### BREATH ACETONE AS AN INDICATOR OF KETOSIS WHILE CONSUMING A KETOGENIC DIET

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Nutritional Science University of Toronto

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### ABSTRACT

### Breath Acetone as an Indicator of Ketosis While Consuming a Ketogenic Diet Master of Science, 1999 Kathy Musa Department of Nutritional Sciences, Faculty of Medicine University of Toronto

The ketogenic diet is a high fat, low carbohydrate diet used to treat children with intractable epilepsy. The maintenance of blood  $\beta$ -hydroxybutyrate above 2 mM and acetoacetate above 0.6 mM is important to the diet's effectiveness. The objectives of this thesis were to determine whether the Ketostix urinary test for ketosis reflects plasma ketones and whether breath acetone is a better measure of plasma ketones than the urinary ketone test. It was hypothesised that breath acetone would be a more reliable indicator of the plasma ketones than the urinary ketone test. In adults in acute ketosis, urinary acetoacetate was significantly correlated with plasma acetoacetate ( $r^2 = 0.72$ , p = 0.0003) and plasma  $\beta$ -hydroxybutyrate ( $r^2 = 0.52$ , p = 0.0057). Breath acetone was significantly correlated with plasma  $\beta$ -hydroxybutyrate ( $r^2 = 0.66$ , p < 0.0001). In rats in chronic ketosis, breath acetone was significantly correlated with plasma  $\beta$ -hydroxybutyrate ( $r^2 = 0.73$ , p < 0.0001). Breath acetone and Ketostix urinary acetoacetate are both significantly correlated with the plasma ketone significantly correlated with plasma  $\beta$ -hydroxybutyrate ( $r^2 = 0.73$ , p < 0.0001). Breath acetone and Ketostix urinary acetoacetate are both significantly correlated with the plasma ketones.

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## **ABBREVIATIONS**

AcAc	Acetoacetate

AED(s)	Anti-epileptic drug(s)			
AIN	American Institute of Nutrition			
AMP	Adenosine monophosphate			
β-ΗΒΑ	β-hydroxybutyrate			
β-HBDH	β-hydroxybutyrate dehydrogenase			
BMI	Body Mass Index			
CHO(s)	Carbohydrate(s)			
FA(s)	Fatty acid(s)			
HMG-CoA	3-hydroxy-3-methylglutaryl CoA			
HMS	Hexose monophosphate shunt			
KD	Ketogenic Diet			
LCFA(s)	Long-chain fatty acid(s)			
MCFA(s)	Medium-chain fatty acid(s)			
MCT(s)	Medium chain triglyceride(s)			
MJ(s)	Myoclonic jerks			
PTZ	Pentylenetetrazol			
REE	Resting energy expenditure			
SCFA(s)	Short-chain fatty acid(s)			
TCA	Tricarboxylic acid			

### **CHAPTER 1. INTRODUCTION**

The ketogenic diet (KD) is a very high fat, very low carbohydrate (CHO) diet that is used clinically to treat epilepsy in children who are either unresponsive to anti-epileptic drugs (AEDs) or experience intolerable side effects from them and are not candidates for surgical treatment (Wheless, 1995). The diet is "ketogenic" because it causes the patient to remain in a constant state of ketosis (Prasad et al., 1996). While in a state of ketosis, the body primarily oxidizes fat for energy rather than glucose because glycogen stores are depleted (Prasad et al., 1996). Two intermediates in fatty acid oxidation that the body can further oxidize to meet its energy needs are the ketones,  $\beta$ -hydroxybutyrate ( $\beta$ -HBA) and acetoacetate (AcAc). While ketogenesis occurs mainly within the mitochondria of liver cells, ketone body metabolism can occur in the brain, and to a lesser extent, in the heart, kidney cortex, and skeletal muscle (Mitchell et al., 1995).

Although the anticonvulsant effects of the KD are recognized clinically, the precise mechanism of this effect is still obscure (Freeman et al., 1996; Nordli et al., 1997; Prasad et al., 1996; Swink et al., 1997). Regardless of the mechanism of the KD, its anticonvulsant effect in humans seems to be dependent upon the maintenance of blood  $\beta$ -HBA above 2 mM and of AcAc above 0.6 mM (Huttenlocher, 1976). A reliable measure/indicator of either plasma  $\beta$ -HBA or AcAc is important in monitoring the efficacy of the diet but is not in routine clinical use at present.

A third ketone body which is often overlooked in the literature is acetone. Acetone is generated by the spontaneous decarboxylation of AcAc and is responsible for the sweet odor of the breath of individuals in ketosis (Mitchell et al., 1995). Unlike  $\beta$ -HBA and AcAc,

acetone is not an energy-rich compound (Mitchell et al., 1995); nevertheless, its concentration in the breath may be a useful, non-invasive indicator of ketosis.

Ketone body concentrations can be determined in urine (as  $\beta$ -HBA, AcAc, or acetone), in plasma (as  $\beta$ -HBA, AcAc, or acetone), or in breath (as expired acetone). In the home setting, the most practical current method available to parents for monitoring their child's degree of ketosis is the urinary test for ketosis. Unfortunately, the urinary test for ketosis is qualitative and is thought to not reflect the actual blood ketone concentration (Livingston, 1972). In support of the unreliability of the ketone body urinary test, Schwartz et al. (1989) observed that in a short-term clinical study on the effects of the KD in the treatment of epilepsy, there were fluctuations in the concentrations of urinary ketone bodies which did not appear to be related to alterations in the diet. Thus, although the urinary test for ketosis is simple and convenient, it does not appear to accurately estimate blood ketone concentrations.

Although plasma measures of  $\beta$ -HBA and AcAc are accurate, they cannot easily be determined in a home setting as would be required for the KD. Currently, there is a portable machine called Ketosite® (GDS Diagnostics) that resembles a glucometer, but which measures  $\beta$ -HBA rather than blood glucose. Although whole blood can be used and only a small quantity of blood (25µL) is required, the test is still invasive and parents and patients may feel uncomfortable about frequently measuring  $\beta$ -HBA, since each measure would require a finger-prick.

Freund (1965) demonstrated that breath acetone concentrations rise during fasting as well as during the consumption of a KD. Although Freund (1965) correlated breath acetone with plasma acetone, he did not correlate breath acetone with either plasma  $\beta$ -HBA or

plasma AcAc. If a correlation between breath acetone and plasma  $\beta$ -HBA or AcAc does exist, then in the future, a portable breath acetone analyzer could be constructed that would enable parents to monitor their child's state of ketosis safely, non-invasively, and as frequently as needed. Thus, the primary purpose of the experiments described in this thesis was to determine whether a significant, positive correlation exists between breath acetone and plasma  $\beta$ -HBA and AcAc. This relationship was investigated in a rat model of the KD, followed by investigations in adult human volunteers. Also, in collaboration with Dr. Burnham's group (Department of Pharmacology, University of Toronto), seizure protection in this rat model of the KD was assessed (see Appendices A, B, and C for experiments involving seizure testing).

### **CHAPTER 2. LITERATURE REVIEW**

#### 2.1 GENERAL

Epilepsy is a chronic condition characterized by a predisposition to recurrent, spontaneous seizures (Guberman and Bruni, 1997). Epilepsy is as common a disorder as juvenile-onset diabetes mellitus; its prevalence is approximately 1% and those affected are often children (Guberman and Bruni, 1997). Most epileptic children obtain complete or partial control of their seizures with anti-epileptic drugs (AEDs); however, 20%-30% of all children with epilepsy cannot rely on AEDs because either their seizures are intractable/refractory (unresponsive to anticonvulsant drugs) or the medications prescribed cause intolerable side effects (Wheless, 1995).

#### 2.2 HISTORY OF THE KETOGENIC DIET

Throughout history, the value of fasting in controlling epileptic seizures has been recognized. For instance, in the 5<sup>th</sup> century B.C., Hippocrates documented an epileptic man who became completely seizure-free upon abstaining from food and drink (Prasad et al., 1996). Also, in biblical times, Jesus is reported to have cured several epileptic children by asking them to pray and fast (Prasad et al., 1996). Although the benefits of fasting in controlling epileptic seizures were recognized anecdotally, so too were the dangers inherent

in completely abstaining from food. Thus, in 1921, in an attempt to mimic the physiological effects of fasting, Wilder developed the KD (Schwartz et al., 1989).

