetics in the steady state was presented and applied to experimental data generated from our laboratory. The model
yielded fasting plasma glucose $R_a$ values comparable to those reported in the canine literature (calculated using a single pool model). All calculated flows increased in the post-prandial state, reflecting the increased availability of glucose. There was a considerable range of post-prandial kinetic parameters that may be explained, in part, by differences in experimental methodology.
Table 6.6. Plasma glucose rate of appearance (R\textsubscript{a}, μmol/min/kg) and glucose oxidation (Ox, μmol/min/kg) reported in the canine literature (data means are presented).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Experimental groups (conditions)</th>
<th>BW, kg</th>
<th>R\textsubscript{a},</th>
<th>Ox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Altszuler et al., 1975</td>
<td>2-3H [Basal; Methylprednisolone]</td>
<td>21.1</td>
<td>22; 40.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3-3H [Basal; Methylprednisolone]</td>
<td>21.1</td>
<td>14.4; 18.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>U-14C [Basal; Methylprednisolone]</td>
<td>21.1</td>
<td>14.4; 19.3</td>
<td>-</td>
</tr>
<tr>
<td>Bailhache et al., 2003</td>
<td>Healthy; Obese</td>
<td>12.2</td>
<td>15.6; 13.9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2003</td>
<td>17.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Christopher et al., 1994</td>
<td>Clamped euglycemia [0; 7; 40; 120 mU/kg/h constant insulin infusion]</td>
<td>22</td>
<td>19.1; 17.9; 54.7;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Clamped hyperglycemia [0; 7; 40; 120 mU/kg/h constant insulin infusion]</td>
<td>22</td>
<td>36; 46.1; 96.1;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1994</td>
<td></td>
<td>101.7</td>
<td></td>
</tr>
<tr>
<td>Christopher et al., 2005</td>
<td>Healthy [saline; AICAR\textsuperscript{2}; methylpalmitoxirate; AICAR &amp; methylpalmitoxirate]</td>
<td>20.1</td>
<td>13; 17.5; 14.5; 16</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Diabetic [saline; AICAR; methylpalmitoxirate; AICAR &amp; methylpalmitoxirate]</td>
<td>20.1</td>
<td>32.4; 31.6; 23.6;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2005</td>
<td></td>
<td>21.9</td>
<td></td>
</tr>
<tr>
<td>Hsu et al., 2010</td>
<td>Saline infusion; Pulsatlie; Constant octanoate infusion</td>
<td>-</td>
<td>16.7; 18.3; 14.4</td>
<td>-</td>
</tr>
<tr>
<td>Kim et al., 2003</td>
<td>Baseline; 6 wk; 12 wk moderate-fat feeding</td>
<td>27.5</td>
<td>14.4; 18.3; 18.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2003</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paul et al., 1966</td>
<td>Saline; Nicotinic Acid</td>
<td>-</td>
<td>15; 24</td>
<td>6; 14</td>
</tr>
<tr>
<td>Rantzau et al., 2008</td>
<td>Basal</td>
<td>20.4</td>
<td>12.7</td>
<td>-</td>
</tr>
<tr>
<td>Strack et al., 1994</td>
<td>Basal [U-14C; 3-3H]</td>
<td>15.2</td>
<td>8.7; 13.9</td>
<td>-</td>
</tr>
<tr>
<td>Weber et al., 1996</td>
<td>Basal</td>
<td>-</td>
<td>24.3</td>
<td>-</td>
</tr>
<tr>
<td>Wolfe and Shaw, 1986</td>
<td>Basal [Saline; high FFA\textsuperscript{3}; low FFA infusion]</td>
<td>-</td>
<td>15.3; 15.3; 15.3</td>
<td>5; 4.8; 8.9</td>
</tr>
<tr>
<td></td>
<td>Epinephrine infusion [Saline; high FFA; low FFA infusion]</td>
<td>-</td>
<td>15; 15.8; 15.3</td>
<td>4.9; 5.2; 9.7</td>
</tr>
<tr>
<td></td>
<td>Propanolol &amp; phentolamine infusion [Saline; high FFA; low FFA infusion]</td>
<td>-</td>
<td>15.2; 15.0; 15.2</td>
<td>5.1; 5.1; 9.8</td>
</tr>
<tr>
<td></td>
<td>Overall Mean and SE</td>
<td>20.9 ± 26.8 ± 4.3</td>
<td>7.1 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} Basal refers to fasted rest
\textsuperscript{2} AICAR, 5-Aminoimidazole-4-carboxamide ribonucleotide
\textsuperscript{3} FFA, free fatty acids
CHAPTER 7
GENERAL CONCLUSIONS

The aim of this work was to evaluate the metabolic effects of MH, a proposed novel nutraceutical for dogs. More specifically, MH effects on fasting and post-prandial macronutrient metabolism and EE in healthy adult dogs were investigated. MH was expected to limit glucose as a substrate for energy production and consequently increase fat utilization. Daily transient increases in fat oxidation were expected to lead to a reduction in body fat mass without altering energy intake. However, the effects of MH on EE and macronutrient metabolism were inconsistent between studies (Table 7.1). No study observed a difference in REE. Given that the largest predictor of REE is fat free mass, we did not necessarily expect to see a difference (Johnstone et al., 2005). MH was expected to decrease dietary thermogenesis and this reduction would be associated with increases in the activation of AMPK in skeletal muscle. In agreement, MH decreased EE in Chapter 4. However, changes in EE were not mediated by AMPK. In contrast, MH increased dietary thermogenesis (5 – 10 h post-prandial) in Chapter 2 in dogs fed a diet high carbohydrate relative to fat concentrations. As a competitive inhibitor of hexokinases, we expected MH to limit glucose utilization. In agreement, MH decreased post-prandial RQ in experiment 2 suggesting greater fat relative to carbohydrate oxidation. However, MH did not affect fed state plasma glucose or lipid kinetics. The discrepant findings are likely due to differences in feeding paradigms. Changes in RQ were noted after ingestion of a single meal, whereas, glucose and lipid kinetics were assessed during repeated meal feeding. The repeated meal feeding technique was used to elicit a physiological steady state enabling steady state kinetic modeling. However, the small meals delivered a smaller bolus of MH per meal than the single meal.
Table 7.1. Summary of experimental findings.

<table>
<thead>
<tr>
<th>Outcome&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Chapter 3: 2 mg/kg BW</th>
<th>Chapters 4 &amp; 5: 4 mg/kg BW</th>
<th>Chapter 2: 8 mg/kg BW</th>
<th>Low CHO:Fat</th>
<th>High CHO:fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE</td>
<td>↔ REE</td>
<td>↔ REE</td>
<td>↔ REE</td>
<td>↔ REE</td>
<td>↔ REE</td>
</tr>
<tr>
<td></td>
<td>↔ dietary</td>
<td>↓ dietary</td>
<td>↔ dietary</td>
<td>↑ dietary</td>
<td>↑ dietary</td>
</tr>
<tr>
<td>thermogenesis</td>
<td>thermogenesis</td>
<td>thermogenesis</td>
<td>thermogenesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↓ fast AMPK</td>
<td>↔ fast or fed AMPK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓ physical activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RQ</td>
<td>↓ fast</td>
<td>↓ post-prandial</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Glucose</td>
<td>↑ fast ↓ fed oxidation</td>
<td>↔ fast or fed [glucose]</td>
<td>↔ fast or fed [glucose]</td>
<td>↔ fast or fed [glucose]</td>
<td>↔ fast or fed [glucose]</td>
</tr>
<tr>
<td></td>
<td>↔ Rₜ or tissue uptake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid</td>
<td>not measured</td>
<td>↔ lipolysis</td>
<td>not measured</td>
<td>not measured</td>
<td>not measured</td>
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</tbody>
</table>
|<sup>1</sup>Primary outcomes were energy expenditure (EE), respiratory quotient (RQ), glucose metabolism (Glucose), and lipid metabolism (Lipid). The arrows, ↓, ↑, ↔, indicate a decrease, increase, or no change, respectively.

Interestingly, 4 mg/kg MH decreased voluntary physical activity, especially on weekends when there was reduced human-animal interaction. Activity, as measured in Chapter 4, represents the mean activity count (locomotion) over a 12-h period. Locomotion encompasses complex behaviours (i.e. spontaneous activity, exploration and exercise) that are elicited by a wide range of internal (i.e. circadian rhythms) and external (i.e. environment) stimuli. Furthermore, locomotion does not describe the frequency, duration, or intensity of the
exercise. Physical activity has been shown to induce weight loss and provide other health benefits. Conversely, exercise may be stimulated by stress (i.e. pacing) or be indicative of cognitive deficit. A more critical analysis of physical activity and related behaviours is necessary to fully understand the effects of MH.

The inability of MH to elicit changes in fasting metabolism may have been related to the transience of MH effects. Indeed, in Chapter 2, plasma MH concentrations were shown to return to non-detectable levels within 24 h of ingestion. The inability of MH to impact post-prandial metabolism may be related to several factors, including the test diets, dietary dose, bioavailability, and the animal model used. The effectiveness of dietary MH as a competitive inhibitor of hexokinases was likely reduced due to the abundance of dietary derived carbohydrate (glucose). Furthermore, ingestion of dietary carbohydrate has been shown to activate glucokinase in the liver (Panserat et al., 2014). Dietary carbohydrate source also impacted our ability to observe MH effects. All test diets used had a high inclusion of soluble fibres, including those from sorghum grain, which has been demonstrated to minimize post-prandial glucose and insulin responses in dogs (Carciofi et al. 2008). Such dietary ingredients would make it difficult to observe notable changes in glucose metabolism associated with MH feeding. The high carbohydrate relative to fat diet used in Chapter 2 was high in corn which contains high amounts of fructose. Fructose has been shown to acutely activate hepatic glucokinase (Panserat et al., 2014).

The doses of MH used in the current experiments are considerably less than doses published in the literature (2 – 8 mg/kg BW vs. >1000 mg/kg BW administered i.v.). Doses used in the current studies represent cost-effective doses that would be incorporated into commercial pet foods. However, it is likely that much higher doses of MH are needed to see notably changes in metabolism. A dose response is necessary to determine the optimal dose of dietary MH. Within the dose response study, a maximum dietary dose of 1000 mg/kg BW
should be used as this dose is the only dose that has been demonstrated to elicit changes in metabolism. Future studies which examine increasing dietary doses of MH in the presence of different carbohydrate concentrations and sources are also warranted to determine the optimal dietary delivery of MH.

While ingested MH appears in peripheral circulation (Chapters 2–5), it is unknown how much MH is lost to first pass metabolism or whether MH is bioconverted to other metabolites. In humans, the bioavailability of plant polyphenols is very low, and many are bioconverted by microbial enzymes in the gut to other metabolites (Walle, 2011). Another issue concerning MH is its bioavailability in the diet versus an oral supplement. It is possible that dietary MH could be become bound to protein during the extrusion process limiting its availability. Furthermore, the differences in MH extracts used between studies could impact MH metabolic effects. In Chapter 2, a whole fruit extract was used (peel, pit and flesh), whereas, in Chapters 3–5 a flesh only extract was used. A flesh-only extract was used due to concerns over potentially toxic compounds (i.e. persin) found in the pit and peel of avocados. Detailed characterizations of the extracts are needed to identify factors which could influence the bioavailability of MH.

It is unknown the amount of MH reaching body tissues (tissue specific availability). In the current experiments we sampled skeletal muscle to quantify phosphorylated and total AMPK protein content. It is also unknown the amount of MH reaching body tissues. Skeletal muscle is predominantly comprised of hexokinase II which is saturated at low concentrations of glucose. Therefore, it is possible that AMPK in skeletal muscle is not be as sensitive to changes in glucose supply as it would be in tissues that express hexokinase IV for example. However, sampling liver and pancreas was not possible under the current Animal Care and Use Guidelines of Procter and Gamble Pet Care.
Finally, in the current experiments we were trying to notice changes in energy and glucose metabolism in healthy lean adult dogs that are provided the best veterinary care available. Using this animal model, we would need to greatly increase our sample size to have the power to look for modest changes in metabolism. Repeating these experiments in overweight dogs would provide more insight into the role of MH as a potential nutraceutical.

Irrespective of MH, these experiments provide pertinent information related macronutrient metabolism and EE in dogs. Historically, dogs have served as an important model for studying glucose kinetics using isotope tracer methodologies. Fasting plasma glucose turnover has been determined in dogs under a wide range of physiological conditions (reviewed in Appendix A). However, very few studies have assessed glucose oxidation or post-prandial glucose kinetics. Furthermore, no studies have assessed post-prandial lipid kinetics in dogs. The data generated in Chapters 3 and 5 provide a quantitative basis for future research into post-prandial glucose and lipid metabolism in dogs. Interestingly, we found dogs fed a diet low in dietary carbohydrate relative to fat (30.6 % CHO, 27.9 % fat) had higher circulating glucose post-prandially than dogs fed a diet high in carbohydrate relative to fat (53.6 % CHO, 11.2 % fat). In order to fully understand the effects of feeding a diet high in fat relative to CHO on insulin sensitivity and glucose tolerance, more sensitive measures are needed, partnered with a longer dietary adaptation period. In addition, measuring serum lipids, including triglycerides and free fatty acids, would provide insight into the metabolic adaptations that occur with high fat feeding.

In general, there is a paucity of published scientific evidence quantifying EE in dogs. Currently energy intake recommendations are based on REE estimates derived from predictive equations. The accuracy of these equations to predict REE is arguable, especially given the variation in breed, body size, health status, and environment that exists. Indirect calorimetry provided the animals are adequately acclimated to the equipment, offers
meaningful estimates of REE and is the only means of isolating the contribution of adaptive thermogenesis to EE in free-living animals. However, the use of indirect calorimetry to determine REE in dogs is very limited and only one other study has examined dietary thermogenesis in dogs (Pouteau et al., 2002). In Chapter 2, we demonstrated that dogs are able to adapt macronutrient oxidation to diet macronutrient composition, as evidenced by changes in RQ. As mentioned, the contribution of dietary thermogenesis to weight maintenance is unclear and warrants additional research. Understanding how diet alters dietary thermogenesis would be of particular interest in dogs as they appear to be uniquely adapted to fat metabolism.