#### 2.3 HOW THE KETOGENIC DIET MIMICS STARVATION

The KD is a very high fat, very low CHO diet. Although not immediately apparent, there are many physiological similarities between fasting and the consumption of a KD (Nordli et al., 1997; Figure 2.1). In both cases, the body must rely on the oxidation of fatty acids (FAs) in order to meet its energy needs (Swink et al., 1997). While fasting, these FAs are derived from the body's fat stores (Swink et al., 1997); however, during the consumption of a KD, these FAs are derived mainly from dietary fats. In either case, the acetyl CoA that is produced via  $\beta$ -oxidation of the FAs either enters the tricarboxylic acid (TCA) cycle where it is completely oxidized or it is used in the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) pathway for the production of ketones (Swink et al., 1997). The body then enters a state of ketosis because the hepatic production of the ketones  $\beta$ -HBA, AcAc, and acetone is substantially increased (Mitchell et al., 1995). Although the precise mechanism is poorly understood, the ketosis that develops is assumed to be important in seizure control.

Because ketone bodies are derived from fat, dietary fats are potentially ketogenic. On the other hand, because CHOs and, to a lesser extent, proteins are the sources of glucose, they are antiketogenic. Wilder calculated that by weight, the ratio of ketogenic foods to antiketogenic foods must be 1.5 or greater to produce ketonemia and ketonuria, and 3 or greater to optimize seizure control (Prasad et al., 1996).



Figure 2.1 Physiological Events Occurring as a Result of Fasting or Fat-feeding

During a fast or the consumption of a ketogenic diet, fatty acids are metabolized to acetyl CoA. Acetyl CoA is then used as a substrate for ketone body production in the 3-hydroxy-3-methylglutaryl-CoA pathway or for ATP production in the tricarboxylic acid cycle. In either case, the body enters a state of ketosis as the production of ketones is increased.

#### 2.4 KETONE BODY METABOLISM

#### 2.4.1 General Information on Ketone Bodies

AcAc and acetone are, by definition, ketones since they each contain one ketone group (Mitchell et al., 1995).  $\beta$ -HBA is not technically a ketone since it does not contain a ketone group (Mitchell et al., 1995). The three molecules are presented in Figure 2.2.

AcAc is the key molecule from which the other two ketone bodies,  $\beta$ -HBA and acetone, are derived (Mitchell et al., 1995). Acetone is produced by the spontaneous decarboxylation of AcAc and is responsible for the sweet odor of the breath of ketotic individuals (Mitchell et al., 1995). AcAc and  $\beta$ -HBA can be interconverted in the presence of the enzyme  $\beta$ -hydroxybutyrate dehydrogenase ( $\beta$ -HBDH; Mitchell et al., 1995). All *r*these ketones can be found in blood and urine; however, in breath, only acetone can be detected.

 $\beta$ -HBA and AcAc are energy-rich compounds that can replace glucose as the primary energy substrates in the brain, and to a lesser extent, in the heart, kidney cortex, and skeletal muscle (Mitchell et al., 1995). Although  $\beta$ -HBA is considered to be an energy-rich compound, it is really a "dead-end" metabolite in that its only known metabolic role is interconversion with AcAc (Mitchell et al., 1995). Thus, the concentration of AcAc is extremely important since it is the substrate that is used for energy production as well as lipogenesis (Mitchell et al., 1995).

Normally, in the postprandial state, the blood concentration of  $\beta$ -HBA + AcAc is less than 0.2 mM (Mitchell et al., 1995). Hyperketonemia exists when the blood concentration of



### Figure 2.2 Structure of the Ketone Bodies

AcAc is the key ketone body from which the other ketone bodies are produced. Acetone is produced from the spontaneous decarboxylation of AcAc, while  $\beta$ -HBA is produced from the reduction of AcAc. AcAc and  $\beta$ -HBA can be interconverted. Abbreviations: AcAc, acetoacetate;  $\beta$ -HBA,  $\beta$ -hydroxybutyrate. (Adapted from Mitchell et al., 1995).

 $\beta$ -HBA + AcAc is greater than 0.2 mM (Mitchell et al., 1995). The ratio of  $\beta$ -HBA:AcAc is approximately 1 in the postprandial state, but rises to 5 to 6 after a week of fasting (Mitchell et al., 1995). The ratio of  $\beta$ -HBA:AcAc is important because it is related to the redox state of hepatic mitochondria.  $\beta$ -HBA and AcAc are interconverted via the following reaction:

$$\beta$$
-HBA + NAD<sup>+</sup>  $\leftrightarrow$  AcAc + NADH  
 $\beta$ -HBDH

A high ratio of  $\beta$ -HBA: AcAc is indicative of a reduced mitochondrial matrix (i.e. the ratio of NADH:NAD<sup>+</sup> is high) and a reduced mitochondrial matrix is normally indicative of a defect or deficiency in the respiratory chain (Mitchell et al., 1995).

#### 2.4.2 Causes of Ketosis

Under normal physiologic conditions, the concentration of ketone bodies in the blood is low (Swink et al., 1997). Moderate increases in ketone body concentrations normally occur during fasting, during and especially following prolonged exercise, and during the consumption of a KD (Swink et al., 1997). Mild ketosis also develops late in pregnancy and during the neonatal period of the suckling infant (Swink et al., 1997). In all of these instances, the ketosis that develops is considered to be a normal physiological response (Swink et al., 1997). Increases in ketone body concentrations can also occur in response to various other medical conditions (i.e. weight loss, diabetes, and cancer; Mitchell et al., 1995) which are beyond the scope of this thesis.

#### 2.4.3 Ketone Body Production

Ketogenesis occurs mainly within the mitochondria of liver cells (Mitchell et al., 1995). Unless medium-chain fatty acids (MCFAs; 6 – 12 carbons) or short-chain fatty acids (SCFAs; 1 – 4 carbons) are present, long-chain fatty acids (LCFAs; 14 + carbons) are the primary precursors of ketone bodies (Swink et al., 1997). When there is a decrease in blood glucose and a concomitant decrease in plasma insulin (i.e. during starvation, fat-feeding, or prolonged, strenuous exercise), free LCFAs are released from adipose tissue triacylglycerol stores. These LCFAs are transported as free FAs in plasma bound to albumin. They cross the cell membrane of the liver as free LCFAs by binding to cytoplasmic FA binding proteins; the amount that is extracted by the liver is dependent upon the plasma concentration. In the liver, LCFAs undergo one of two events: Either they are re-esterified to form phospholipids and triacylglycerol or they enter hepatic mitochondria via the carnitine acyltransferase system to undergo  $\beta$ -fatty acid oxidation. The acetyl CoA that results can either enter the TCA cycle and undergo complete oxidation or can be converted to AcAc via the HMG-CoA pathway.  $\beta$ -HBDH then catalyzes the production of  $\beta$ -HBA from AcAc (Figure 2.3).

Although LCFAs serve as the primary precursors for ketone body production, MCFAs and SCFAs are also potentially important precursors for ketogenesis (Swink et al., 1997). MCFAs and SCFAs are found in relatively high concentrations in maternal milk and medium chain triglyceride (MCT) oil (Swink et al., 1997). Unlike LCFAs, MCFAs and SCFAs are readily absorbed from the stomach into the portal venous system and cross directly into hepatic mitochondria independently of the carnitine acyltransferase system



Figure 2.3 Fatty Acid Catabolism and Ketone Body Formation in the Liver

Enzymes involved: (1) long-chain fatty acyl-CoA synthetase; (2) glycerol-3-phosphate acyltransferase; (3) carnitine acyltransferase I; (4) carnitine acyltransferase II; (5) carnitine exchange; (6) short- and medium-chain fatty acyl-CoA synthetase; (7) fatty acid oxidation complex; (8) citrate synthase; (9) acetoacetyl-CoA thiolase; (10) 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) synthase; (11) HMG-CoA lyase; (12)  $\beta$ -hydroxybutyrate dehydrogenase. Abbreviations: ATP, adenosine triphosphate; AMP, adenosine monophosphate. (Swink et al., 1997).

(Swink et al., 1997). Like LCFAs, MCFAs and SCFAs are then converted to acetyl CoA via  $\beta$ -oxidation and are either completely oxidized in the TCA cycle or are converted to ketone bodies via the HMG-CoA pathway (Figure 2.3).

#### 2.4.4 Uptake and Utilization of Ketone Bodies

At times of glucose shortage, ketone bodies provide the brain with an alternative source of energy (Figure 2.4). The brain is so efficient at utilizing ketone bodies for energy that during prolonged fasting, up to two-thirds of the brain's energy needs can be supplied by ketone bodies (Mitchell et al., 1995). Other tissues which are capable of utilizing ketones for energy include the heart, kidney cortex, and skeletal muscle.

In general, tissue uptake of ketone bodies is via specific monocarboxylic acid transporters and is dependent on plasma ketones (Swink et al., 1997). The brains of newborns utilize ketone bodies for energy at rates 4 – 5 times those of adult brains (Swink et al., 1997).

In the brain, ketones are important precursors not only for energy metabolism, but also for lipogenesis (Figure 2.4). Ketones are important substrates for the synthesis of cholesterol, fatty acids, and other complex lipids in the brain that are required for brain growth and myelination.