In conclusion, oral MH supplementation at doses ranging from 2 – 8 mg/kg does not substantially alter macronutrient metabolism in healthy adult canines. MH does, however, impact EE, but its effects are inconsistent between studies. At this time, there is not sufficient evidence to support MH use as a novel nutraceutical for canines. Further research aimed at determining the optimal dietary dose and delivery are needed. Regardless of MH, the present research provides important information related to both fasting and post-prandial macronutrient metabolism and EE in healthy adult dogs.


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A.1. Abstract

The maintenance of blood glucose homeostasis is complex and involves several key tissues. Most of these tissues are not easily accessible, making direct measurement of the physiological parameters involved in glucose metabolism difficult. The use of isotope tracer methodology and mathematical modeling allows indirect estimates of \textit{in vivo} glucose metabolism through relatively non-invasive means. The purpose of this paper was to provide a mathematical synthesis of the models developed for describing glucose kinetics. As many of the models were developed using dogs, example data from the canine literature are presented. However, examples from the human and feline literature are also given in the absence of dog data. The glucose system is considered in both the steady and non-steady states, and the models are examined by grouping them into schemes consisting of one, two and three glucose compartments. Non-compartmental schemes are also considered briefly.

\textsuperscript{6} McKnight et al. ISRN Biomathematics Article ID 120974, 16 pages, 2013. doi.org/10.1155/2013/120974
A.2. Introduction

Glucose is a ubiquitous cellular fuel source for all mammalian tissues. As such, the regulation of glucose metabolism has been under extensive investigation for the past century. In healthy animals, several biological mechanisms ensure that the rate of appearance of glucose in the bloodstream tightly matches that of glucose uptake by tissues, resulting in relatively constant blood glucose concentrations irrespective of physiological condition. These control mechanisms are predominantly dictated by the energy status of the animal. In post-absorptive and fasting periods the body largely relies on endogenous glucose production via liver glycogenolysis or gluconeogenesis to maintain glucose homeostasis. In the post-prandial period (or fed state) glucose uptake and utilization by tissues is increased in response to the absorption of glucose in the small intestine, giving rise to the subsequent stabilization of blood glucose concentrations. Both production and uptake are principally hormonally regulated by glucagon and insulin, but substrate availability, circulating free fatty acids (FFA) and catecholamines (released in response to stress and/or exercise) also impact glucose metabolism.

Dysregulation of glucose homeostasis, as seen in metabolic diseases including obesity and diabetes, has become a worldwide epidemic in humans [1] and companion animals [2]. These diseases share the common feature of progressive insulin resistance and hyperglycemia. The rat has been used extensively as a model for studying the etiology of these diseases [reviewed by 3]. However, the dog provides a model more relatable to the human condition due to the similarities in pathophysiology of insulin resistance between dogs and humans. In addition, the dog enables better in vivo access to the key tissues involved in metabolic disease than rodents. Therefore, it is not surprising that the early kinetic models to describe glucose metabolism were developed using dogs. Pancreatectomy and chemical destruction of the pancreas (i.e. alloxan) are the most common methods used to induce dysregulation in glucose
metabolism in dogs [3]. Such models are reflective of overt diabetes, as these procedures significantly impair (or in some cases abolish) insulin secretion resulting in marked hyperglycemia. These invasive procedures cannot, however, be used to investigate the progression of the diabetes. The “pre-diabetes” state, characterized by insulin resistance, is most commonly studied using dietary interventions, including high fat and/or high fructose feeding. These diets have been shown to induce insulin resistance in humans and dogs; however, the etiology of insulin resistance has not been fully elucidated.

The use of stable and/or radioactive isotope tracers allows for the quantification pool sizes and flows between compartments involved in glucose metabolism in vivo at the whole body level. Both hydrogen (2H or 3H) and carbon (13C or 14C) labelled glucose tracers are available and selection is dependent on what aspect of metabolism is of interest. The most commonly used hydrogen tracers are labelled in the 2, 3 or 6 position. Label in the 2 or 3 positions will be lost in glycolysis and incorporated into body water, making these tracers most useful for determining plasma turnover and/or glycolytic flux. Label in the 6 position is not lost in glycolysis but in gluconeogenesis, making it ideal for assessing endogenous glucose production. Carbon tracers include labelling of the 1, 6 or 1-6 (uniformly, U) position(s). The carbon label is lost in the tricarboxylic acid (TCA) cycle as labelled CO2 enabling estimation of whole body glucose oxidation. Carbon label may become incorporated in TCA cycle intermediates and as lactate (via the Cori cycle). This sequestration of carbon is referred to as carbon recycling and should be accounted for in kinetic calculations.

The most common techniques used in glucose tracer kinetics include the bolus injection and the constant infusion [4]. The bolus injection method generally involves venous injection of a bolus dose of isotope resulting in an initial increase in plasma isotope enrichment followed by an exponential decline/decay over time. The most common application is the intravenous glucose tolerance test (IVGTT), where a bolus of unlabelled
glucose is administered with (or without) tracer allowing for the calculation of glucose effectiveness (GE) (the ability of glucose to stimulate its own uptake) and insulin sensitivity (IS) (the ability of insulin to stimulate glucose uptake). In the constant infusion method, isotope is infused at a constant rate until an isotopic equilibrium is reached. In this situation the rate of appearance of glucose in the plasma is equal to the rate of disappearance. This methodology is commonly used during “clamp” studies, where either unlabelled glucose, insulin, free fatty acids or a combination thereof is also infused at a constant rate (“clamped”). A common example would be the euglycemic hyperinsulinemic clamp (EHC), where insulin concentrations are clamped at a supraphysiological level and glucose is infused at a variable rate to maintain euglycemia. The rate of glucose infusion is used as an index of insulin sensitivity. As a result of the slow turnover rate of glucose relative to its pool size, it takes several hours to reach isotopic equilibrium using the constant infusion technique. A bolus (priming) dose of isotope in combination with constant infusion can be used to reduce the time to achieve isotopic steady state. Either methodology allows for the calculation of glucose kinetics under physiological steady state conditions. However, the constant infusion technique (± prime) enables the most accurate description of non-steady state kinetics. The premise being that once isotopic equilibrium is reached any changes in isotope enrichment will reflect those induced by the metabolic perturbation (i.e. exercise).

Interpretation of the kinetic data generated from isotope tracer studies requires the use of mathematical modeling. Several mathematical models of varying complexity have been published to describe in vivo whole body glucose kinetics. The purpose of this paper was to provide a mathematical synthesis of these models. As many of the models were developed using dogs, example data from the canine literature are presented. However, examples from the human and feline literature are also given in the absence of canine data. The glucose system is considered in both the steady and non-steady states, and the models are examined
by grouping them into schemes consisting of one, two and three glucose compartments. Non-compartmental schemes are also considered briefly. Mathematical notation is defined in Table A.1. Software implementations are not reviewed as this paper is a mathematical synthesis rather than a typical review. Although reviews and accounts of whole-body glucose models have recently been published (5, 6), these are not specific to kinetic modelling (i.e. resolving isotope tracer data).

**Table A.1. Mathematical notation**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D, D^*$</td>
<td>Dose of total and labelled glucose, respectively, injected into primary pool [mmol]</td>
</tr>
<tr>
<td>$F_{ij}$</td>
<td>Flow of total glucose to pool $i$ from pool $j$; $F_{i0}$ represents external flow of glucose into pool $i$ and $F_{oi}$ flow of glucose from pool $i$ out of the system [mmol/min]</td>
</tr>
<tr>
<td>$\phi$</td>
<td>Constant rate of labelled glucose infusion into primary pool [mmol/min]</td>
</tr>
<tr>
<td>$G, G^*$</td>
<td>Total and labelled glucose concentration, respectively, in primary pool [mmol/mL]</td>
</tr>
<tr>
<td>$I, I'$</td>
<td>Plasma and remote insulin concentrations, respectively [pmol]</td>
</tr>
<tr>
<td>$k_{ij}$</td>
<td>Relative rate constant for flow from pool $i$ from pool $j$ [per min]</td>
</tr>
<tr>
<td>$Q_i$</td>
<td>Quantity of total glucose in pool $i$ [mmol]</td>
</tr>
<tr>
<td>$q_i$</td>
<td>Quantity of labelled glucose in pool $i$ [mmol]</td>
</tr>
<tr>
<td>$s_i$</td>
<td>Enrichment (or specific activity) of pool $i$ [mmol labelled glucose/mmol total glucose]</td>
</tr>
<tr>
<td>$t$</td>
<td>Time since isotope first administered [min]</td>
</tr>
<tr>
<td>$V_i$</td>
<td>Volume of pool $i$; $V_1$ represents primary (plasma) pool volume [mL]</td>
</tr>
</tbody>
</table>
A.3. Models with one glucose compartment

A.3.1. Insulin-independent representations

The minimal scheme is shown in Figure A.1 with example data given in Table A.2. This scheme contains one pool (venous plasma glucose), one inflow (rate of glucose appearance, Ra), and one outflow (rate of glucose disappearance, Rd). Isotope (either stable or radio-active) is administered by constant infusion or by single dose injection and plasma glucose concentration and enrichment (or specific activity) are monitored following isotope administration. The fundamental equations follow.

For total (plasma) glucose:

\[
\frac{dQ}{dt} = F_{10} - F_{01} \quad (1)
\]

For labelled glucose:

\[
\frac{dq_i}{dt} = \phi - s_i F_{01}; \quad q_i(0) = 0 \text{ (no priming), for constant infusion} \quad (2a)
\]

\[
= -s_i F_{01}; \quad q_i(0) = D^*, \text{ for single dose injection} \quad (2b)
\]

Further:

\[
s_i = \frac{q_i}{Q_i}; \quad G = \frac{Q_i}{V_i} \quad (3)
\]

If multiple exits should exist, the rate of disappearance \( F_{01} \) is the sum of all the exits; likewise for \( F_{10} \) and multiple entrances.

If the plasma glucose pool is in steady state, such as after an overnight fast, the derivative \( dQ_i/dt \) becomes zero, and the constant \( Q_i \) can be calculated from Equation (3) as \( GV_i \). Likewise \( dq_i/dt \) becomes zero for constant infusion. Equations (1) and (2a) then reduce to two simultaneous equations in two unknowns \( (F_{01} \text{ and } F_{10}) \), which can readily be solved to give:

\[
F_{10} = \frac{F_{01} = \phi / s_i}
\]
Under single dose injection, assuming outflow obeys mass action kinetics (i.e. \( F_{01} = k_{01}Q_1 \)),

Equation (2b) becomes:

\[
\frac{dq}{dt} = -s, k_{01}Q_1; \quad q_1(0) = D^* 
\]

Dividing both sides by constant \( Q_1 \) yields:

\[
\frac{ds}{dt} = -k_{01} s_1; \quad s_1(0) = D^* / Q_1 
\]

Integrating \( ds_1/dt \):

\[ s_1 = s_1(0)e^{-k_{01}t} \quad (4) \]

The rate constant \( k_{01} \) can be estimated by regression analysis of the isotope dilution curve (Equation 4), allowing determination the rate of glucose disappearance (and hence the rate of appearance).

The model can be solved for the plasma glucose pool in non-steady state by combining the fundamental equations using \( ds_1/dt \) as follows:

\[
\frac{ds_1}{dt} = \frac{d(q/Q_1^{-1})}{dt} = Q_1^{-1}\frac{dq}{dt} - q_1Q_1^{-2}\frac{dQ}{dt} 
\]

i.e.,

\[
Q_1\frac{ds_1}{dt} = \frac{dq}{dt} - s_1\frac{dQ}{dt} 
\]

Substituting for \( dq/dt \) and \( dQ/dt \) using Equations (1) and (2a):

\[
Q_1\frac{ds_1}{dt} = \phi - s_1F_{10} 
\]

Re-arranging:

\[
F_{10} = \left( \phi - Q_1\frac{ds_1}{dt} \right) / s_1 
\]

i.e.

rate of glucose appearance \( = \left( \phi - G(t)V_i\frac{ds_1}{dt} \right) / s_1 \); \( s_1(0) = 0 \) \quad (5)
The derivative \( ds_i/dt \), which varies over time, can be estimated numerically from the isotope dilution curve. This non-steady state model was first proposed by Steele [7] in studying hepatic glucose output using \(^{14}\)C glucose, with one modification:

\[
F_{i0} = \left( \phi - pV_iG \frac{ds_i}{dt} \right) / s_i
\]

where \( pV_i \) is interpreted as the ‘effective’ glucose volume; a fraction \( p \) of \( V_i \), the ‘total’ glucose distribution volume. The generally chosen value of \( p \) is 0.65. If isotope administration is by single injection rather than constant infusion then Equation (5) is replaced by:

rate of glucose appearance \( = -GV_i \frac{ds_i}{dt} / s_i \); \( s_i(0) = D^* / Q_i(0) \)

For IVGTT, where a significant dose of glucose is injected following an overnight fast, Equation (1) becomes:

\[
\frac{dQ_i}{dt} = F_{i0} - k_{i0}Q_i
\]

where \( F_{i0} \) is assumed constant and glucose disappearance follows mass action kinetics.

Dividing by constant \( V_i \):

\[
\frac{dG}{dt} = \frac{F_{i0}}{V_i} - k_{i0}G; \ G(0) = G_b + \frac{D}{V_i}
\]

where \( G_b \) is glucose concentration in the fasted (basal) state. The differential equation can be integrated analytically, using the integrating factor method [8], to give:

\[
G = \frac{F_{i0}}{k_{i0}V_i} + \left[ G(0) - \frac{F_{i0}}{k_{i0}V_i} \right] e^{k_{i0}t}
\]

The unknown parameters \( (k_{01}, F_{01}) \) can be determined by regression analysis of the glucose concentration curve post injection. If a labelled IVGTT is performed, the resultant isotope dilution curve is described by Equation (4). Of some interest is the partial derivative of Equation (6) with respect to \( G \):
as it gives a measure of the effect of glucose per se on its own disposition (see next section).

Bergman et al. [9] proposed this model of glucose disappearance (among several others) in examining the feasibility of using an unlabelled IVGTT (α-D-glucose) for estimating peripheral insulin sensitivity in dogs. Bergman et al. [9] also proposed an identical model for this purpose, except that glucose utilization is a saturable, rather than mass-action process that obeys Michaelis-Menten kinetics, i.e.

\[ k_0 G \rightarrow \frac{(v_0 / V_i) G}{K_G + G} \]

in Equation (6), where \( v_0 \) is maximum velocity and \( K_G \) an affinity constant. The resulting differential equation is now non-linear and has to be handled numerically using specialist software for integration and parameter estimation. The partial derivative of Equation (6) becomes:

\[
\frac{\partial (dG / dt)}{\partial G} = -\frac{v_0 V_i^{-1}}{K_G + G} \left( 1 - \frac{G}{K_G + G} \right)
\]
Figure A.1. Insulin-independent minimal model for describing glucose kinetics. Arrowed solid lines represent flows, hollow arrow represents glucose application (infusion or dose), and broken line sampling.
Table A.2. Example literature values of insulin-independent minimal model for describing glucose kinetics.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population (Species); n per group; body weight (kg)</th>
<th>Experimental Groups/Conditions</th>
<th>Isotope (U-14C, 3-3H, 6,6-2H)</th>
<th>Plasma Rate of Appearance (mg kg⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[22]</td>
<td>Canine (Beagles); 6; 15.2 ± 0.70</td>
<td>Basal (C), total (T1) and peak (T2) in response to single mixed meal</td>
<td>U-14C 1.57; 3-3H 2.50 †</td>
<td>C 2.30 †; T1 3.68 †; T2 -; T3 -</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Same as above</td>
<td></td>
<td></td>
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<tr>
<td>[23]</td>
<td>Canine (Beagles); 7; 12.2 ± 1.0 (C); 17.5 ± 1.7 (T1)</td>
<td>Healthy (C) vs. Obese (T1)</td>
<td>6,6-2H 2.60 ± 0.30</td>
<td>C 3.03 †; T1 3.30 †; T2 3.30 ± 0.50; T3 -</td>
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<tr>
<td>[24]</td>
<td>Canine (Mongrel); 6; 27.5 ± 1.5 (C); 29.0 ± 1.70 (T2)</td>
<td>Basal (C). after 6 (T1) and 12 (T2) weeks of moderate-fed feeding</td>
<td>3-3H 2.60 ± 0.30</td>
<td>C 3.05 ± 0.50; T1 3.30 ± 0.30; T2 -</td>
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<tr>
<td>[25]</td>
<td>Canine (Mongrel); 6; -22</td>
<td>Basal (C), 7 (T1), 40 (T2) and 120 (T3) mU/kg/h constant insulin infusion during clamped euglycemia</td>
<td>3-3H 3.43 ± 0.32</td>
<td>C 3.22 ± 0.32; T1 9.85 ± 1.67; T2 18.3 ± 2.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Same parameters as above during clamped hyperglycemia</td>
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<tr>
<td></td>
<td></td>
<td>6.48 ± 0.30; 17.3 ± 0.97</td>
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<tr>
<td>[26]</td>
<td>Canine (Mongrel); 6; 21.1 ± 1.13</td>
<td>Basal (C) vs. Methylprednisolone (T1)</td>
<td>2-3H 3.96 ± 0.34</td>
<td>C 3.22 ± 0.45; T1 17.7 ± 0.26; T2 -</td>
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<tr>
<td>[27]</td>
<td>Canine (Labrador Retriever); 3; BW not reported</td>
<td>Basal (C) vs. Exercise intensities of 40% (T1), 60% (T2) and 85% (T3) VO₂max</td>
<td>3-3H 4.38 ± 1.62 †; 2.81 †</td>
<td>C 18.9 ± 1.62; T1 17.7 ± 1.62; T2 3.49 †</td>
</tr>
<tr>
<td>[28]</td>
<td>Canine (Mongrel); 6; -22</td>
<td>Basal (C); exercise at 60% VO₂max (T1); Mild diabetic after exercise (T2); Diabetic treated with Phlorizin after exercise (T3)</td>
<td>3-3H 2.14 ± 0.25 †; 0.11</td>
<td>C 5.17 ± 0.25; T1 3.83 ± 0.17; T2 0.36</td>
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<tr>
<td>[29]</td>
<td>Canine (Mongrel); 6; 20.4 ± 1.6</td>
<td>Basal (C), exercise at 60% VO₂max (T1); primed constant infusion of AICAR (T2) or free fatty acids (T3)</td>
<td>3-3H 2.29 ± 0.34 †; 0.16</td>
<td>C 4.95 ± 0.25; T1 2.07 ± 0.14; T2 0.22</td>
</tr>
<tr>
<td>[30]</td>
<td>Canine (Mongrel); 8 healthy; 3 diabetic; 20.1 ± 1.6</td>
<td>Basal (C), primed constant infusion of AICAR (T1), Methyllumoxirate (T2) or both (T3)</td>
<td>3-3H 2.34 ± 0.34 †; 0.36</td>
<td>C 3.15 ± 0.25; T1 2.88; T2 0.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Same as above in diabetic dogs (alloxan induced)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[31]</td>
<td>Canine (Mongrel); 8; BW not reported</td>
<td>Saline (C); pulsatlie (T1) or constant (T2) octanoate infusion</td>
<td>3-3H 5.83 ± 0.36</td>
<td>C 5.69 ± 0.45; T1 4.25 ± 0.34; T2 3.94 ± 0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Same as above</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C, control group; T1-3, treatment groups 1-3; All flows are expressed in mg glucose kg⁻¹ min⁻¹ to aid comparison; † represents non-steady state conditions; “Basal” refers to fasted rest; Data are presented as mean ± SE.
A.3.2. Insulin-dependent representations

Figure A.2 shows the insulin-dependent minimal model, again due to Bergman et al. [9] and promulgated by Cobelli et al. [10], for glucose disappearance during an IVGTT. It builds on work by Bolie [11], the first to introduce insulin dependence into the minimal glucose model. The model contains two pools (plasma glucose and remote insulin). The glucose pool has one inflow (rate of glucose appearance), and two outflows (rate of glucose disappearance to liver and disappearance to peripheral tissues). All glucose flows are affected by remote insulin concentration. A single dose of cold glucose with (or without) labeled glucose is administered by injection, and plasma glucose concentration, its enrichment (or specific activity) and plasma insulin concentration are monitored following dosing. The remote insulin pool has one inflow (from plasma insulin) and one outflow. The fundamental equations follow. For total plasma glucose:

\[
\frac{dQ_1}{dt} = V_1 \frac{dG}{dt} = F_{01} - F_{01}^{(l)} - F_{01}^{(p)}; \quad Q_1(0) = G_0 V_1 + D
\]

For labelled plasma glucose:

\[
\frac{dq_{1l}}{dt} = V_1 \frac{dG^*}{dt} = -s_1 \left( F_{01}^{(l)} + F_{01}^{(p)} \right); \quad q_{1l}(0) = D^*
\]

For remote insulin:

\[
\frac{dQ_2}{dt} = V_2 \frac{dI'}{dt} = F_{02} - F_{02}; \quad Q_2(0) = 0
\]

Further:

\[
G = Q_1 / V_1; \quad G^* = q_{1l} / V_1; \quad I' = Q_2 / V_2
\]

If the outflows follow mass action kinetics, then:

\[
F_{01}^{(l)} = k_{01}^{(l)} Q_1; \quad F_{01}^{(p)} = k_{01}^{(p)} Q_1; \quad F_{02} = k_{02} Q_2
\]

where the \(k\)'s are relative rate parameters. The fundamental equations now yield:
\[ \frac{dG}{dt} = F_{10} V_{i}^{-1} - \left( k_{01}^{(f)} + k_{01}^{(p)} \right) G; \quad G(0) = G_b + D V_{i}^{-1} \]

\[ \frac{dG^*}{dt} = -\left( k_{01}^{(f)} + k_{01}^{(p)} \right) G^*; \quad G^*(0) = D^* V_{i}^{-1} \]

\[ \frac{dl'}{dt} = F_{20} V_{2}^{-1} - k_{02} l'; \quad l'(0) = 0 \]

All flows out of the plasma glucose pool are assumed to be directly affected by remote insulin concentration, and flow into the remote insulin pool by the concentration of plasma insulin. These assumptions, together with the assumption of constant glucose appearance rate, give rise to the following equations:

\[ k_{01}^{(f)} = p_1 + p_2 l'(t) \]

\[ k_{01}^{(p)} = p_3 + p_4 l'(t) \]

\[ F_{10} V_{i}^{-1} = p_5 \]

\[ F_{20} V_{2}^{-1} = p_6 \left[ I(t) - I_b \right] \]

where \( p_1, p_2, p_3, p_4, p_5, p_6 \) are constants and \( I_b \) is basal plasma insulin concentration. The fundamental equations can therefore be re-parameterized to give Equations (7)-(9):

\[ \frac{dG}{dt} = p_5 - \left[ p_1 + p_3 + (p_2 + p_4) l' \right] G; \quad G(0) = G_b + D V_{i}^{-1} \quad (7) \]

\[ \frac{dG^*}{dt} = -\left[ p_1 + p_3 + (p_2 + p_4) l' \right] G^*; \quad G^*(0) = D^* V_{i}^{-1} \quad (8) \]

\[ \frac{dl'}{dt} = p_6 \left[ I(t) - I_b \right] - k_{02} l'; \quad l'(0) = 0 \quad (9) \]

Glucose effectiveness (GE or \( S_G \)), following Bergman et al. [9], is defined as the enhancement of glucose disappearance due to an increase in plasma glucose concentration:

\[ GE = -\frac{\partial}{\partial G} \left( \frac{dG}{dt} \right) \]
Insulin sensitivity (IS or S\textsubscript{i}) is then defined as the ability of insulin to increase glucose effectiveness:

\[ IS = -\frac{\partial^2 (dG / dt)}{\partial I \partial G} \]

For the insulin-dependent minimal model, the definitions yield in the basal steady state:

\[ GE = p_1 + p_3 \]
\[ IS = p_e (p_2 + p_4)/k_{02} \]

These parametric formulae may be derived as follows. Differentiating Equation (7) with respect to \( G \):

\[ \frac{\partial (dG / dt)}{\partial G} = -\left[ p_1 + p_3 + (p_2 + p_4)I' \right] \]
\[ = -(p_1 + p_3) \text{ in the basal steady state.} \]

Differentiating with respect to \( I' \):

\[ \frac{\partial^2 (dG / dt)}{\partial I' \partial G} = -(p_2 + p_4) \]

In steady state, Equation (9) gives:

\[ I' = p_e (I - I_b)/k_{02} \]
\[ \frac{\partial I'}{\partial I} = p_e/k_{02} \]

Therefore:

\[ \frac{\partial^2 (dG / dt)}{\partial I \partial G} = \frac{\partial^2 (dG / dt)}{\partial I' \partial G} \times \frac{\partial I'}{\partial I} = -p_e (p_2 + p_4)/k_{02} \]

In applying the insulin-dependent minimal model, integration and parameter estimation is undertaken numerically.

There are numerous applications of the model in the literature (see Table A.3) [reviewed by 12], the most common being the modified insulin or tolbutamide (stimulates endogenous insulin secretion) IVGTT (miIVGTT and mtIVGTT, respectively).
application, either insulin or tolbutamide is infused into the plasma to stimulate the remote insulin pool. A novel application was introduced by [13], who used a modified version of the minimal model to analyze EHC data on lean and obese cats obtained using an infusion of cold and $^3$H-glucose. The primary novelty is the addition of a pool of labelled water to the model to determine the rate of glycolysis, and the introduction of a Michaelis-Menten type inhibition of glucose appearance by plasma insulin characterized by a parameter defined as the supra-basal concentration at which glucose appearance is inhibited by 50%.