Note that whenever the term "ketones" or "ketone bodies" is used in reference to energy metabolism or lipogenesis, it is important to understand that only AcAc participates directly in these pathways (Mitchell et al., 1995). Although  $\beta$ -HBA can be taken up by the brain and other organs, it must first be converted to AcAc before it can be used for energy metabolism and/or lipogenesis (Mitchell et al., 1995).





Enzymes involved: (1) acetoacetyl-CoA synthetase; (2) HMG-CoA synthase; (3) HMG-CoA reductase; (4) kinases; (5) decarboxylase; (6) isomerase; (7) transferase; (8) transferase; (9) squalene synthase; (10) squalene monoxygenase; (11) acetoacetyl-CoA thiolase; (12) citrate lyase; (13) acetyl CoA carboxylase; (14) βhydroxybutyrate dehydrogenase; (15) 3-oxoacid-CoA transferase; (16) mitochondrial acetoacetyl-CoA thiolase; (17) pyruvate dehydrogenase; (18) lactate dehydrogenase. Abbraviations: AcAc, acetoacetate; β-HBA, βhydroxybutyrate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; TCA, tricarboxylic acid cycle; HMS, hexose monophosphate shunt. (Adapted from Swink et al., 1997.)

#### 2.5 TYPES OF KETOGENIC DIETS

The original or "classical" KD proposed by Wilder (1921) and later by Livingston (1972) involved the consumption of long-chain saturated fats (dairy fats) with a fat to (protein + CHO) ratio of 4:1. Because the classical KD is unpalatable, difficult to prepare, and rigid (due to the severe CHO restriction it imposes), various researchers have attempted to modify it.

Triglycerides of octanoic and decanoic acids (medium-chain triglycerides, MCTs) were proposed as the basis for an alternative KD (Huttenlocher et al., 1971). MCTs have many advantages over dairy fats. MCTs are rapidly absorbed from the gastro-intestinal tract and are considerably more ketogenic per gram than dairy fats (Huttenlocher et al., 1971); thus, the palatability of the KD and, consequently, patient compliance, improve because more protein and CHOs can be added to the diet. Also, since MCT oil is water-miscible and tasteless, the preparation of ketogenic meals is much easier (Huttenlocher et al., 1971). Furthermore, in children on the MCT diet for a duration of 3 months to 4 years, no elevations in serum cholesterol and only modest increases in serum total fatty acids were observed, in contrast to the marked hyperlipidemia observed in children consuming the classical KD (Huttenlocher, 1976).

Despite the benefits of the MCT diet, it is associated with several GI side effects including nausea, abdominal cramps, occasional vomiting, and diarrhea (Huttenlocher et al., 1971). In an attempt to exploit the benefits of the MCT diet but reduce its side effects, Schwartz et al., devised a modified MCT diet (the Radcliffe Diet) which incorporates both long and medium-chain fatty acids (1989). The compositions of the classical, MCT, and modified MCT diets are shown in Table 2.1 (Schwartz et al., 1989).

# 2.6 EFFICACY OF THE KETOGENIC DIET IN TREATING INTRACTABLE EPILEPSY

As long as the appropriate levels of ketosis are achieved (>2 mM  $\beta$ -HBA in blood, >0.6 mM AcAc in blood), the anticonvulsant effects of the KD appear to be independent of the dietary fat used (Schwartz et al., 1989). In general, when children with intractable epilepsy are placed on a KD, 30% to 40% have a greater than 90% decrease in seizure frequency, 30% to 40% have a greater than 50% decrease in seizure frequency, and 20% to 30% experience little or no difference in seizure control (Swink et al., 1997). Although younger children seem to respond best to the KD and may have better brain uptake of ketones (Livingston, 1972; Freeman et al., 1996; and Swink et al., 1997), a lack of dietary compliance in older children and adults may explain why results in these populations have not been as favourable (Prasad et al., 1996). Males and females respond equally well to the KD. Although the diet is particularly effective in controlling myoclonic, absence, and atonic (drop) seizures, it has been successful in children with virtually any type of seizure (Freeman et al., 1996).

## Table 2.1

KETOGENIC DIET	CALCULATION BASED ON	MCT OIL <sup>1</sup>	LONG-CHAIN SATURATED FATS	PROTEIN	CARBOHYDRATE
Classical (4:1)	75 kcals/kg bodyweight; 1g protein/kg bodyweight	36 kcals from fat to 4 kcals from protein and carbohydrate			
MCT <sup>1</sup>	RDI <sup>2</sup>	60%	11%	10%	19%
Modified MCT <sup>1</sup>	RDI <sup>2</sup>	30%	41%	10%	19%

<sup>1</sup> MCT - medium chain triglyceride

<sup>2</sup> RDI - recommended daily intake

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#### 2.7 MECHANISMS OF THE KETOGENIC DIET

Several theories exist regarding the mechanism(s) by which the KD ameliorates seizures. Hyperlipidemia, dehydration, alterations in electrolytes, and acidosis have all been implicated in the anticonvulsant effects of the KD (Nordli and DeVivo, 1997). Another mechanism of the KD involves its effects on cerebral metabolism with a change from dependence on glucose as a primary energy substrate to the direct utilization of the ketones  $\beta$ -HBA and AcAc (Huttenlocher, 1976). Although definitive evidence regarding the precise mechanism(s) of the KD is currently lacking, an alteration in cerebral metabolism is plausible because in children with defective glucose transport across the blood-brain barrier, the seizure control attained upon consumption of the KD is most likely due to the ability of the brain to take up and metabolize ketones instead of glucose (DeVivo et al., 1991). Whether the KD has anticonvulsant properties due to mechanisms other than alterations in cerebral metabolism needs to be determined. Interestingly, there is currently no study that compares the KD to a standard AED as the primary treatment (Prasad et al., 1996). Since the KD is prescribed as a last resort (i.e. only after AEDs have failed to elicit seizure control), it is very likely that the mechanism(s) of the KD is/are not comparable to the mechanisms of current AEDs.

#### 2.8 ANIMAL STUDIES INVOLVING THE KETOGENIC DIET

In an attempt to elucidate the mechanism(s) of the KD, researchers have tried to demonstrate seizure protection in various animal models of the KD. Uhlemann and Neims (1972) demonstrated that mice (pups) fed a KD (70% lipid based on raw pork sausage and caesin) for 10 days exhibited significant resistance to maximal electroshock and hydration threshold electroshock-induced seizures, but no protection against electroshock-threshold and pentylenetetrazol-induced seizures. Similarly, Nakazawa et al. (1983) showed that mice fed a KD (in the form of MCT milk powder) for 14 days exhibited a higher maximal electroconvulsive threshold than control mice fed a commercially available diet. Appleton and DeVivo (1974) showed that adult rats fed a 4:1 KD (consisting of corn oil, lard, caesin, glucose, salt mixture, and vitamin mixture) for 10-20 days had a significantly higher electroconvulsive shock threshold compared to control rats. Hori et al., (1997) using a kindling model of epilepsy, demonstrated that adult rats fed a KD (70% lipid) for 35 days had an increase in afterdischarge threshold and stage 5 seizure threshold compared to control rats (1997). In contrast, 5-6 week old magnesium-deficient rats fed a KD for 17 days experienced an increase in the incidence and severity and a decrease in the latency of audiogenic seizures (Mahoney et al., 1983). Rats fed a KD and challenged with injections of kainic acid experienced more severe seizures and a greater incidence of mortality compared to control rats (Bough et al., 1998).

The results of the animal studies conducted thus far are somewhat confusing. In some instances, the KD has shown anticonvulsant effects while in other instances it has shown either no effects or proconvulsant effects. There are many differences from study to study, and any one of these variables may have affected the outcome of the experiment. For instance, the animal (species and age) used in the study may be an important predictor of the efficacy of the KD. The composition of the KD as well as the animal's food intake, age at the time of diet onset, and duration of the diet may be important. Finally, the stimulus used to induce the seizures may not appropriately challenge the anticonvulsant effects of the KD (i.e. the convulsant may not be presented at the most optimal time during the feeding period, may surpass the threshold of the KD, or may operate via a mechanism entirely different from that of the KD). Until the confusion surrounding the issues outlined above is resolved, there will continue to be a lack of uniformity in future animal studies of the KD.

#### 2.9 CURRENT METHODS FOR MONITORING KETONE LEVELS

Despite a lack of understanding regarding the mechanism(s) of the KD, its clinical effectiveness in controlling seizures seems to be dependent upon the maintenance of blood  $\beta$ -HBA above 2 mM and of AcAc above 0.6 mM (Huttenlocher, 1976). Thus, a reliable measure/indicator of plasma  $\beta$ -HBA and AcAc might be useful in monitoring the efficacy of the KD, both in clinical and home settings. Although plasma measures of  $\beta$ -HBA and AcAc are accurate, one must remember that blood sampling from children is particularly invasive – especially when frequent monitoring of ketones is desired. The urinary dipstick test for ketosis is non-invasive and convenient; however, it is qualitative and impractical because it appears not to reflect actual blood ketone concentrations (Livingston, 1972; Scwartz et al., 1989). Thus, there is need for a reliable, noninvasive measure of plasma ketones.

#### 2.10 BREATH ACETONE AS AN INDICATOR OF KETOSIS

An indicator of ketosis that has been largely overlooked in the literature is the acetone in expired, alveolar air. Acetone, which is produced spontaneously from the decarboxylation of AcAc, is a normal breath constituent and its concentration varies from individual to individual (Table 2.2).

Although there is some information in the literature regarding breath acetone in humans, there is very little regarding changes in breath acetone in response to prolonged fasting or the consumption of a KD. In fact, to my knowledge, only Freund (1965) and Freund and Weinsier (1966) have examined changes in breath acetone in response to these dietary manipulations.

Freund (1965) demonstrated that levels of breath acetone rise while fasting and consuming a KD, but drop immediately upon the consumption of a CHO-rich meal. In a subsequent study (1966), Freund and Weinseir showed that:

- i.) After an over-night fast and the ingestion of 25 ml of MCT oil, breath acetone rises rapidly, reaches a maximum 6 h after ingestion, and returns to base-line values 18 h after ingestion;
- ii.) Ingestion of increasing amounts of MCT results in increased acetone;
- iii.) The acetone response is suppressed by the ingestion of sucrose; and
- iv.) Maximal acetone increases with prolongation of fasting before ingestion of MCT.

Although Freund (1965) and Freund and Weinseir (1966) demonstrated that breath acetone increases and decreases in response to dietary manipulations, they did not examine correlations between breath acetone and plasma  $\beta$ -HBA and AcAc. If a correlation between breath acetone and plasma  $\beta$ -HBA and AcAc does exist, then parents may be able to rely on breath acetone measurements to quickly and non-invasively monitor their child's degree of ketosis. Breath acetone could potentially become the safest and most reliable measure of ketosis for parents and clinicians who have had to otherwise rely on unreliable (urine) or

## Table 2.2

## Breath Acetone Concentrations of Healthy Humans

AUTHORS	NUMBER OF SUBJECTS	ACETONE (RANGE) (nM)
Crofford et al., 1977	35	25 (5 - 142)
Euler et al., 1996	50	15
Freund, 1965	4	17
Jones, 1988	9	26 (15 – 73)
Kupari et al., 1995	24	19 (8 - 47)
Mitsui et al., 1999	6	12 (6 - 23)
Phillips, 1987	15 .	23 (10 - 48)

invasive (blood) tests.

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Correlations between breath acetone and plasma  $\beta$ -HBA and AcAc were examined in a rat model of the KD as well as in adult human volunteers in acute ketosis. The relationship between urinary AcAc and the plasma ketones was also examined in adult human volunteers in acute ketosis. In collaboration with Dr. Burnham's group (Department of Pharmacology, University of Toronto), seizure protection in rats consuming a KD was assessed (see Appendices A, B, and C for experiments involving seizure testing).
## **CHAPTER 3. OBJECTIVES**

#### 3.1 OVERALL OBJECTIVE

To determine whether breath acetone reliably predicts plasma ketone levels in an animal and a human model of ketosis.

## 3.2 SPECIFIC OBJECTIVES

- Develop a system for breath acetone collection and analysis in a rat model of the KD.
- Examine correlations between breath acetone and plasma AcAc and  $\beta$ -HBA in a rat model of the KD.
- Develop a system of breath acetone collection and analysis for humans.
- Examine correlations between breath acetone and plasma AcAc and  $\beta$ -HBA in adult volunteers in acute/short-term ketosis.
- Examine correlations between urinary AcAc and plasma AcAc and  $\beta$ -HBA in adult volunteers in acute/short-term ketosis.

## **CHAPTER 4. HYPOTHESES**

- In rats and humans consuming a KD, a significant, positive correlation will be observed between breath acetone and the plasma ketones, AcAc and  $\beta$ -HBA.
- Breath acetone will be a better indicator of plasma AcAc and β-HBA than the urinary test for ketosis.

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## **CHAPTER 5. METHODS**

#### 5.1 OVERVIEW OF EXPERIMENTS

An overview of the experiments that have been conducted is presented in Table 5.1

5.2 DIETS

#### 5.2.1 Rats

The diets used to feed the control and ketotic rats of experiment 1 were purchased from Dyets Inc. (Bethlehem, Pennsylvania). The AIN 93G diet that was fed to control rats did not require any modification; however, the 3.5:1 (fat: protein + CHO) KD was prepared by adding some CHO (in the form of maltose dextrin) and more fat to a modified AIN 93G diet. The fat source added was flaxseed oil (purchased from a local health food store). The percent composition (by weight) of the control diet and the flaxseed oil KD is shown in Table 5.2.

The modified AIN 93G diet was developed so that when the appropriate amount of fat (flaxseed oil) was added to it, it would contain exactly the same concentration of ingredients (protein, vitamins, minerals, cellulose, salt, and soybean oil) in g/kg diet as the AIN 93G diet. When flaxseed oil was added to the modified AIN 93G diet, the diet took on a very runny consistency and the oil separated from the rest of the diet. To overcome this dilemma, more cellulose was added to the KD. In addition, sucaryl was added to enhance the taste and texture of the KD. Due to the addition of cellulose and sucaryl to the KD, the nutrients of this diet were diluted compared to the control diet (Table 5.3).

## An Overview of Experiments that have been Conducted

EXPERIMENT	SPECIES/ SUBJECTS	EXPERIMENTAL GROUP(S)	OBJECTIVE(S)
1 The Relationship Between Breath Acetone and Plasma β- HBA in a Rat Model of the KD	20 d old male albino Wistar rats	Control (n = 6) Flaxseed (n = 6)	To correlate breath acetone and plasma $\beta$ -HBA.
2 Ketosis in Healthy Adults Following the Ingestion of Ketogenic Meals	Healthy adults	Ketotic group (n = 6)	<ol> <li>To determine whether a correlation exists between plasma ketones (AcAc and β-HBA) and breath acetone and plasma ketones and urinary AcAc during short-term ketosis in adults;</li> <li>To determine if breath acetone measures are more reliable than urinary AcAc measures for predicting plasma levels of each of the ketones.</li> </ol>

MACRONUTRIENT	CONTROL DIET	<b>KETOGENIC DIET</b> <sup>1</sup>	
Carbohydrate	69.8	1.6	
Fat	7.7	76.2	
Protein	22.5	22.2	

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<sup>1</sup> Values are expressed as percentages by weight.

## Composition of Control and Ketogenic Rodent Diets

INGREDIENT	CONTROL DIET 1	<b>KETOGENIC DIET</b> WITHOUT THE ADDITION OF CELLULOSE AND SUCARYL <sup>1</sup>	KETOGENIC DIET WITH THE ADDITION OF CELLULOSE AND SUCARYL <sup>1</sup>
Casein	20.4	20.1	15.4
Cornstarch	40.5	0	0
Dextrose	13.4	1.5	1.2
Sucrose	10.2	0	0
Cellulose	5.1	5.0	19.8
Soybean Oil	7.1	7.0	5.6
t-Butylhydroquinone	0.001	0.001	0.001
Salt Mix <sup>2</sup>	2.8	2.7	2.2
Vitamin Mix <sup>2</sup>	0.03	0.03	0.02
L-cystine	0.3	0.3	0.2
Choline Bitartrate	0.3	0.3	0.2
Sucaryl	0	0	3.2
Flaxseed Oil	0	63.1	52.2

<sup>1</sup> All values are expressed as percentage of diet, by weight.

<sup>2</sup> Salt and vitamin mixes were sugar-free in both the control and ketogenic diets.

another four days (age 24 d - 27 d). At 28 d of age, the rats were fed the final 3.5:1 flaxseed oil KD until the termination of the experiment. Control and experimental rats were allowed to consume food *ad lib*. In order to acclimitize the rats to the KD, the rats were fed a 1:1 flaxseed oil KD for four days after being weaned (age 20 d - 23 d) and then were fed a 2:1 flaxseed oil KD for

#### 5.2.2 <u>Humans in an Acute State of Ketosis</u>

Total energy needs for each subject were determined using various calculations (Table 5.4). First, using the subject's weight, height, age, and gender, the subject's resting energy expenditure (REE; kcal/day) was estimated using a Harris-Benedict equation. Then, the REE was multiplied by a physical activity factor. Finally, the thermic effect of food was calculated by taking 10% of the sum of the REE and physical activity calories. Each subject's total energy requirements were calculated as the sum of the REE, physical activity energy needs, and thermic effect of food energy needs. Clinically the KD is calorie-restricted (Freeman et al., 1996); thus, only 75% of the subject's total energy needs were provided.