**Figure A.2.** Insulin-dependent minimal model for describing glucose kinetics. Arrowed solid lines represent flows, valves indicate flows affected by remote insulin concentration, hollow arrow represents glucose application (infusion or dose), and broken line sampling.
Table A.3. Example literature values of insulin-dependent minimal model for describing glucose kinetics.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population (Species); n per group; body weight (kg)</th>
<th>Experimental Groups/Conditions</th>
<th>Test</th>
<th>Isotope</th>
<th>GE (min⁻¹)</th>
<th>IS (µU⁻¹ min⁻¹ mL × 10⁻⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[9]</td>
<td>Canine (Mongrel); 5 (C); 8 (T1); ~25</td>
<td>Low (C) vs high (T1) dose</td>
<td>IVGTT</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>T</td>
<td>13.9 ± 5.37</td>
<td>7.00 ± 0.59</td>
</tr>
<tr>
<td>[10]</td>
<td>Canine (Beagle); 6; 11 ± 2</td>
<td>Cold (no isotope) (C) vs. labelled (T1)</td>
<td>IVGTT</td>
<td>2⁻³H</td>
<td>0.042 ± 0.009</td>
<td>0.009 ± 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>T</td>
<td>4.52 ± 1.39</td>
<td>6.41 ± 0.91</td>
</tr>
<tr>
<td>[32]</td>
<td>Canine (Mixed); 8; 27.2 ± 1.1</td>
<td>Healthy (C) vs. Acepromazine (T1)</td>
<td>IVGTT</td>
<td>-</td>
<td>0.04 ± 0.01</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>T</td>
<td>6.6 ± 1.0</td>
<td>7.7 ± 2.3</td>
</tr>
<tr>
<td>[33]</td>
<td>Canine (Mixed); 6; 22 ± 4.4 (C); 45 ± 18.4 (T1)</td>
<td>Healthy (C) vs Obese (T1)</td>
<td>IVGTT</td>
<td>-</td>
<td>3.0 ± 0.8</td>
<td>6.5 ± 1.5</td>
</tr>
<tr>
<td>[34]</td>
<td>Canine (Mongrel); 9; 35.9 ± 1.4 (C); 42.4 ± 2.1 (T1)</td>
<td>Chow diet © vs. High Fat Diet (T1)</td>
<td>tmIVGTT</td>
<td>-</td>
<td>0.031 ± 0.40</td>
<td>0.020 ± 0.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>T</td>
<td>13.6 ± 1.74</td>
<td>5.87 ± 1.21</td>
</tr>
<tr>
<td>[35]</td>
<td>Canine (Mongrel); 6; 28.2 ± 1.7</td>
<td>Chow diet © vs. High Fat Diet (T1)</td>
<td>imIVGTT</td>
<td>-</td>
<td>0.041 ± 0.004</td>
<td>0.051 ± 0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>T</td>
<td>3.98 ± 0.54</td>
<td>1.40 ± 0.55</td>
</tr>
<tr>
<td>[36]</td>
<td>Canine (Mixed); 8, ~13</td>
<td>Healthy © vs. Hypothyroid (T1)</td>
<td>imIVGTT</td>
<td>-</td>
<td>0.043 ± 0.009</td>
<td>0.043 ± 0.011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>T</td>
<td>23.6 ± 5.3</td>
<td>4.9 ± 1.2</td>
</tr>
<tr>
<td>[13]</td>
<td>Feline (Domestic Shorthair); 10; 3.74 ± 0.36 (C); 6.6 ± 0.79 (T1)</td>
<td>Healthy (C) vs. Obese (T1)</td>
<td>EHC</td>
<td>3⁻²H</td>
<td>0.016</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>T</td>
<td>2.58 × 10⁻⁵ †</td>
<td>1.02 × 10⁻⁵ †</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>T</td>
<td>6.27 × 10⁻⁵ †</td>
<td>1.13 × 10⁻⁵ †</td>
</tr>
</tbody>
</table>

C, control group; T, treatment group; GE, glucose effectiveness; IS, insulin sensitivity; IVGTT, intravenous glucose tolerance test; tmIVGTT, tolbutamide modified intravenous glucose tolerance test; imIVGTT, insulin modified glucose tolerance test; EHC, euglycemic hyperinsulinemic clamp; * refers to per unit of concentration; † units of min⁻¹ pmol⁻¹ L; Data are presented as mean ± SE.
A.4. Models with two glucose compartments

A.4.1. Insulin-independent representations

Single pool models of glucose kinetics assume that glucose is uniformly distributed in the extracellular fluid space. In attempt to overcome this simplification of glucose distribution, a two compartment approach was proposed by Radziuk et al. [14]. The general scheme is shown in Figure A.3. It contains two glucose pools; the first encompassing plasma and the second being interstitial fluid. The external inflow to pool 1 is from hepatic glucose production/output (HGO) (appearance) and irreversible loss from this pool is due to insulin independent tissue uptake (i.e. brain and liver). There is a flow out of the system from each pool, and a bi-directional flow between the two pools. Irreversible loss from pool 2 represents insulin stimulated glucose uptake. Isotope is administered by constant infusion or by single dose injection, and then plasma glucose concentration and isotope enrichment/specific activity monitored. The fundamental equations follow. For total glucose:

\[
\frac{dQ_1}{dt} = V_1 \frac{dG}{dt} = F_{t0} + F_{12} - F_{01} - F_{21} \quad (10)
\]

\[
\frac{dQ_2}{dt} = F_{21} - F_{02} - F_{12} \quad (11)
\]

For labelled glucose:

\[
\frac{dq_1}{dt} = V_1 \frac{dG}{dt} = \phi + s_2 F_{12} - s_1 (F_{01} + F_{21}); \quad q_1(0) = 0 \text{ (no priming)}, \text{ for constant infusion} \quad (12a)
\]

\[
= s_2 F_{12} - s_1 (F_{01} + F_{21}); \quad q_1(0) = D^*, \text{ for single dose injection} \quad (12b)
\]

\[
\frac{dq_2}{dt} = s_1 F_{21} - s_2 (F_{02} + F_{12}); \quad q_2(0) = 0 \quad (13)
\]

If the system is in non-isotopic steady state, the derivatives \(dQ_1/dt\) and \(dQ_2/dt\) are zero and, if isotope is administered by constant infusion, \(dq_1/dt\) and \(dq_2/dt\) become zero and \(s_2\) equilibrates with \(s_1\). Equations (10)-(13) then reduce to a set of 3 simultaneous equations...
(Equation 13 becomes redundant when \( s_1 \) equals \( s_2 \)) in 5 unknowns which can be solved uniquely only for \( F_{10} \):

\[
F_{10} = \frac{\phi}{s_1}
\]

\[
F_{02} = F_{21} - F_{12}
\]

\[
F_{01} = F_{10} - F_{02}
\]

For single dose injection, assuming mass action kinetics, the differential equations for labelled glucose become:

\[
\frac{dq_1}{dt} = k_{12}q_2 - (k_{01} + k_{21})q_1; \quad q_1(0) = D^*
\]

\[
\frac{dq_2}{dt} = k_{21}q_1 - (k_{02} + k_{12})q_2; \quad q_2(0) = 0
\]

Integration of these two equations can be accomplished through their Laplace transformation \([8, 15]\):

\[
q_1/D^* = H_1 e^{-c_1 t} + H_2 e^{-c_2 t}
\]

\[
q_2/D^* = K_1 e^{-c_1 t} + K_2 e^{-c_2 t}
\]

\[
k_{11} = k_{01} + k_{21}
\]

\[
k_{22} = k_{02} + k_{12}
\]

\[
k_{11} = H_1 c_1 + H_2 c_2
\]

\[
k_{11} + k_{22} = c_1 + c_2
\]

\[
k_{11} k_{22} - k_{12} k_{21} = c_1 c_2
\]

where the \( H \)'s and \( K \)'s are coefficients of integration (dimensionless) and \( c \)'s are rate parameters (min\(^{-1}\)).

Radziuk et al. [14] developed this model to describe glucose kinetics in the non-steady state (and therefore the differential equations have to be solved numerically). Two versions of
the model were used for analysis, the first version with a single irreversible loss emanating from the primary pool with a varying relative rate parameter, i.e.
\[ k_{01} = k_{01}(t); \quad k_{02} = 0 \]
and the second with a loss from both pools with a single varying rate parameter, i.e.
\[ k_{02}(t) = k_{01}(t) \]

Cobelli et al. [16] propose a version of the model with 3 time-varying parameters:
\[ k_{01} = \text{constant}; \quad k_{02} = k_{02}(t); \quad k_{12} = k_{12}(t); \quad k_{21} = k_{21}(t) \]
to describe the transition between basal and final state of an EHC. The irreversible loss from the primary pool is interpreted as insulin-independent utilization, and the irreversible loss from pool 2 as insulin-dependent utilization. They also propose a simplified version to interpret glucose kinetics during an IVGTT:
\[ k_{01} = k_{01}(t); \quad k_{02} = k_{02}(t); \quad k_{12} = \text{constant}; \quad k_{21} = \text{constant} \]

In another version of the model, Hoenig et al. [13] propose a third pool, the water distribution space, to study 3-3H-glucose and 3H-H2O kinetics in cats to estimate the glycolytic fraction of glucose disappearance. Literature data are presented in Table 4.
**Figure A.3.** Insulin-independent two-compartment model for describing glucose kinetics. First compartment contains the vascular space. Arrowed solid lines represent flows, hollow arrow represents glucose application (infusion or dose), and broken line sampling.
Table A.4. Example literature values of insulin-independent two pool model for describing glucose kinetics.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population (Species); n per group; body weight (kg)</th>
<th>Experimental Groups/Conditions</th>
<th>Isotope</th>
<th>$V_1$ (mL kg$^{-1}$)</th>
<th>$F_{01}$ (mg kg$^{-1}$ min$^{-1}$)</th>
<th>Fractional Rate Constant (min$^{-1}$) $k_{01}$</th>
<th>$k_{11}$</th>
<th>$k_{12}$</th>
<th>$k_{02}$</th>
<th>GE (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[16]</td>
<td>Canine (Mongrel); 4; ~13</td>
<td>IVGTT</td>
<td>6-$^3$H</td>
<td>122</td>
<td>2.38</td>
<td>0.141</td>
<td>0.054</td>
<td>0.064</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IVGTT</td>
<td>6-$^3$H</td>
<td>122</td>
<td>1.85</td>
<td>0.061</td>
<td>0.049</td>
<td>0.072</td>
<td>0.061</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IVGTT</td>
<td>1-$^{14}$C</td>
<td>117</td>
<td>2.23</td>
<td>0.170</td>
<td>0.048</td>
<td>0.056</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IVGTT</td>
<td>1-$^{14}$C</td>
<td>117</td>
<td>1.77</td>
<td>0.072</td>
<td>0.041</td>
<td>0.066</td>
<td>0.072</td>
<td>-</td>
</tr>
<tr>
<td>[39]</td>
<td>Canine (Mongrel); 8; 25.8 ± 1.0</td>
<td>IVGTT</td>
<td>3-$^3$H</td>
<td>132</td>
<td>-</td>
<td>0.021 ± 0.001</td>
<td>0.111 ± 0.023</td>
<td>0.123 ± 0.029</td>
<td>0</td>
<td>0.030 ± 0.002</td>
</tr>
<tr>
<td>[15]</td>
<td>Feline (Domestic Shorthair); 10; Healthy (C) vs.</td>
<td>IVGTT</td>
<td>3-$^3$H</td>
<td>253.4 †</td>
<td>-</td>
<td>0.000</td>
<td>101.6 (C);</td>
<td>101.6 (C);</td>
<td>0.0378</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Obese (T1) during Healthy (C) vs. 3.74 ± 0.36 (C); 6.6 ± 0.79 (T1)</td>
<td></td>
<td></td>
<td>‡</td>
<td>68.2 (T1) ‡</td>
<td>68.2 (T1) ‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>EHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE; C, control group; T, treatment group, GE, glucose effectiveness (min$^{-1}$); IVGTT, intravenous glucose tolerance test; EHC, euglycemic hyperinsulinemic clamp

† units of mL
‡ units of mL min$^{-1}$
A.4.2. Insulin-dependent representations

Figure A.4 shows the insulin-dependent version of the model, due to Caumo and Cobelli [17], for glucose disappearance and HGO during an IVGTT (example data in Table A.5). The model contains two glucose pools and a remote insulin pool. Insulin-independent glucose disposal (removal) and appearance (HPO) takes place from the accessible (venous plasma) pool, and insulin-dependent glucose disposal from the slowly exchanging interstitial pool (pool 2). Inflow to the remote insulin pool is from plasma insulin. A single combined dose of cold and labelled glucose is administered by injection, and plasma glucose concentration, its enrichment (or specific activity) and plasma insulin concentration are monitored following dosing. Pool exits are assumed to follow mass-action kinetics. The fundamental equations for the glucose pools are given by Equations (10), (11), (12b) and (13), and that for the remote insulin pool by:

\[
\frac{dI'}{dt} = F_{30} - F_{03} = a_0 \left[ I(t) - I_0 \right] - k_{03}I' \quad I'(0) = 0
\]

which is analogous to Equation (9). Both relative disposal rates are made time dependent, i.e.