The ketogenic meals that were fed to the subjects had a fat:(CHO + protein) ratio of 3.8:1. The KD was made up of only two ingredients: Beatrice 35% whipping cream (purchased from a local grocery store) and ProMod Protein Supplement (purchased from the Specialty Food Store, the Toronto Hospital for Sick Children). The diet compositions are shown in Table 5.5. Instead of giving one large meal to each subject, each subject received four small meals, one meal every three hours. Each of the four meals was identical in

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Steps Used in Calculating Total Energy Requirements of Experimental Subjects

CALCULATION	FORMULA		
REE	Harris-Benedict Equations Males = 66.5 + 13.8 (weight in kilograms) + 5 (height in centimeters) - 6.76 (age in years) Females = 655 + 9.56 (weight in kilograms) + 1.85 (height In centimeters) - 4.68 (age in years)		
Effect of Physical Activity <sup>1</sup>	Sedentary $^2 = 0.30 \text{ X REE}$ Light activity $^3 = 0.60 \text{ X REE}$ Moderate activity $^4 = 0.70 \text{ X REE}$ Heavy activity $^5 = 0.90 \text{ X REE}$		
Thermic Effect of Food <sup>1</sup>	= (0.10) (REE + Effect of Physical Activity)		
Total Energy Requirements <sup>1</sup>	= REE + Effect of Physical Activity + Thermic Effect of Food		
Total Energy Requirements while Consuming a Ketogenic Diet <sup>1</sup>	<ul> <li>= (0.75) (REE + Effect of Physical Activity + Thermic Effect of Food)</li> </ul>		

<sup>1</sup> Measured in kilocalories/day.

<sup>2</sup> Sedentary - mostly sitting.

<sup>3</sup> Light activity - mostly sitting, but with a daily walking program.

<sup>4</sup> Moderate activity - involved in some sort of daily, vigorous physical activity.

<sup>5</sup> Heavy activity - prolonged, vigorous daily physical activity.

Abbreviations: REE, resting energy expenditure.

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Percent Macronutrient Composition of the Ketogenic Diet Used in the Human Study

MACRONUTRIENT	COMPOSITION	CONTRIBUTION FROM 35% WHIPPING CREAM <sup>1</sup>	CONTRIBUTION FROM PROMOD PROTEIN SUPPPLEMENT POWDER <sup>1</sup>	AMOUNT CONSUMED <sup>2</sup>
Carbohydrate	8.2	7.4	0.8	0.4
Fat	79.2	78.5	0.7	3.5
Protein	12.6	4.4	8.2	0.6

<sup>1</sup> Values are expressed as percentages, by dry weight.

<sup>2</sup> Values are expressed in g/kg body weight/day.

composition and caloric density, and altogether, the four meals provided the subject with 75% of his/her caloric requirements.

#### 5.3 BLOOD COLLECTION TECHNIQUES

#### 5.3.1 Rats

On each sampling day, approximately 200  $\mu$ l of venous blood were collected from each rat. Blood was collected by inserting a 23 G 1 needle (Becton Dickinson Company; Franklin Lakes, New Jersey) into one of the veins of the tail and 75 mm heparinized capillary tubes were used to collect the blood and keep it flowing continuously. The needle that was inserted was first cut (using pliers) to a length of about 2 cm and was left in the tail while blood was being collected. Blood samples were then dispensed into 2.5 ml heparinized microfuge tubes and kept on ice until centrifugation. During centrifugation, blood samples were spun at 2500 rpm for 9 min in order to separate the plasma from the other blood components. Plasma samples were then dispensed into 500  $\mu$ l Ependorf tubes and stored at -20°C until analysis.

#### 5.3.2 <u>Humans</u>

Human blood was collected from fingertips after pricking a fingertip with a Glucometer Elite lancet pen (Bayer, Etobicoke, Ontario). 75 mm heparinized capillary tubes were used to collect 375  $\mu$ l of blood. Blood samples were then dispensed into 2.5 ml

heparinized microfuge tubes and kept on ice until centrifugation. During centrifugation, blood samples were spun at 2500 rpm for 9 min in order to separate the plasma from the other blood components. Plasma samples were then analyzed immediately for AcAc and  $\beta$ -HBA.

#### 5.4 BREATH COLLECTION TECHNIQUES

#### 5.4.1 <u>Rats</u>

Ten 4 L pickling jars were purchased from a retail store specializing in restaurant supplies. The exact volume of each jar was measured by weighing the mass of water that it could hold. Results indicated that the volume of each of the ten pickling jars was approximately 4.2 L.

The lid of each jar was made of metal and when screwed onto the jar, an airtight system for rat breath collection was created. Since it was necessary to draw breath samples from each jar via a syringe, a septum had to be installed in each of the jar lids. First, a 2 mm hole was drilled into the centre of each lid. Then, a septum was secured onto the outside of the lid by attaching a triangular piece of metal (also with a 2 mm hole drilled into its centre) over each septum. In turn, the triangular piece of metal was secured to the outside of the lid by three screws.

A system of rat breath collection and analysis had to be developed. Several issues had to be addressed. The amount of time that the rats should be left in the air-tight pickling jars was uncertain. If the rats were not left in the pickling jars for a sufficient amount of time, breath acetone would not accumulate to detectable levels; however, if the rats were left in the pickling jars for too long, too much moisture would develop within the jar, and acetone would be lost in this moisture; as well, there was some concern over the levels of  $CO_2$  that might be accumulating in the gas-tight jars. The type of acetone standard that should be used (liquid vs. gaseous), as well as the accuracy of the acetone standard (especially over time) were also issues that had to be addressed.

In order to collect breath samples from the rats, each rat was left in an airtight pickling jar. In the beginning of the experiment when the rats were quite small, the rats were left in the jars for a total of 20 min. As the rats gained weight towards the end of the experiment, the rats were left in the pickling jars for 10 min. Breath acetone concentrations were expressed in nM/kg/min because the concentration of acetone increased linearly with time (i.e. the acetone concentration detected after 10 min was roughly half of the acetone concentration detected after 20 min).

For breath acetone analysis, a 1 ml breath sample was taken from the jar and injected into the gas chromatograph. Measures were taken to ensure that  $CO_2$  within the jars was not accumulating to dangerous levels. A 450 g male albino Wistar rat was kept in one of the jars for 20 min. Breath samples were drawn and stored in vacutainers every 2 min and were later analyzed for  $CO_2$  by isotope ratio mass spectroscopy. Results showed that after 20 min,  $CO_2$ levels rose to 5000 ppm, which is the upper acceptable limit (www.vaisala.com). It is important to note that the 450 g rat used in this  $CO_2$  experiment was approximately 100 g heavier than the heaviest rat that was breath sampled in the actual experiment. Thus, the possibility of  $CO_2$  building up within the jars and affecting the respiratory rates of smaller rats was ruled out. The acetone standard used to quantify the acetone peaks on the chromatograms was a gaseous standard. A gaseous (as opposed to a liquid) acetone standard was used because the gaseous standard more closely mimicked the experimental situation. The acetone standard was created by injecting a known volume of acetone into one of the pickling jars. The acetone standard had to be prepared on each day of breath acetone analysis because, over time, the acetone concentration decreased, probably due to its adsorption into the glass walls and/or the metal lid of the pickling jar.

#### 5.4.2 <u>Humans</u>

As with the rats, a system of breath acetone collection and analysis had to be developed for humans. Human subjects were asked to exhale into a gas collection bag (purchased from Quintron Instrument Company, Milwaukee, Wisconsin). The bag has two 0.5 cm diameter holes in it. Initially, the alveolar portion of the breath samples was collected by inserting a 1 mm glass, gas-tight syringe into one of these holes. Immediately after the breath sample was collected, it was injected into the gas chromatograph for acetone analysis. Unfortunately, using this method, too much moisture was collected in the sample, and a water peak interfered with the acetone peak obtained on the chromatogram. The flow rate and temperature settings were manipulated in an attempt to separate the water and acetone peaks; however, the attempts were unsuccessful.

The gas collection bags were still used to collect breath samples; however, instead of collecting the breath sample directly from the bag into the syringe, the breath sample was collected from the bag into an evacuated 11.5 mm glass tube via a vacutainer needle equipped on the gas collection bag. Collecting the breath sample into a glass tube eliminated

the problem of having too much moisture in the breath sample; however, a new problem arose. Breath samples could not be stored in the glass tubes for longer than 6 min without losing a significant amount of the acetone. We hypothesized that this was probably because as the temperature of the breath sample dropped in the glass tube, the acetone became adsorbed on the glass walls. Several attempts were made to release the acetone from the glass walls. Since the boiling point of acetone is 56.5 °C (Handbook of Chemistry and Physics, 1986), the glass tubes were heated to try to release the acetone from the walls. The tubes were incubated in an incubator (65°C), were blow-dried with a hair-dryer, and were kept under 65°C water, but the full acetone concentration was never retrieved, and what was retrieved was not consistent. Thus, breath samples were collected in evacuated glass tubes and were analyzed immediately for acetone.

The acetone standard used to quantify the acetone peaks obtained on the chromatograms was a liquid (rather than a gaseous) standard. A gaseous acetone standard prepared in an 11.5 mm evacuated tube would have been ideal; however, there was too much error inherent in preparing this gaseous acetone standard (i.e. too many serial dilutions would have been necessary and acetone cannot be stored in these glass tubes). Thus, a liquid acetone standard was prepared by diluting a known volume of acetone in a known volume of water. Again, the standard was prepared on each day of breath analysis because, over time, the concentration of the acetone standard decreased.

#### **5.5 Analytical Methods**

#### 5.5.1 Blood Glucose

Blood glucose measurements were made using the Glucometer Elite glucometer and Glucometer Elite test strips (Bayer, Etobicoke, Ontario).

#### 5.5.2 Plasma β-HBA

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Plasma  $\beta$ -HBA was determined using an enzymatic assay kit (Sigma, St. Louis, Missouri). The enzymatic reaction involved in the assay is as follows:

#### $\beta$ -HBA + NAD<sup>+</sup> $\rightarrow$ AcAc + NADH $\beta$ -HBDH

In the presence of the enzyme,  $\beta$ -hydroxybutyrate dehydrogenase ( $\beta$ -HBDH),  $\beta$ -HBA is oxidized to AcAc. During this oxidation, an equimolar amount of NAD<sup>+</sup> is reduced to NADH. Since NADH absorbs light at 340 nm, the increase in absorbance at 340 nm is directly proportional to the  $\beta$ -HBA concentration in the sample.

The materials provided within the  $\beta$ -HBA enzymatic assay kit include:

- β-HBA Reagent (lyophilized preparation containing NAD, 4.6mM oxamic acid and buffer, pH 7.6);
- ii.)  $\beta$ -HBDH (50 units/ml, with buffer and stabilizers, pH 7.6); and
- iii.)  $\beta$ -HBA Calibrator Solution (50 mg/dl, with preservatives).

To analyze the  $\beta$ -HBA in each of the plasma samples, 3.0 ml of  $\beta$ -HBA Reagent and 50  $\mu$ l of plasma were pipetted into 4.5 ml acrylic cuvettes. As well, calibrators were prepared by pipetting 50  $\mu$ l of the  $\beta$ -HBA Calibrator Solution and 3.0 ml of  $\beta$ -HBA Reagent into 4.5 ml acrylic cuvettes. A blank was prepared by pipetting 50  $\mu$ l of deionized water and 3.0 ml of  $\beta$ -HBA Reagent into a 4.5 ml acrylic cuvette. Initial absorbance readings were measured at a wavelength of 340 nm, using water as a reference. 50  $\mu$ l of  $\beta$ -HBDH were then added to each of the cuvettes (except the reference) and 15 min later, final absorbance readings were measured measured, again at a wavelength of 340 nm.

To determine  $\beta$ -HBA concentrations, the following calculations were made:

i.) 
$$[\beta$$
-HBA] (mM) = ( $\Delta$ A sample -  $\Delta$ A blank) X 104 X 0.096

ii.) 
$$[\beta$$
-HBA] (mM) = ( $\Delta A \text{ sample - } \Delta A \text{ blank}$ ) X 50 • 0.096  
( $\Delta A \text{ calibrator - } \Delta A \text{ blank}$ )

iii.) Final [
$$\beta$$
-HBA] (mM) = Average of i.) and ii.)

## 5.5.3 Plasma AcAc

The enzymatic assay for plasma AcAc was developed by Harano et al (1985). The enzymatic reaction involved in the assay is as follows:

$$\begin{array}{c} \leftarrow \\ \beta \text{-HBA} + \text{NAD}^{+} \rightarrow \text{AcAc} + \text{NADH} \\ \beta \text{-HBDH} \end{array}$$

Normally, in the presence of  $\beta$ -HBDH,  $\beta$ -HBA is oxidized to AcAc with a concomitant, equimolar reduction of NAD<sup>+</sup> to NADH. Although the forward reaction is thermodynamically favored, the reaction can be forced in the opposite direction by increasing

the pH and the concentration of NADH. Due to Le Chatelier's principle, an increase in the pH and the concentration of NADH would result in the oxidation of NADH to NAD<sup>+</sup> with a concomitant, equimolar reduction of AcAc to  $\beta$ -HBA. As long as the concentration of NADH far exceeds that of AcAc, AcAc would be the limiting reactant in the reaction. Since NADH absorbs light at a wavelength of 340 nm, the decrease in absorbance at 340 nm would be directly proportional to the AcAc concentration in the plasma sample.

The reagents required for the AcAc enzymatic assay include:

- i.) 0.25 M Triethanolamine Buffer (pH 7)
- ii.) NADH
- iii.) Oxamic acid
- iv.)  $\beta$ -HBDH (55.6 units/ml)

All of the above reagents were purchased from Sigma Diagnostics except the enzyme which was purchased from Toyobo Company Ltd., Tokyo Japan. The following calculations illustrate how the reagents with the required concentrations were prepared:

- A 0.5 g/dl solution of oxamic acid was prepared by dissolving 0.05 g of oxamic acid in 10 ml of deionized water.
- ii.) A 3.0 mM solution of NADH was prepared by dissolving 0.0106 g of NADH in 5 ml of deionized water.
- iii.) A 2.8 M solution of ammonium sulfate was prepared by dissolving 3.7 g of ammonium sulfate in 10.0 ml of deionized water.
- iv.) A 55.6 units/ml enzyme solution was prepared by dissolving 500 units of β-HBDH in
   9.0 ml of 2.8 M ammonium sulfate solution.

Prior to analyzing the AcAc content in each of the plasma samples, a standard curve

showing the relationship between AcAc and change in absorbance at 340 nm had to be constructed. Thus, the AcAc concentrations shown in Table 5.6 were prepared using lithium acetoacetate and deionized water.

To analyze the AcAc in each of the solutions shown in Table 5.6, 386 µl of triethanolamine buffer, 200 µl of oxamic acid, 100 µl of NADH, and 50 µl of each of the LiAcAc standard solutions were pipeted into 1.5 µl acrylic cuvettes. Each LiAcAc solution was analyzed three times (i.e. 50 µl of each LiAcAc solution were dispensed into three different cuvettes). A blank was prepared by replacing the 50 µl of LiAcAc with 50 µl of deionized water. Initial absorbance readings were measured at a wavelength of 340 nm, using the blank as a reference. 20  $\mu$ l of  $\beta$ -HBDH were then added to each of the cuvettes (except the blank), and 15 min later, final absorbance readings were measured, again at a wavelength of 340 nm. The changes in absorbance were plotted against the actual AcAc concentrations, and a smooth curve (described by a polynomial, fourth order equation) was generated. (Note: The plasma samples were analyzed in exactly the same way as the LiAcAc samples were analyzed, only the 50 µl of LiAcAc were replaced with 50 µl of plasma.) The AcAc standard curve was constructed on several occasions using freshly prepared LiAcAc solutions and reagents. Each time, a polynomial fourth order equation best described the relationship between AcAc concentration and change in absorbance. Although the relationship was reproducible, the generation of a slightly curvelinear relationship as opposed to a linear one is unclear. Nevertheless, the relationship was linear for the range of AcAc (0 mM - 1.5 mM) that was expected (Figure 5.1).

Acetoacetate Solutions Used to Construct a Standard Curve

CONENTRATION OF AcAc <sup>1</sup>	VOLUME OF STOCK <sup>2</sup> SOLUTION <sup>3</sup>	VOLUME OF DEIOINIZED WATER <sup>3</sup>
0	0	50
0.125	50	9950
0.250	50	4950
0.500	150	7350
0.750	150	4850
1.000	150	3600
1.500	180	2820
2.000	300	3450
3.000	450	3300
4.000	500	2625
5.000	700	2800

<sup>1</sup> Measured in mmol/L.

<sup>2</sup> Stock solution had a concentration of 25 mM.

<sup>3</sup> Measured in  $\mu$ L.

•





Points and error bars represent means  $\pm$  SEM; n = 3 per point. The relationship was linear when the concentration of AcAc was below 2.0 mM. When the concentration of AcAc was > 2.0 mM, the relationship was curvilinear. Because an AcAc concentration > 2.0 mM was never detected in the study subjects of Experiment 4, the equation of the line depicted above was used to calculate all AcAc concentrations. Abbreviations: AcAc, acetoacetate.

An unfortunate discovery made after Experiment 1 had been completed was that plasma samples that were spiked with AcAc, frozen, and analyzed later lost approximately 92% of their AcAc. It seems that the enzymatic assay for AcAc described in this section is only good for fresh plasma samples; that is, plasma samples had to be analyzed immediately in order to assess their AcAc content because AcAc is a very volatile compound. Hence, reliable plasma AcAc values are available only for the subjects of Experiment 2.