\[
k_{01} = k_{01}(t) = a_1 + \frac{F_{01}^{(b)}}{V_G(t)}
\]

\[
k_{02} = k_{02}(t) = a_2 + a_3I'(t)
\]

where the \(a\)'s are constants and \(F_{01}^{(b)}\) is the value of \(F_{01}\) in the basal state. All the other relative rates (\(k\)'s) are all assumed constant, and the model is solved numerically. To aid parameter estimation, Caumo and Cobelli [17] employ the constraint that insulin-independent glucose disposal is three times insulin-dependent glucose disposal in the basal state.
Figure A.4. Insulin-dependent two-compartment model for describing glucose kinetics. First compartment contains the vascular space. Arrowed solid lines represent flows, valves indicate flows affected by remote insulin concentration, hollow arrow represents glucose application (infusion or dose), and broken line sampling.
Table A.5. Example literature values of insulin dependent two pool model for describing glucose kinetics.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population</th>
<th>Experimental Conditions</th>
<th>Test Isotope</th>
<th>$V_1$ (\text{mL kg}^{-1})</th>
<th>$F_{10}$ (\text{mg kg}^{-1} 	ext{mi}^{-1})</th>
<th>$F_{30}$ (\mu U mL^{-1} n^{-1})</th>
<th>Fractional Rate Constant (\text{min}^{-1})</th>
<th>GE (\text{min}^{-1})</th>
<th>IS (\mu U^{-1} 	ext{min}^{-1} \text{mL} \times 10^{-4})</th>
<th>HGO (\text{mg kg}^{-1} \text{min}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>[17]</td>
<td>Human; 6; BW not reported</td>
<td>N/A</td>
<td>IGVTT (2^{-3}H &amp; 6,6^{-2}H_2)</td>
<td>124</td>
<td>1</td>
<td>-</td>
<td>(0.007)</td>
<td>(0.052)</td>
<td>(0.040)</td>
<td>(0.031)</td>
</tr>
<tr>
<td>[38]</td>
<td>Human; 10; 62 ± 3</td>
<td>IVGTT</td>
<td>U-(^13)C</td>
<td>155 ± 10</td>
<td>139 ± 3.7</td>
<td>1</td>
<td>-</td>
<td>(0.01)</td>
<td>(0.070 \pm 0.008)</td>
<td>(0.080 \pm 0.014)</td>
</tr>
<tr>
<td>[39]</td>
<td>Canine (Mongrel); 8; 35.6 ± 1.3</td>
<td>No insulin</td>
<td>IVGT T</td>
<td>3-(^3)H</td>
<td>157 ± 0.8</td>
<td>2.65 ± 0.78</td>
<td>(0.002)</td>
<td>(0.025)</td>
<td>(0.041)</td>
<td>(0.501)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insulin injected into portal vein</td>
<td>imlIV GT T</td>
<td>158 ± 0</td>
<td>3.5</td>
<td>0</td>
<td>(0.011 \pm 0.001)</td>
<td>(0.111 \pm 0.012)</td>
<td>(0.182 \pm 0.023)</td>
<td>(0.018 \pm 0.002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insulin injected into femoral vein</td>
<td>imlIV GT T</td>
<td>155 ± 0</td>
<td>2.81</td>
<td>0</td>
<td>(0.012 \pm 0.003)</td>
<td>(0.114 \pm 0.020)</td>
<td>(0.211 \pm 0.043)</td>
<td>(0.010 \pm 0.002)</td>
</tr>
<tr>
<td>[40]</td>
<td>Human; 10; 62 ± 3</td>
<td>N/A</td>
<td>imlIV GTT</td>
<td>6,6-(^3)H</td>
<td>191 ± 1</td>
<td>1</td>
<td>-</td>
<td>(0.012)</td>
<td>(0.068 \pm 0.012)</td>
<td>(0.106 \pm 0.024)</td>
</tr>
</tbody>
</table>

GE, glucose effectiveness; IS, insulin sensitivity; HGO, hepatic glucose output; IGVTT, intravenous glucose tolerance test; imlIVGTT, insulin modified glucose tolerance test; Data are presented as mean ± SE
A.5. Other schemes

A.5.1. Models with three glucose compartments

The general scheme for three glucose pools is shown in Figure A.5. Pool 1 is the accessible venous pool encompassing plasma. The external inflow to pool 1 is glucose production (appearance). Pools 2 and 3 represent peripheral compartments in rapid and slow equilibrium, respectively, with the accessible pool. Pool 2 is associated with insulin independent tissues, such as brain, liver, kidneys, and pool 3 with insulin dependent tissues, including muscle and adipose tissue. There is a bidirectional flow between pools 1 and 2, and between pools 1 and 3. Irreversible losses occur from pools 2 and 3 accounting for insulin-independent and insulin-dependent glucose utilization respectively. Isotope is administered by constant infusion or by single dose injection, and then plasma glucose concentration and isotope enrichment/specific activity monitored in plasma. The fundamental equations follow. For total glucose:

\[
\frac{dQ_1}{dt} = V_1 \frac{dG}{dt} = F_{10} + F_{12} + F_{13} - F_{01} - F_{21} - F_{31} \tag{14}
\]

\[
\frac{dQ_2}{dt} = F_{21} - F_{02} - F_{12} \tag{15}
\]

\[
\frac{dQ_3}{dt} = F_{31} - F_{03} - F_{13} \tag{16}
\]

For labelled glucose:

\[
\frac{dq_1}{dt} = V_1 \frac{dG^*}{dt} = \phi + s_2 F_{12} + s_3 F_{13} - s_1 \left( F_{01} + F_{21} + F_{31} \right); \quad q_1(0) = 0 \text{(no priming)}, \\
\text{for constant infusion} \tag{17a}
\]

\[
= s_2 F_{12} + s_3 F_{13} - s_1 \left( F_{01} + F_{21} + F_{31} \right); \quad q_1(0) = D^*, \text{ for single dose injection} \tag{17b}
\]

\[
\frac{dq_2}{dt} = s_2 F_{21} - s_2 \left( F_{02} + F_{12} \right); \quad q_2(0) = 0 \tag{18}
\]

\[
\frac{dq_3}{dt} = s_3 F_{31} - s_3 \left( F_{03} + F_{13} \right); \quad q_3(0) = 0 \tag{19}
\]
If the system is in non-isotopic steady state, the derivatives \(\frac{dQ_1}{dt}, \frac{dQ_2}{dt}, \frac{dQ_3}{dt}\) are zero and, if isotope is administered by constant infusion, \(\frac{dq_1}{dt}, \frac{dq_2}{dt}, \frac{dq_3}{dt}\) become zero and both \(s_2\) and \(s_3\) equilibrate with \(s_1\). Equations (14)-(19) then reduce to a set of 4 simultaneous equations (Equations 18 and 19 become redundant when \(s_1 = s_2 = s_3\)) in 7 unknowns which can be solved uniquely only for \(F_{10}\):

\[
F_{10} = \phi / s_1
\]

\[
F_{02} = F_{21} - F_{12}
\]

\[
F_{03} = F_{31} - F_{13}
\]

\[
F_{01} = F_{10} - F_{02} - F_{03}
\]

For single dose injection, assuming mass action kinetics, the differential equations for labelled glucose become:

\[
\frac{dq_1}{dt} = k_{12}q_2 + k_{13}q_3 - (k_{01} + k_{21} + k_{31})q_1; \quad q_1(0) = D^*
\]

\[
\frac{dq_2}{dt} = k_{21}q_1 - (k_{02} + k_{12})q_2; \quad q_2(0) = 0
\]

\[
\frac{dq_3}{dt} = k_{31}q_1 - (k_{03} + k_{13})q_3; \quad q_3(0) = 0
\]

Integration of these three equations can be accomplished through their Laplace transformation:

\[
q_1 / D^* = H_1 e^{-c^*t} + H_2 e^{-c^*t} + H_3 e^{-c^*t}
\]

For further details, see Shipley & Clark [13], Appendix II.

This model was originally proposed by Cobelli et al. [18] to analyze EHC data (Table A.6). They constrained insulin-independent glucose disposal to be three times insulin-independent disposal in the basal state in order to facilitate parameter estimation.
Figure A.5. Insulin-independent three-compartment model for describing glucose kinetics.

First compartment is the accessible space (plasma volume). Arrowed solid lines represent flows, hollow arrow represents glucose application (infusion or dose), and broken line sampling.
### Table A.6. Example literature values of the three pool model for describing glucose kinetics

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population</th>
<th>Experimental Groups/Conditions</th>
<th>Isotope</th>
<th>$V_1$</th>
<th>Fractional Rate Constant (min$^{-1}$)</th>
<th>Compartment Size (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[18]</td>
<td>Human; 6; BW not reported</td>
<td>Clamped isotope euglycemia</td>
<td>$^{3-3H}$</td>
<td>4.13</td>
<td>1.09 ± 0.46 ± 0.019 ± 0.006 ± 0.12 ± 0.047 ± 40 ± 8 91 ± 30 96 ± 24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>EHC isotope euglycemia</td>
<td>$^{3-3H}$</td>
<td>4.56 ± 1.03 ± 0.61 ± 0.027 ± 0.035 ± 0.29 ± 0.026 ± 40 ± 10 61 ± 12 190 ± 74</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.06</td>
<td>0.58</td>
<td>0.3</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*EHC, euglycemic hyperinsulinemic clamp; Data are presented as mean ± SE*
A.5.2. Non-compartmental schemes (dispersal models)

In this type of model, the glucose system is considered a region governed by diffusion and convection. The fundamental equations are statements of the general equation for diffusion, convection and chemical reaction [19]. To illustrate the approach, consider glucose occupying a region bounded by a regular cylinder of radius \( r \) and indeterminate length (Figure A.6). An impulse dose of labeled glucose is applied at time zero at the point \( x = 0 \) \((-\infty \leq x \leq \infty\)). The general diffusion-convection-reaction equation simplifies to:

\[
\frac{\partial G^\ast(x,t)}{\partial t} = \Omega \frac{\partial^2 G^\ast}{\partial x^2} - v \frac{\partial G^\ast}{\partial x} - k G^\ast; \quad G^\ast(0,0) = \frac{D^\ast}{\pi r^2} \tag{20}
\]

where \( \Omega \) [cm\(^2\)/h] is the diffusion coefficient, \( v \) [cm/h] the velocity of convection (i.e. blood flow) which is assumed to be in the direction of increasing \( x \), and \( k \) [per h] is the relative rate of glucose disappearance (uptake). Instantaneous disappearance of label is:

\[
\int_{-\infty}^{\infty} k G^\ast(x,t) \times \pi r^2 \, dx = k \pi r^2 \int_{-\infty}^{\infty} G^\ast(x,t) \, dx
\]

Labeled glucose remaining at time \( t \) (as a fraction of the impulse dose) is therefore:

\[
\frac{q(t)}{D^\ast} = \frac{G^\ast V^{-1}}{D^\ast} = 1 - \frac{k \pi r^2}{D^\ast} \int_{t}^{\infty} \int_{x=0}^{\infty} G^\ast(x,t-\tau) \, dx \, d\tau \tag{21}
\]

Solutions to the model are obtained by solving Equations (20) and (21) numerically. Equation (21) is a convolution integral. This model is a very simple (arguably simplistic) illustration of a dispersal model of the glucose system and is meant to be instructive rather than realistic. Though dispersal models assume less structural knowledge, their equations are clearly more complex and their solutions require advanced numerical methods. A more physiologically-based example of a dispersal model for glucose kinetics is given by Radziuk et al. [14], who consider two regions. A first region, in which cold and labeled glucose appear and are sampled,
is well-mixed and exchanges with a second region in which diffusion and convection are allowed. Disappearance of glucose takes place in both regions. The equations of the model are more complex than the illustrative example described above. McGuinness and Mari [20] also describe a dispersal model with two regions but use a markedly different kinetic analysis. Their model, designed for EHC application, is comprised of heart-lung and periphery blocks. The impulse responses of the two blocks are modeled as multi-exponential functions then converted to linear differential equations. The differential equations of the heart-lung and periphery blocks are subsequently combined to obtain the differential equations of the circulatory model.

Figure A.6. Simple dispersal model for describing glucose kinetics. Glucose occupies a region represented by a regular cylinder of indeterminate length and radius $r$. Arrow $v$ represents convective flow through the cylinder, unlabelled arrows of varying thickness represent isotope disappearance, hollow arrow isotope application (impulse dose), and broken line sampling.
A.6. Discussion

The maintenance of blood glucose homeostasis is complex and involves several key tissues. Most of these tissues are not easily (non-invasively) accessible, making direct measurement of the physiological parameters involved in glucose metabolism difficult. As such, several mathematical models have been developed to describe in vivo whole body glucose metabolism. The application of single pool models dominate the literature, while examples of two pool models are limited and three pool models are rare.

The wide spread use of single pool models is likely due to their simplicity both technically and mathematically. Technically, they require the insertion of a venous catheter for isotope, glucose and/or insulin infusion and repeated blood sampling from a single site, often a vein (i.e. jugular or cephalic), and glucose and/or insulin is measured in the plasma or serum. Such methodology is relatively straightforward, inexpensive and non-invasive in most animals and humans. Mathematically, the underlying assumption with one pool models is that blood glucose rapidly and uniformly distributes in all physiological pools (i.e. plasma, interstitial and intracellular). However, most agree that glucose cannot be adequately explained so simply [4]. Plasma glucose exchanges with the interstitial fluid pool where it is then taken up by the tissues through facilitated diffusion. In humans, plasma accounts for 4.5 % of body weight and interstitial fluid ~16 % of body weight, meaning changes in whole body plasma glucose appearance will not be immediately reflected by changes in the entire interstitial fluid pool [4]. Furthermore, the rate of diffusion from the interstitial pool to the intracellular pool differs between tissues and exchange between these pools is bidirectional in gluconeogenic tissues like the liver. As one pool models group all these factors together, the rate of plasma glucose turnover is assumed to be reflective of all glucose pools and due to the combined abilities of
glucose and insulin to stimulate glucose disposal and suppress glucose production (GE and IS). Such assumptions may be valid if the system is in steady state or a large perturbation in plasma glucose is made.

As the one pool insulin independent model is unable to separate the contributions of glucose and insulin to plasma glucose Rd, it is most often applied to steady state conditions. Steady state, or “clamp”, conditions may take several hours of continuous isotope infusion to achieve. Such experiments often aim to determine acute (i.e. AICAR infusion) or chronic (i.e. after several weeks of high fat feeding) changes in plasma glucose turnover from basal, or control, conditions (Table 2).

Large perturbations to the plasma glucose pool, as made in the IVGTT, presume a rapid exchange (within minutes) among glucose pools. In these experiments glucose Rd is assumed to be monoexponential and the first few data time points are often excluded from model calculations. Addition of the remote insulin pool to the model allows for indirect estimates of GE and insulin mediated glucose disposal/production (IS). It was developed in healthy canines, however, its application to pancreatectomized dogs revealed that it could not accurately predict GE or IS in this population. This limitation is due to the model assumption that insulin secretion in response to glucose infusion rises above a certain basal level. Therefore, if insulin secretion is impaired, as in the case of diabetes, the measurement of GE may be overestimated and IS underestimated. In order to rectify this problem, the addition of an exogenous bolus of insulin or tolbutamide was added to the IVGTT and was shown to improve estimates. The application of the imIVGTT is widespread and is often done without the use of isotope tracers (likely due to the cost of isotopes). Without the use of isotopes, one cannot distinguish between HGO and hepatic glucose uptake. Instead it describes net hepatic glucose flux. As such, the relative contribution
of HGO to IS cannot be determined and is known to vary depending on the degree of insulin resistance [21]. However, even when isotopes are used with the imIVGTT, this model can produce unrealistic HGO values [17].

The two pool insulin dependent model provides more meaningful estimates of HGO [17]. Unlike the single pool version, it takes into account the fact that glucose is not uniformly distributed, by separating glucose into fast and slow exchanging pools. The fast or accessible plasma pool describes tissues such as the brain, which requires a constant rate of glucose uptake independent of insulin. The other component of the fast pool is the ability of glucose to stimulate its own uptake (diffusion) independent of insulin (i.e. liver). Insulin dependent glucose uptake is considered to take place in the slow interstitial pool, the key tissues being muscle and adipose. This model does rely on the assumption that in the basal insulin independent glucose disposal is three times insulin dependent glucose disposal Rd at time zero is fixed to at 1 mg/kg/min. These values were attained experimentally by Cobelli et al. [18] in six human subjects. Despite these assumptions, model estimates correlate well with direct measures of IS and HGO. The necessity for isotope tracers is likely the reason why this model has not been universally adopted.

Two and three pool insulin independent models of glucose turnover were developed to describe glucose turnover in non-steady state conditions. Application of these models requires constant infusion of isotope and frequent blood sampling over several hours. Both models require previous knowledge of parameters, either measured in a separate experiment or prior to initiation of experimental conditions. The three pool model in particular is computationally difficult and has received limited attention.
Dispersal models, except for Radzuik et al. [14], have received virtually no consideration in the study of whole body glucose metabolism, despite their theoretical appeal. Such models have the advantages of requiring less structural knowledge and representing key processes such as convective flow, diffusion and uptake explicitly. Their lack of adoption could be due to biologists finding them computationally challenging and mathematically obscure.

In conclusion, the use of isotope tracer methodology and mathematical modeling has been used extensively to determine indirect estimates of various aspects of in vivo glucose metabolism through relatively non-invasive means. Overall, minimal model schemes dominate the literature, likely due to their technical and mathematical ease.
A.7. References


APPENDIX B

Models for the study of whole-body insulin kinetics: a mathematical synthesis

B.1. Abstract

Insulin is an anabolic hormone that plays a central role in glucose homeostasis. Synthesis, secretion and appearance of insulin in portal and peripheral circulation are complex and tightly regulated. As many of the tissues involved are not directly accessible, kinetics models have been developed to describe insulin kinetics. This paper presents a mathematical synthesis of models for the study of insulin kinetics and is meant as a supplemental to our recent review of glucose kinetics models, some of which encompass insulin sub-models. Models are described in steady and non-steady state and are grouped into compartmental and non-compartmental schemes.

7 McKnight et al. CABI Reviews, In Press
B.2. Introduction

Insulin is a critical regulator of macronutrient metabolism and growth. Of particular importance is the role of insulin in the maintenance of glucose homeostasis. Elevated blood glucose increases the transcription, translation, synthesis and secretion of insulin from the pancreatic beta cells, whereas, low circulating glucose inhibits these processes. Insulin acts in a tissue specific manner to stimulate glucose uptake, utilization and storage (muscle, adipose and liver) and inhibit gluconeogenesis (liver and kidney).

The biosynthesis and secretion of insulin from the pancreatic beta cells is complex and tightly regulated. The translation of insulin mRNA produces preproinsulin. In the endoplasmic reticulum (ER) the ‘pre’ portion is enzymatically cleaved and the resultant proinsulin is transported to the golgi apparatus. Proinsulin is packaged in granules where it is further processed to insulin and C-peptide (in equimolar concentrations). C-peptide can thus serve as an index of insulin biosynthesis [1]. Insulin granules divide into two types, readily releasable and reserve types. The readily releasable type represents a small pool (5%) that is immediately released (first phase insulin secretion). The majority of insulin (95%) lies in the reserve pool, where it must be mobilized prior to its pulsatile release (second phase insulin secretion) [1] resulting in the commonly seen biphasic serum insulin response to meal feeding. Glucose stimulates insulin release through its own metabolism. Glucose enters the pancreatic beta cells through the GLUT-2 receptor and undergoes glycolysis. Glycolysis increases the ATP: ADP ratio which ultimately results in changes in ion ratios and depolarization of the beta cell membrane causing insulin release.

Insulin released from the pancreatic beta cells travels the portal vein to the liver where approximately 50% of it is removed [1]. In liver, insulin negatively regulates the transcription of
several gluconeogenic enzymes. Peripherally, the kidney is responsible for roughly half of the insulin clearance, in addition to C-peptide clearance. Remaining insulin acts on skeletal muscle and adipose. Specifically, insulin increases glucose uptake in skeletal muscle and adipose tissue by stimulating GLUT-4 translocation to the plasma membrane.

Dysregulation(s) of insulin synthesis, secretion or action are often seen in diabetes. Type 1 diabetes is an auto-immune disease where the beta cells are destroyed. These patients require exogenous insulin, administered by subcutaneous injection, to maintain glucose homeostasis. In type 2 diabetes, insulin secretion and action are compromised. While the causes are unclear, lifestyle factors including poor diet and/or over nutrition and inactivity, appear to contribute to the pathology [2]. Impaired insulin action on target tissues (insulin resistance) is the underlying feature of several other metabolic diseases including obesity, hypertension, and hyperlipidemia [2]. While exogenous insulin therapy is not always necessary for the treatment of insulin resistance, understanding insulin action, specifically secretion and metabolism is of great importance.

Measurement of in vivo insulin metabolism presents a challenge, given the aforementioned complexity of its release and systemic effects. Nevertheless, kinetic studies have been used to describe insulin metabolism. Kinetic studies require an exogenous input of insulin, generally a radioactive isotope of iodine or unlabelled insulin. This paper presents a mathematical synthesis of models for the study of insulin kinetics per se at the whole-body level and is meant as a supplemental to our recent review [3] of glucose kinetics models, some of which encompass insulin sub-models. Hence it is similar in structure to [3], but involves no duplication. The insulin system is considered in both the steady and non-steady states, and the models are examined by grouping them into schemes consisting of one, two and three insulin
compartments. Embedded compartmental and non-compartmental schemes are also considered.

The paper starts with the simplest (minimal) model and adds complexity progressively. Example values, predominantly from the canine and human literature, are presented where available. Mathematical notation is defined in Table B.1. Models of the insulin system have recently been reviewed by Cobelli et al. [4], but as part of a biomedical engineering review of diabetes control not as a mathematical synthesis of models specific to resolving insulin kinetic data.

**Table B.1. Mathematical notation**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( D )</td>
<td>Dose of labelled insulin injected into the primary pool [pmol]</td>
</tr>
<tr>
<td>( F_{ij} )</td>
<td>Flow of total (labelled and unlabelled) insulin to pool ( i ) from pool ( j ); ( F_{i0} ) represents external flow of insulin into pool ( i ) and ( F_{0i} ) flow of insulin from pool ( i ) out of the system [pmol/min]</td>
</tr>
<tr>
<td>( \varphi )</td>
<td>Constant rate of labelled insulin infusion into the primary pool [pmol/min]</td>
</tr>
<tr>
<td>( I )</td>
<td>Total insulin concentration in the primary pool [pmol/mL]</td>
</tr>
<tr>
<td>( k_{ij} )</td>
<td>Relative rate constant for flow from pool ( i ) from pool ( j ) [per min]</td>
</tr>
<tr>
<td>( X_i )</td>
<td>Quantity of total insulin in pool ( i ) [pmol]</td>
</tr>
<tr>
<td>( x_i )</td>
<td>Quantity of labelled insulin in pool ( i ) [pmol]</td>
</tr>
<tr>
<td>( s_i )</td>
<td>Specific activity (or enrichment) of pool ( i ) [pmol labelled insulin/pmol total insulin]</td>
</tr>
<tr>
<td>( t )</td>
<td>Time since isotope first administered [min]</td>
</tr>
<tr>
<td>( V_i )</td>
<td>Volume of pool ( i ); ( V_1 ) represents primary pool volume [mL/kg]</td>
</tr>
</tbody>
</table>
**B.3. Models with one insulin compartment**

The minimal scheme is shown in Figure B.1. It contains one pool (an accessible pool e.g., plasma), one inflow (rate of insulin appearance), and one outflow (rate of insulin disappearance). Isotope is administered by constant infusion or by single dose injection, and insulin concentration and specific activity (or enrichment) are monitored following isotope administration. The fundamental equations follow. For total (labeled plus unlabelled) insulin:

$$\frac{dX_1}{dt} = F_{10} - F_{01} \quad (1)$$

For labelled insulin:

$$\frac{dx}{dt} = \phi - s_1 F_{01} ; \quad x(0) = 0, \text{for non-primed constant infusion}$$

$$= -s_1 F_{01} ; \quad x(0) = D, \text{for single dose injection} \quad (2a, b)$$

Further:

$$s_1 = \frac{x_1}{X_1} ; \quad I = \frac{X_1}{V_1} \quad (3)$$

If multiple exits should exist, the rate of disappearance $F_{01}$ is the sum of all the exits; likewise for $F_{10}$ and multiple entrances.

If the plasma insulin pool is in steady state, the derivative $dX_1/dt$ becomes zero, and the constant $X_1$ can be calculated from Equation (3) as $IV_1$. Likewise $dx_1/dt$ becomes zero for constant infusion. Equations (1) and (2a) then reduce to two simultaneous equations in two unknowns ($F_{01}$ and $F_{10}$), which can readily be solved to give:

$$F_{10} = F_{01} = \frac{\phi}{s_1} \quad (4)$$

Under single dose injection, assuming outflow obeys mass action kinetics (i.e., $F_{01} = k_{01}X_1$), Equation (2b) becomes:
\[
\frac{dx_i}{dt} = -s_i k_{01} X_i; \quad x_i(0) = D
\]  
(5)

Dividing both sides by constant \(X_1\) yields:

\[
\frac{ds_1}{dt} = -k_{01} s_1; \quad s_1(0) = \frac{D}{X_1}
\]  
(6)

Integrating \(ds_1/dt\):

\[
s_1 = s_1(0)e^{-k_{01}t}
\]  
(7)

The rate constant \(k_{01}\) can be estimated by regression analysis of the isotope dilution curve (Equation 7), allowing determination of the rate of insulin disappearance and hence the rate of appearance (post hepatic insulin secretion rate).

The model can be solved for the insulin pool in non-steady state by combining the fundamental equations using \(ds_1/dt\) as follows:

\[
\frac{ds_1}{dt} = \frac{d(x_i X_i^{-1})}{dt} = X_i^{-1} \frac{dx_i}{dt} - x_i X_i^{-2} \frac{dX_i}{dt}
\]  
(8)

i.e.,

\[
X_i \frac{ds_1}{dt} = \frac{dx_i}{dt} - s_1 \frac{dX_i}{dt}
\]  
(9)

Substituting for \(dx_1/dt\) and \(dX_1/dt\) using Equations (1) and (2a) for constant infusion:

\[
X_i \frac{ds_1}{dt} = \phi - s_1 F_{10}
\]  
(10)

Re-arranging:

\[
F_{10} = \frac{\left(\phi - X_i \frac{ds_1}{dt}\right)}{s_1}
\]  
(11)

i.e.,
rate of insulin appearance = \frac{\left(\phi - I(t)V_1 \frac{ds_1}{dt}\right)}{s_1}; \; s_1(0) = 0 \quad (12)

The derivative $\frac{ds_1}{dt}$, which varies over time, can be estimated numerically from the isotope dilution curve.

This minimal model of insulin kinetics (Figure B.1) was first applied by Transberg and Dencker [5] to investigate plasma insulin disappearance in man. Single doses of $^{125}$I-insulin were administered intra-portal and peripherally, and plasma clearance rate and first-pass hepatic uptake estimated. The model was also one of eight evaluated by Toffolo et al. [6] to interpret intra-venous glucose tolerance tests (IVTT) performed on dogs. Plasma samples were assayed for glucose and insulin, and the insulin data used (in conjunction with the models) to determine post hepatic delivery and plasma clearance rates. Polansky et al. [7] used the model to calculate the systemic delivery rate of insulin in normal man from peripheral insulin concentration data. Endogenous insulin secretion was suppressed by somatostatin, and porcine regular insulin infused exogenously via a periphery vein. The cited models were solved numerically, generally using non-linear regression techniques.
Figure B.1. Minimal model for describing whole-body insulin kinetics. Arrowed solid lines represent flows, hollow arrow represents insulin application (infusion or dose), and broken line sampling.