#### 5.5.4 Urinary AcAc

Urinary AcAc was measured using the methods described for plasma AcAc, only 50  $\mu$ l of urine replaced the 50  $\mu$ l of plasma. Again, the enzymatic assay for urinary AcAc was used only on fresh (not frozen) urine samples. Also, urinary AcAc was measured qualitatively using Ketostix dipsticks (Bayer, Etobicoke, Ontario). The principle behind the Ketostix dipstick urinary test is that if AcAc is present in the urine, it will react with nitroprusside, resulting in the development of colours ranging from buff-pink to maroon. The colour that develops is then matched (as best as possible) to a colour found on a colour chart and a corresponding AcAc concentration (from an exponential scale) is then allotted.

#### 5.5.5 Breath Acetone

Breath acetone in rat and human breath samples was analyzed using a Hewlett Packard gas chromatograph (model 5890) equipped with a column purchased from Supelco, Pennsylvania. The glass column, 6 ft in length with an internal diameter of 4 mm, was packed with 80/100 carbopack<sup>TM</sup> C/0.1% SP<sup>TM</sup> - 1000.

The following parameters were set on the gas chromatograph:

- i.) Helium pressure: 55 lb/in<sup>2</sup>
- ii.) Air pressure: 40-50 lb/in<sup>2</sup>
- iii.) Hydrogen pressure: 20 lb/in<sup>2</sup>
- iv.) Flow rate: 20 ml/min
- v.) Oven temperature: 75-100°C
- vi.) Injection temperature: 150°C
- vii.) Detection temperature: 200°C

The acetone peaks obtained from the breath samples were calibrated against either a gaseous acetone standard (Experiment 1) or a liquid acetone standard (Experiment 2). In either case, the acetone standard was prepared on each day of breath analysis because, over time, the acetone concentration decreased, probably due to its adsorption into the glass walls of the vessel.

#### 5.5.6 Statistical Analyses

To compare group means in Experiment 1 a repeated measures two-way analysis of variance (ANOVA) was conducted. A repeated measures one-way ANOVA was conducted to compare group means in Experiment 2. The software used to conduct the ANOVA tests was SPSS. When the ANOVA test indicated that group means were significantly different, a post-F (Tukey's Honestly Significant Difference) test was conducted using the software,

Statistica, to determine where these significant differences existed. For correlation analyses, linear and nonlinear regression analyses were conducted using the software, Prism.

#### CHAPTER 6. RESULTS

# 6.1 EXPERIMENT 1: THE RELATIONSHIP BETWEEN BREATH ACETONE AND PLASMA β-HBA IN A RAT MODEL OF THE KD

#### 6.1.1 Overview of Experiment 1

Twelve 17 d old male albino Wistar pups arrived at the facility with their dam. The rats were given thee days to acclimatize to their new environment and at 20 d of age, the rats were weaned and randomly divided into either a control or ketogenic diet group. Rats were housed individually in plastic cages with wood chip bedding and were placed on a 12 h light/12 h dark cycle (lights were turned on at 7:00 AM). At 20 d old, the control rats were given the AIN 93G diet and consumed it *ad lib*. In order to assist them in adjusting to the 3.5:1 (fat: protein + CHO) KD, rats in the ketogenic diet group were gradually weaned onto the diet; that is, at 20 d of age, these rats were given unlimited access to a 1:1 KD, and then at 24 d of age, they were given unlimited access to a 2:1 KD. *Ad lib* feeding of the 3.5:1 KD did not occur until the rats were 28 d old. The added fat source in the 1:1, 2:1, and 3.5:1 KDs was flaxseed oil.

The rats were weighed at 20 d of age and approximately every four days thereafter until the rats were 53 d old. Blood glucose, plasma  $\beta$ -HBA, and breath acetone were measured approximately every four days, beginning when the rats were 28 d old and ending when the rats were 49 d old. Food intakes were measured at various time points throughout the experiment. At 53 d of age, the rats underwent a PTZ seizure test. For details on this seizure test, refer to Appendix A.

#### 6.1.2 <u>Results of Experiment 1</u>

Control rats differed significantly from ketotic rats in terms of weight and weight gain (p<0.05; see Figures 6.1 and 6.2). By the end of the experiment (rats were 53 d old), control rats weighed an average of 271 g  $\pm$  15 g while ketotic rats weighed an average of 233 g  $\pm$  27 g. Control rats gained an average of 224 g  $\pm$  12 g while ketotic rats only gained an average of 186 g  $\pm$  23 g. Food intakes of control and ketotic rats also differed significantly (p<0.05; see Table 6.1) even though food was offered to all rats *ad lib*. By weight, ketotic rats consumed only 69%  $\pm$  9% of what control rats consumed. In terms of energy intake, however, ketotic rats consumed 132%  $\pm$  18% of what control rats consumed.

Blood glucose (Figure 6.3) differed significantly between control and ketotic rats as did plasma  $\beta$ -HBA (Figure 6.4) and breath acetone (Figure 6.5). In general, ketotic rats had a lower blood glucose and a higher plasma  $\beta$ -HBA and breath acetone. Ketotic rats attained the lowest blood glucose and the highest plasma  $\beta$ -HBA and breath acetone between the ages of 37 d and 41 d.

Plasma AcAc was measured; however, these values have not been included because the plasma samples had been frozen, and this is not appropriate for the AcAc enzymatic assay.

A nonlinear regression analysis revealed that breath acetone and plasma  $\beta$ -HBA were significantly correlated ( $r^2 = 0.73$ , p < 0.0001; Figure 6.6). The relationship between plasma  $\beta$ -HBA and breath acetone was best described by a one phase exponential equation. The equation of the line was  $y = 7.4071 \cdot (1-\exp(-0.0017 \cdot breath acetone, nM/kg/min))$ .



Figure 6.1 Weights of Control and Ketotic Rats

Plotted points represent means  $\pm$  SEM; n = 6 rats/group. Different letters (a & b) denote weights which were significantly different at the same time point (p < 0.05). From age 20 d - 23 d, ketotic rats were fed a 1:1 flaxseed oil diet and from age 24 d - 27 d, ketotic rats were fed a 2:1 flaxseed oil diet. A 3.5:1 flaxseed oil diet was fed to ketotic rats from age 28 d until the termination of the experiment. Control rats were fed the AIN 93G diet throughout the entire experiment. Food was offered to all rats ad lib.



Figure 6.2 Weight Gain of Control and Ketotic Rats

Plotted points represent means  $\pm$  SEM; n = 6 rats/group. Different letters (a & b) denote weight gains which were significantly different at the same time point (p < 0.05). From age 20 d - 23 d, ketotic rats were fed a 1:1 flaxseed oil diet and from age 24 d - 27 d, ketotic rats were fed a 2:1 flaxseed oil diet. A 3.5:1 flaxseed oil diet was fed to ketotic rats from age 28 d until the termination of the experiment. Control rats were fed the AIN 93G diet throughout the entire experiment. Food was offered to all rats *ad lib*.

## Table 6.1

AGE (d) <sup>1</sup>	CONTROL GROUP <sup>2</sup>	KETOTIC GROUP <sup>2</sup>	% WEIGHT INTAKE OF CONTROL	% ENERGY INTAKE OF CONTROL <sup>3</sup>
31 - 32	21 ± 2	$12 \pm 3$	59	113
32 – 33	19 ± 1	16 ± 4	84	162
39 - 40	23 ± 3	17 ± 2	74	144
40 - 41	28 ± 2	18 ± 2	66	127
45 – 46	28 ± 4	17 ± 2	62	119
48 – 49	26 ± 4	17±4	67	129
		MEAN	69	132
		SD .	9	18

Mean Food Intakes of Control and Ketotic Rats Measured at Various Time Points

<sup>1</sup> During these measurements, ketotic rats were consuming the 3.5:1 ketogenic diet.

<sup>2</sup>Food intakes were measured in g.

<sup>3</sup> The AIN 93G control diet had a caloric density of 3.87 kcal/g while the 3.5:1 flaxseed oil ketogenic diet had a caloric density of 7.48 kcal/g.



Figure 6.3 Blood Glucose in Control and Ketotic Rats

Points and error bars represent means  $\pm$  SEM; n = 6 rats/group. Different letters (a & b) denote blood glucose concentrations which were significantly different at the same time point (p < 0.05). From age 20 d - 23 d, ketotic rats were fed a 1:1 flaxseed oil diet and from age 24 d - 27 d, ketotic rats were fed a 2:1 flaxseed oil diet. A 3.5:1 flaxseed oil diet was fed to ketotic rats from age 28 d until the termination of the experiment. Control rats were fed the AIN 93G diet throughout the entire experiment. Food was offered to all rats ad lib.