B.4. Models with two insulin compartments

The general scheme is shown in Figure B.2. It contains two insulin pools; the first encompassing an accessible pool such as plasma. The external inflow to pool 1 is insulin appearance. There is a flow out of the system from each pool, and a bi-directional flow between the two pools. Isotope is administered by constant infusion or by single dose injection, and then plasma insulin concentration and isotope specific activity (or enrichment) monitored. The fundamental equations follow. For total insulin:

\[
\frac{dX_1}{dt} = F_{10} + F_{12} - F_{01} - F_{21} \tag{13}
\]

\[
\frac{dX_2}{dt} = F_{21} - F_{02} - F_{12} \tag{14}
\]

For labelled insulin:
\[
\frac{dx_1}{dt} = \phi + s_2 F_{12} - s_1 \left( F_{01} + F_{21} \right) x_1(0) = 0, \text{ for non-primed constant infusion} \\
= s_2 F_{12} - s_1 \left( F_{01} + F_{21} \right) x_1(0) = D, \text{ for single dose injection} \\
\]

\[
\frac{dx_2}{dt} = s_1 F_{21} - s_2 \left( F_{02} + F_{12} \right) x_2(0) = 0 \\
\]

If the system is in steady state with respect to total insulin, the derivatives \( \frac{dX_1}{dt} \) and \( \frac{dX_2}{dt} \) are zero and, if isotope is administered by constant infusion, \( \frac{dx_1}{dt} \) and \( \frac{dx_2}{dt} \) become zero and \( s_2 \) equilibrates with \( s_1 \). Equations (13)-(16) then reduce to a set of 3 simultaneous equations (Equation 16 becomes redundant when \( s_1 \) equals \( s_2 \)) in 5 unknowns which can be solved uniquely only for \( F_{10} \):

\[
F_{10} = \frac{\phi}{s_1} \\
F_{02} = F_{21} - F_{12} \\
F_{01} = F_{10} - F_{02}
\]

For single dose injection, assuming mass action kinetics, the differential equations for labelled insulin become:

\[
\frac{dx_1}{dt} = k_{12} x_2 - \left( k_{01} + k_{21} \right) x_1; x_1(0) = D \\
\frac{dx_2}{dt} = k_{21} x_1 - \left( k_{02} + k_{12} \right) x_2; x_2(0) = 0
\]

Integration of these two equations can be accomplished through their Laplace transformation [8, 9]:

\[
x_1/D = H_1 e^{-ct} + H_2 e^{-ct} \\
x_2/D = K_1 e^{-ct} + K_2 e^{-ct}
\]

\[
k_{11} = k_{01} + k_{21}
\]
The equation for \( x_2/D \) is effectively redundant as pool 2 is likely inaccessible for measurement.

The two-compartment scheme for insulin kinetics (Figure B.2) was first used by Frost et al. [10] to study diabetes mellitus in man. The metabolism of unlabelled mono-component human insulin was investigated by a priming-dose constant-infusion technique. Disappearance was assumed to occur from the plasma pool alone (i.e., \( F_{02} = 0 \)), and its associated fractional rate parameter \( k_{01} \) (per h) described by a Michaelis-Menten-type equation. Fractional rate constants \( k_{21}, k_{12} \) (per h) were assigned to bi-directional exchange between the two pools. The two-pool model was solved numerically (Table B.2). This saturable mechanism for plasma insulin clearance was also investigated by Thorsteinssen et al. [11] in normal men. Similar assumptions to those of Frost et al. [10] were made in the two-compartment model used by Morishima et al. [12] to assess the accuracy with which insulin appearance rates in the peripheral circulation can be measured in the non-steady state. Conscious dogs were infused simultaneously with porcine insulin and somatostatin at known variable rates, and tritiated insulin (B1-[^3H-Phe]-insulin) infused concurrently at a constant rate. Constant fractional transfer rates \( k_{21}, k_{12} \) and a time-dependent fractional clearance rate \( k_{01}(t) \) were assumed in the model. Tranberg and Dencker [5] compared the scheme proposed by Frost et al. [10], but with \( k_{01} \) assumed constant, with the minimal model (Figure B.1) in investigating plasma insulin disappearance in humans (Table B.2). Additional investigations using this two-compartment scheme were undertaken by Tranberg [13] and Tranberg and Thorell [14]. Toffolo et al. [6] and Polansky et al. [7] also
compared this scheme (constant $k_{01}$) with the minimal model using dogs and human subjects respectively. Lotz et al. [15] proposed a model-based method for insulin sensitivity testing. Two insulin pools, plasma and interstitial fluid, with bidirectional exchange are represented. Exogenous and endogenous inputs to pool 1 are specified. Two irreversible losses from pool 1 (liver clearance and kidney clearance) and one from pool 2 (cellular clearance) are assumed to occur, with liver clearance following Michaelis-Menten-type kinetics. Parameter values are prescribed and the differential equations are integrated numerically.

Only post-hepatic insulin release, not pancreatic secretion, can be determined from plasma insulin concentration data. The problem of determining pancreatic insulin secretion can be overcome by measuring plasma concentration of connecting peptide (C-peptide). C-peptide is secreted from the pancreas in equimolar concentration with insulin but extracted only to a negligible extent by the liver, whilst approximately half of the insulin secreted is degraded by the liver. Eaton et al. [16] used a two-compartment model of C-peptide removal from plasma (Figure 2 with fractional rates $k_{01}$ constant and $k_{02}$ zero) to evaluate pre-hepatic production of insulin in normal man (Table B.2). Pools 1 and 2 were nominated intra- and inter-vascular C-peptide, respectively, and the model solved using the SAAM computer program, which is a resource facility for kinetic analysis of biomedical problems and widely used in tracer and pharmacokinetic studies [17]. Polansky et al. [18] used the same model to examine the accuracy of plasma C-peptide as a marker of insulin secretion (Table B.2). The peripheral kinetics of biosynthetic human C-peptide (BHCP) were studied in normal volunteers and insulin-dependent subjects. Each subject received intra-venous bolus injections of BHCP as well as constant and variable infusions. The model was solved numerically. If measurements of both insulin and C-
peptide concentrations in plasma are taken, the model can be used to calculate percentage hepatic extraction, \( E \), as follows:

\[
E \, (\%) = \frac{F_{01}^{(\text{pep})} - F_{01}^{(\text{ins})}}{F_{01}^{(\text{pep})}} \times 100
\]  

(29)

where \( F_{01}^{(\text{pep})} \) and \( F_{01}^{(\text{ins})} \) are the values of \( F_{01} \) determined by the model using the C-peptide and insulin plasma concentration data, respectively.
Figure B.2. Two-compartment model for describing whole-body insulin kinetics. First compartment contains the vascular space. Arrowed solid lines represent flows, hollow arrow represents insulin application (infusion or dose), and broken line sampling.
Table B.2. Compartmental models to describe insulin kinetics.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population</th>
<th>Experimental Conditions</th>
<th>Volume Distributions (mL/kg)</th>
<th>Fractional Rate Constants (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>( V_1 )</td>
<td>( V_2 )</td>
</tr>
<tr>
<td>Two-pool Models</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Healthy adults (n = 6)</td>
<td>Primed constant insulin infusion</td>
<td>74.7 ± 7.48</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>&amp; Diabetic adults (n = 7)</td>
<td></td>
<td>146 ± 19.4</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>5 Surgical patients (non-diabetic) (n = 62)</td>
<td></td>
<td>60.7 (85.1)</td>
<td>83.7</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Peripheral insulin under clamped euglycemia</td>
<td>78.9 (84.5)</td>
<td>384</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Portal insulin under clamped hyperglycemia</td>
<td>92.0 (112)</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Portal insulin under clamped hyperglycemia</td>
<td>77.7 (86.6)</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Peripheral and portal under euglycemia</td>
<td>58.8 (70.0)</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>&amp; Diabetic adults (n = 8)</td>
<td>C-peptide injection</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>16 Healthy adults (n = 10)</td>
<td>C-peptide injection</td>
<td>65.5 ± 2.5</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>&amp; Diabetic adults (n = 7)</td>
<td></td>
<td>65.5 ± 2.0</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>Three-pool Models</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 Healthy adults (n = 6)</td>
<td>181-I-Insulin bolus injection</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&amp; Diabetic adults (n = 8)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&amp; Non-diabetic adults with renal failure (n = 6)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19 Healthy adults (n = 16)</td>
<td>Pulsatile infusion of unlabelled insulin</td>
<td>45</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>Five-pool Model</td>
<td>Hyperinsulinemic euglycemic clamp</td>
<td>45.05</td>
<td>150</td>
<td>4.95</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------------------------</td>
<td>-------</td>
<td>-----</td>
<td>------</td>
</tr>
<tr>
<td>Adult diabetics (n = 10)</td>
<td>45.05</td>
<td>150</td>
<td>4.95</td>
<td>0.047 ± 0.016</td>
</tr>
<tr>
<td>Adult diabetics after placebo treatment (n = 10)</td>
<td>45.05</td>
<td>150</td>
<td>4.95</td>
<td>0.058 ± 0.022</td>
</tr>
<tr>
<td>Adult diabetics (n = 10)</td>
<td>45.05</td>
<td>150</td>
<td>4.95</td>
<td>0.049 ± 0.025</td>
</tr>
<tr>
<td>Adult diabetics after chloroquine treatment (n = 10)</td>
<td>45.05</td>
<td>150</td>
<td>4.95</td>
<td>0.047 ± 0.016</td>
</tr>
</tbody>
</table>

*#(value) were determined using a one pool model*

*Data are presented as mean ± SE*

*# values calculated using a one pool model under the same experimental conditions*
B.5. Models with three (or more) insulin compartments

The general scheme for three insulin pools is shown in Figure B.3. Pool 1 is the accessible pool (e.g., plasma). The external inflow to pool 1 is insulin appearance. Pools 2 and 3 represent compartments (e.g., extra-vascular pools) in rapid and slow equilibrium, respectively, with the accessible pool. There is a bi-directional flow between pools 1 and 2, and between pools 1 and 3. Insulin disappearance is assumed to occur from all three pools. Isotope is administered by constant infusion or by single dose injection, and then insulin concentration and isotope enrichment/specific activity monitored in the accessible pool. The fundamental equations follow. For total insulin:

\[
\frac{dX_1}{dt} = F_{10} + F_{12} + F_{13} - F_{01} - F_{21} - F_{31} \quad (30)
\]

\[
\frac{dX_2}{dt} = F_{21} - F_{02} - F_{12} \quad (31)
\]

\[
\frac{dX_3}{dt} = F_{31} - F_{03} - F_{13} \quad (32)
\]

For labelled insulin:

\[
\frac{dx_1}{dt} = \phi + s_2 F_{12} + s_3 F_{13} - s_1 \left( F_{01} + F_{21} + F_{31} \right); \quad x_1(0) = 0, \text{ for non-primed constant infusion} \quad (33a, b)
\]

\[
= s_2 F_{12} + s_3 F_{13} - s_1 \left( F_{01} + F_{21} + F_{31} \right); \quad x_1(0) = D, \text{ for single dose injection}
\]

\[
\frac{dx_2}{dt} = s_1 F_{21} - s_2 \left( F_{02} + F_{12} \right); \quad x_2(0) = 0 \quad (34)
\]

\[
\frac{dx_3}{dt} = s_1 F_{31} - s_3 \left( F_{03} + F_{13} \right); \quad x_3(0) = 0 \quad (35)
\]

If the system is in steady state with respect to total insulin, the derivatives \( \frac{dX_1}{dt}, \frac{dX_2}{dt}, \frac{dX_3}{dt} \) are zero and, if isotope is administered by constant infusion, \( \frac{dx_1}{dt}, \frac{dx_2}{dt}, \frac{dx_3}{dt} \) become
zero and both \( s_2 \) and \( s_3 \) equilibrate with \( s_1 \). Equations (30)-(35) then reduce to a set of 4 simultaneous equations (Equations 34 and 35 become redundant when \( s_1 = s_2 = s_3 \)) in 7 unknowns which can be solved uniquely only for \( F_{10} \):

\[
F_{10} = \frac{\phi}{s_1} \quad (36)
\]

\[
F_{02} = F_{21} - F_{12} \quad (37)
\]

\[
F_{03} = F_{31} - F_{13} \quad (38)
\]

\[
F_{01} = F_{10} - F_{02} - F_{03} \quad (39)
\]

For single dose injection, assuming mass action kinetics, the differential equations for labelled insulin become:

\[
\frac{dx_1}{dt} = k_{11}x_1 + k_{13}x_3 - (k_{01} + k_{21} + k_{31})x_3; \quad x_1(0) = D \quad (40)
\]

\[
\frac{dx_2}{dt} = k_{21}x_1 - (k_{02} + k_{12})x_2; \quad x_2(0) = 0 \quad (41)
\]

\[
\frac{dx_3}{dt} = k_{31}x_1 - (k_{03} + k_{13})x_3; \quad x_3(0) = 0 \quad (42)
\]

Integration of these three equations can be accomplished through their Laplace transformation, giving:

\[
\frac{x_1}{D} = H_1e^{-\epsilon\theta} + H_2e^{-\gamma\theta} + H_3e^{-\delta\theta} \quad (43)
\]

For further details, see [8], Appendix II.

The three-compartment model (Figure B.3 with \( F_{02} = F_{03} = 0 \)) was used by Sherwin et al. [19] to study insulin kinetics in man. Subjects were healthy male volunteers aged 18-45 yr, and infusions of porcine insulin were administered by either pulse injection or a prime plus continuous infusion. Application was via the antecubital vein and measurement via the brachial
artery. A second three-compartment scheme was also considered. In this alternative scheme, pool 2 in Figure 3 was nominated as the liver, entry into the system occurred via pool 2 alone, and exit was via both pools 1 and 2 (i.e., only $F_{03} = 0$). First-order kinetics were assumed for both schemes and the models were solved numerically using the SAAM software. These two models, and the same experimental protocol, were also used in an investigation by McGuire et al. [20] of insulin kinetics in diabetic, obese and aged (46 – 78) men. A different three-compartment scheme was adopted in a study by Silvers et al. [21] in which $^{131}$I-insulin was administered by rapid intra-venous injection to normal subjects, to patients with maturity-onset diabetes and normal renal function, and to non-diabetic patients with renal failure. The pools in the scheme were nominated: 1) plasma; 2) interstitial fluid; and 3) site of utilization and degradation. Insulin appearance was via pool 1 and disappearance via pool 3. Pools 1 and 2 and pools 2 and 3 were completely interchanging. A simplified scheme in which pools 1 and 2 were merged was also considered. Model solutions were obtained assuming first-order (i.e., simple linear) kinetics and using SAAM. A three-pool scheme was also proposed by Chase et al. [22] to analyze dynamic insulin sensitivity tests (DIST) on 17 human subjects, DIST being a low-dose insulin-modified IVGTT. The pools in their model were nominated: 1) plasma; 2) hepatic, and 3) interstitium. Insulin appearance was via pool 2 (hepatic) and disappearance via pools 1 and 2. Pools 1 and 2 and pools 1 and 3 were completely interchanging.

Schemes with more than three compartments have also been proposed. Sherwin et al. [19] explored a four-pool representation as part of their investigation. A fourth pool, representing hepatic insulin and fully interchanging with plasma (i.e., pool 1), was added to the scheme depicted in Figure 3. Endogenous entry into the scheme was confined to pool 4 and exit was permitted from both pools 1 and 4. Hovorka et al. [23] used a five-compartment model of
insulin kinetics to investigate the action of chloroquine on carbohydrate metabolism in patients with non-insulin-dependent diabetes mellitus. The five insulin pools represented were: 1) plasma; 2) free interstitial; 3) free hepatic; 4) receptor-bound interstitial; and 5) receptor-bound hepatic. Exogenous infusion was into pool 1, endogenous secretion into pool 3, and the routes of degradation were from pools 1, 4 and 5. The model was used in conjunction with SAAM to resolve hyperinsulinemic euglycemic clamp data (Table 2). A total of 15 compartments were represented in the final model generated in SAAM by Berman et al. [24] to study insulin binding to receptors and degradation in rabbits. Tracer amounts of high affinity (pork) and low affinity (guinea pig) insulin, one $^{125}$I- and the other $^{131}$I-labelled, were injected simultaneously into rabbits either in the basal state or after a large bolus of unlabelled pork insulin, and the kinetic curves generated and subsequently analyzed numerically.
**Figure B.3.** Three-compartment model for describing whole-body insulin kinetics. First compartment is the accessible space (plasma volume). Arrowed solid lines represent flows, hollow arrow represents insulin application (infusion or dose), and broken line sampling.

**B.6. Models with embedded compartments**

Consider the representation shown in Figure B.4 in which a single insulin pool (pool 1) is embedded in a system of multiple pools. Pool 1 is an accessible pool (e.g., plasma), the system of multiple insulin pools is undefined, and a single dose \( D \) of tracer is administered to pool 1 at time zero. The fundamental equations for pool 1 follow. For total insulin:

\[
\frac{dX_1}{dt} = F_{10} + F_{1R} - F_{R1} - F_{R1} \tag{44}
\]

For labelled insulin:

\[
\frac{dx_1}{dt} = -s_1(F_{01} + F_{R1}) + u(t); \quad x_1(0) = D, \quad u_1(0) = 0 \tag{45}
\]

where \( u_1 \) is the rate of entry into pool 1 from recycling of labelled insulin at time \( t \).

If pool 1 (i.e., total insulin) is in steady state, Equation (44) is zero and \( X_1 \) is constant (\( M \) say). Equation (45) then becomes:

\[
M \frac{ds_1}{dt} = -s_1(F_{01} + F_{R1}) + u(t); \quad s_1(0) = \frac{D}{M}, \quad u_1(0) = 0 \tag{46}
\]

Thus:

\[
M = \frac{D}{s_1(0)} \tag{47}
\]

At time zero, Equation (46) yields:

\[
\left( \frac{ds_1}{dt} \right)_{t=0} = -\frac{s_1(0)(F_{01} + F_{R1})}{M} \tag{48}
\]
Mean transit time through pool 1, $\bar{t}_1$, is the average interval of time spent by a particle from its entry into the pool to its next exit. Assuming first-order kinetics:

$$\bar{t}_1 = \frac{M}{F_{01} + F_{R1}} \quad (49)$$

Using Equation (48):

$$\bar{t}_1 = -\frac{s_i(0)}{\left(\frac{ds_i}{dt}\right)_{t=0}} \quad (50)$$

Mean total residence time in pool 1, $\bar{T}_1$, is the total amount of time spent by a particle in pool 1 in all its passages through it. If all particles are lost irreversibly from the pool, then $\bar{T}_1 = \bar{t}_1$.

However, if a fraction $r$ of the particles leaving pool 1 return to it, then another fraction $r^2$ will return one more time, and so on. Consequently, the mean total residence time will be given by the mean transit time increased by a fraction of it due to the particles that return once, plus a fraction due to the particles than return twice, and so on. Thus:

$$\bar{T}_1 = (1 + r + r^2 + ... )\bar{t}_1 \quad (51)$$

The bracketed series on the right-hand side of this equation is a geometric progression which can be summed to give:

$$\bar{T}_1 = \frac{\bar{t}_1}{1-r} \quad (52)$$

The denominator $1-r$ is the fraction of particles leaving pool 1 irreversibly and is given by ratio:

$$1-r = \frac{F_{01}}{F_{01} + F_{R1}} \quad (53)$$

(see Figure 4). Substituting for $1-r$ in Equation (52):
\[
\bar{T}_1 = \frac{F_{01} + F_{R1}}{F_{01}} \overline{t}_1 \tag{54}
\]

Using Equation (49) in (54):
\[
\bar{T}_1 = \frac{M}{F_{01}} = \frac{D/s_1(0)}{F_{01}} \tag{55}
\]

The rate at which tracer is irreversibly cleared from pool 1 at time \( t \) is \( s_1F_{01} \). Conservation of mass principles give:
\[
D = \int_0^\infty s_1F_{01}dt = F_{01} \int_0^\infty s_1dt \tag{56}
\]

Therefore:
\[
F_{01} = \frac{D}{\int_0^\infty s_1dt} \tag{57}
\]

Substituting for \( F_{01} \) in Equation (55):
\[
\bar{T}_1 = \frac{\int_0^\infty s_1dt}{s_1(0)} \tag{58}
\]

The recycle flow \( F_{R1} \) and the fraction \( r \) can now be calculated from Equations (49) and (52) respectively:
\[
F_{R1} = \frac{M}{\overline{t}_1} - F_{01} \tag{59}
\]
\[
r = 1 - \frac{\overline{t}_1}{\bar{T}_1} \tag{60}
\]

The mean number of cycles by a particle around pool 1, \( \overline{c}_1 \), is:
\[
\overline{c}_1 = \frac{\overline{T}}{\overline{t}_1} - 1 = \frac{r}{1-r} \tag{61}
\]
The fractional rate at which tracer particles are lost irreversibly at time \( t \) is:

\[
\frac{s_iF_{01}}{\int_0^\infty s_i F_{01} \, dt} = \frac{s_i}{\int_0^\infty s_i \, dt} \quad (62)
\]

Therefore the average residence time in the system of connected compartments (i.e., the expected time a particle spends in pool 1 and the other parts of the system before leaving for the last time), \( \bar{\tau} \), is given by:

\[
\bar{\tau} = \int_0^\infty \left( \frac{s_i}{\int_0^\infty s_i \, dt} \right) t \, dt = \frac{\int_0^\infty s_i t \, dt}{\int_0^\infty s_i \, dt} \quad (63)
\]

Thus the average time spent in the system but outside pool 1 in a single cycle, \( \bar{t}_R \), is:

\[
\bar{t}_R = \bar{\tau} - \bar{r}_1 \quad (64)
\]

In summary, the following information can be extracted from the specific activity decay curve.

Quantity of insulin in pool 1:

\[
M = \frac{D}{s_i(0)} \quad (65)
\]

Transit time through pool 1:

\[
\bar{t}_1 = \left. \frac{ds_i}{dt} \right|_{t=0} \quad (66)
\]

Total residence time in pool 1:
Total residence time in the system:

\[
\tau = \frac{\int_0^\infty s(t)\, dt}{\int_0^\infty s(t)\, dt} \quad (68)
\]

Fraction of particles leaving pool 1 which return to it:

\[
r = 1 - \frac{T_1}{\tau} \quad (69)
\]

Number of cycles around pool 1:

\[
c_1 = \frac{r}{1-r} \quad (70)
\]

Time spent in the system outside pool 1 per cycle:

\[
t_R = \frac{T_1 - \tau}{c_1} \quad (71)
\]

Irreversible removal rate of insulin:

\[
F_{01} = \frac{D}{\int_0^\infty s(t)\, dt} \quad (72)
\]

Recycle rate of insulin:

\[
F_{RI} = \frac{M}{t_1} - F_{01} \quad (73)
\]

The one-embedded-pool scheme was initially proposed by Rescigno and Gurpide [25] for circulating hormones. The analysis can readily be extended to two accessible pools in the system.
in which the tracer is administered as a single dose into either pool 1 or pool 2 at time zero, and the specific activity of both pools monitored. The single pool representation was used by Navalesi et al. [26] to study plasma insulin disappearance in non-ketotic diabetic patients and normal subjects using the tracer $^{125}$I-insulin (Table B.3). Such models with embedded compartments are routinely referred to in the literature as non-compartmental models.

Figure B.4. The single-compartment insulin model embedded in a system of multiple pools. Arrowed solid lines represent flows, hollow arrow represents insulin application (tracer dose), and broken line sampling.
Table B.3. Non-compartmental model to describe insulin kinetics

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population</th>
<th>Experimental Conditions</th>
<th>$F_0$ (mL/min m$^2$)</th>
<th>$F_R$ (mL/min m$^2$)</th>
<th>$t_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>Healthy adults (n = 34)</td>
<td>$^{125}$I-insulin bolus infusion</td>
<td>614 ± 20</td>
<td>195 ± 8</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Diabetic adults (n = 42)</td>
<td></td>
<td>655 ± 23</td>
<td>251 ± 12</td>
<td>0.67 ± 0.04</td>
</tr>
</tbody>
</table>

B.7. Deconvolution

As C-peptide is secreted in equimolar concentration with insulin but extracted to a negligible extent by the liver, C-peptide concentration in plasma reflects C-peptide (and hence insulin) secretion by the pancreas. Thus a more general approach to modelling insulin kinetics is to consider the concentration of C-peptide as the convolution of a unit bolus response and the rate of secretion:

$$C(t) = \int_0^t h(t-\tau)S(\tau)\,d\tau \tag{74}$$

where $C(t)$ is the C-peptide concentration, $S(t)$ the rate of secretion, and $h(t)$ the response in plasma concentration to the injection of a unit bolus of C-peptide. Knowledge of $h(t)$ (termed the impulse function) is a prerequisite, and requires an experiment applying a bolus injection of C-peptide and an accompanying infusion of somatostatin to inhibit endogenous C-peptide secretion. Equation (74) can be solved for $S(t)$ by deconvolution [7] using an algorithm such as that described by Radzuik [27]. Relevant aspects of the deconvolution problem associated with experimental design and computation are discussed by Cobelli et al. [28]. Deconvolution methods have been applied to estimate the insulin secretion profile in humans in various physiopathological states [17] and during both intravenous and oral glucose tolerance tests [29].
**B.8. Discussion**

Insulin is a central regulator of glucose homeostasis. As such, understanding its release and metabolic effects are of utmost importance. Furthermore, how insulin acts under different physiological, pharmacological, and nutritional conditions is of great interest. Given its complex metabolism and tissue specific effects (many tissues are not directly accessible), understanding whole-body *in vivo* insulin is challenging. Tracer kinetic studies enable quantification of pool sizes and flows between compartments involved in insulin metabolism. However, the application of insulin kinetic models is limited for several reasons.

Firstly, insulin kinetic methodology currently relies on intravenous administration of radioactive or unlabelled insulin that can have dangerous side effects such as hypoglycaemia. Therefore, bolus dose methodology must be undertaken with great caution and measures to identify hypoglycaemia be utilized and treatment measures available. ‘Clamp’ methodology, where glucose levels are stabilized by exogenous constant infusion, can be used to circumvent the risk associated with bolus dosing. In addition to minimizing the risk of hypoglycaemia, clamp methodology allows for other biological outcomes to be studied, including insulin sensitivity and glucose effectiveness. The limitations facing this methodology are expense and the necessity for highly trained technicians to execute the research. These studies are almost invariably performed with unlabelled insulin. The limited use of radioactive insulin tracers is likely due to their potentially harmful health effects in animals and people. In addition, radioactive insulin tracers do not always behave as their endogenous counterpart [5], leaving their suitability as a tracer open to question.

Another limitation to insulin kinetic studies lies in the measurement of peripheral (i.e., plasma) insulin concentrations. Insulin secreted from the pancreas is removed by the liver
through first pass metabolism prior to its entry into peripheral circulation. Peripheral insulin
distribution is complex and involves receptor- and nonreceptor-mediated processes [23].
Whether a mono- or multi-compartment model best describes peripheral insulin kinetics is
unclear, as several models have been applied (Table 2). Under physiological insulin
concentrations a one pool model may be adequate. This option is particularly appealing to
clinicians and physiologists as it is less mathematically challenging. However, others argue that
insulin kinetics is better described by a three pool system [19, 21]. Irrespective of the number of
pools, only insulin exiting liver can be estimated, meaning endogenous insulin secretion cannot
be determined. Models of C-peptide kinetics have therefore been employed and provide an
opportunity to examine pancreatic and hepatic insulin flows.

In comparison with the study of glucose kinetics, insulin kinetics has received limited
attention. This fact is likely due to the complexity of insulin secretion and distribution, in
addition to its wide ranging effects on metabolism. Experimentally, insulin infusion has the
potential to induce dangerous side effects. As such, technical expertise is required to execute this
research. Controversy remains as to what mathematical model(s) best describe peripheral insulin
kinetics. Furthermore, one kinetic model that adequately describes the inter-relationships
between insulin secretion and its appearance in hepatic, peripheral and intercellular
compartments is yet to be determined. The literature on insulin kinetics is richer and data are
more abundant prior to year 2000, a reflection in part of the increasing public awareness of
issues pertaining to animal welfare and the rising costs of in vivo isotope experiments.
B.9. References