Figure 6.4 Plasma β-HBA in Control and Ketotic Rats

Points and error bars represent means  $\pm$  SEM; n = 6 rats/group. Different letters (a & b) denote plasma  $\beta$ -HBA concentrations which were significantly different at the same time point (p < 0.05). From age 20 d - 23 d, ketotic rats were fed a 1:1 flaxseed oil diet and from age 24 d - 27 d, ketotic rats were fed a 2:1 flaxseed oil diet. A 3.5:1 flaxseed oil diet was fed to ketotic rats from age 28 d until the termination of the experiment. Control rats were fed the AIN 93G diet throughout the entire experiment. Food was offered to all rats *ad lib*. Abbreviations:  $\beta$ -HBA,  $\beta$ -hydroxybutyrate.



Figure 6.5 Breath Acetone in Control and Ketotic Rats

Each rat was left in a gas collection tank for 20 min. Points and error bars represent means  $\pm$  SEM; n = 6 rats/group. Different letters (a & b) denote breath acetone concentrations that were significantly different at the same time point (p < 0.05). From age 20 d - 23 d, ketotic rats were fed a 1:1 flaxseed oil diet and from age 24 d - 27 d, ketotic rats were fed a 2:1 flaxseed oil diet. A 3.5:1 flaxseed oil diet was fed to ketotic rats from age 28 d until the termination of the experiment. Control rats were fed the AIN 93G diet throughout the entire experiment. Food was offered to all rats *ad lib*.



# Figure 6.6 Breath Acetone as a Predictor of Plasma $\beta$ -HBA in a Rat Model of the Ketogenic Diet

The relationship is described by a one phase exponential equation. The equation of the line is  $y = 7.4071 \, \text{(1-exp)(-0.0017 * breath acetone, nM/kg/min)}$ . Abbreviations:  $\beta$ -HBA,  $\beta$ -hydroxybutyrate.

## 6.1.3 Discussion of Experiment 1

Although ketotic rats consumed significantly more kilocalories compared to control rats (Table 6.1), they weighed less (Figure 6.1) and gained significantly less weight (Figure 6.2) than control rats. The vitamin, mineral, and protein contents of the control and ketogenic diets were approximately balanced by weight. Although ketotic rats had a higher energy intake, they consumed significantly less food (in terms of weight) compared to control rats. Thus, ketotic rats consumed significantly lower amounts of protein, vitamins, and minerals. Because the rats were growing, the difference in the amount of protein, vitamin, and mineral consumption may explain why ketotic rats did not gain as much weight as control rats.

Ketotic rats had significantly lower blood glucose (Figure 6.3) and significantly higher plasma  $\beta$ -HBA (Figure 6.4) and breath acetone (Figure 6.5) compared to control rats. The lowest blood glucose and the highest plasma  $\beta$ -HBA and breath acetone were reached between 37 – 41 d of age; afterwards, blood glucose increased and plasma  $\beta$ -HBA and breath acetone decreased until plateaus were reached. The age range between 37 – 41 d corresponds to the time that rats undergo puberty. Pre-pubescent rats are capable of attaining higher ketone levels (Swink et al., 1997); thus, this may explain why ketone levels in post-pubescent rats decreased. However, an unpublished study (Likhodii et al., 1999) that examined changes in ketone levels in adult rats also observed similar trends (i.e. the initial increase in ketones and decrease in blood glucose upon initiation of the KD followed by a reduction in ketones and increase in blood glucose shortly after). Thus, plasma ketones may decline not because of puberty but due to a more efficient uptake and utilization of them, especially by the brain. There may be some adaptation period in which there is an increase in the transcription and translation of the appropriate proteins and enzymes necessary for ketone body uptake and utilization. Thus, plasma ketones may appear high when uptake and utilization are low, and plasma ketones may appear low when uptake and utilization become more efficient.

Breath acetone and plasma  $\beta$ -HBA were significantly correlated (Figure 6.6). The relationship between breath acetone and plasma  $\beta$ -HBA was best described by a one phase exponential equation.

## 6.2 EXPERIMENT 2: KETOSIS IN HEALTHY ADULTS FOLLOWING THE INGESTION OF KETOGENIC MEALS

#### 6.2.1 Overview of Experiment 2

Six individuals participated in this study. The subjects each gave their informed consent prior to participating in the study (see Appendix D). Each study subject was required to undergo a 12-h overnight fast, and upon arrival to the laboratory, fasting blood, breath, and urine samples were taken. The fasting time point was considered to be time = 0 h. Each subject was given a ketogenic meal immediately following the collection of the fasting samples and every three hours thereafter until a total of four ketogenic meals were consumed; that is, a ketogenic meal was consumed shortly after 0 h, 3 h, 6 h, and 9 h. Blood, breath, and urine samples were collected hourly. Each of the ketogenic meals that were consumed by the subject was identical in both composition and caloric value. All together, the ketogenic meals provided the subject with 75% of his/her daily caloric requirements. For details on the composition of the ketogenic meals and the calculation of daily caloric requirements, refer to Tables 5.5 and 5.4, respectively. Subjects were also asked to consume 125 ml of water every hour in order to be able to give an hourly urine sample.

Several physiological measures were made every hour during this experiment. Blood glucose was measured on fresh, whole blood, and after the blood samples were centrifuged, the plasma was immediately analyzed for  $\beta$ -HBA and AcAc. Urinary AcAc was measured both enzymatically and qualitatively using Ketostix dipsticks (Bayer, Etobicoke, Ontario). Breath samples were immediately analyzed for acetone.

#### 6.2.2 <u>Results of Experiment 2</u>

Six subjects participated in this study. Their profiles are depicted in Table 6.2.

Blood glucose at each time point and changes in blood glucose over time are depicted in Figure 6.7. As the figure shows, each time a ketogenic meal was ingested, blood glucose was driven slightly lower. The fasting blood glucose of the six subjects was 5.4 mM  $\pm$  0.4 mM. By the end of the study day, blood glucose fell significantly to 4.6 mM  $\pm$  0.3 mM (p < 0.001). Because blood glucose cannot drop indefinitely, there must be a point at which blood glucose becomes constant. Unfortunately, the 12-h dietary period assessed was not long enough to determine, with certainty, the approximate time point and concentration at which blood glucose levels off.

Figure 6.8 depicts plasma  $\beta$ -HBA at each time point and changes in plasma  $\beta$ -HBA over time. The fasting plasma  $\beta$ -HBA was 0.23 mM  $\pm$  0.15 mM. By the end of the study, plasma  $\beta$ -HBA increased significantly to 0.59 mM  $\pm$  0.26 mM (p < 0.01). With the ingestion of each ketogenic meal, plasma  $\beta$ -HBA became transiently elevated. The elevations in plasma  $\beta$ -HBA were not described by a smooth line but by a jagged one comprised of several peaks and valleys. In general, the peaks appeared to correspond to the hour following the consumption of a ketogenic meal while the valleys seemed to correspond to the second or third hour following the consumption of a ketogenic meal.

Plasma AcAc at each time point and changes in plasma AcAc over time are depicted in Figure 6.9. Similar to plasma  $\beta$ -HBA, plasma AcAc increased with the consumption of each ketogenic meal. The fasting plasma AcAc of the six subjects was 0.12 mM  $\pm$  0.11 mM. By the
#### Table 6.2

SUBJECT	GENDER	AGE	BMI <sup>2</sup>	SMOKER?
1	F	41	18.2	Y <sup>3</sup>
2	М	29	31.2	N
3	М	38	25.8	N
4	F	24	21.5	N
5	F	24	19.4	Y <sup>4</sup>
6	М	24	30.9	N
Mean $\pm$ SD	3 M/3 F	30 ± 8	24.5 ± 5.7	2 Y/4 N

#### Profiles of Subjects Involved in Experiment 2

<sup>1</sup> Age was measured in years.

<sup>2</sup> BMI was measured in  $kg/m^2$ .

<sup>3</sup> Subject 1 smoked heavily; she smoked during the day of the study but not within 30 minutes of blood, breath, and urine sampling.

<sup>4</sup> Subject 5 smoked occasionally but did not smoke the night before and the day of the study.

Abbreviations: F, female; M, male; BMI, body mass index; Y, yes; N, no.



Figure 6.7 A Blood Glucose as a Function of Time

#### Figure 6.7 B Changes in Blood Glucose Over Time

Points and bars represent means and SEM; n = 6/point. Dashed lines indicate the times at which a ketogenic meal was consumed. \* represents a blood glucose value/change that was significantly different from the fasting blood glucose at time = 0 h (p < 0.05).

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### Figure 6.8 A Plasma B -HBA as a Function of Time

#### Figure 6.8 B Changes in Plasma 6 -HBA Over Time

Points and error bars represent means  $\pm$  SEM; n = 6 /point. Dashed lines indicate the times at which a ketogenic meal was consumed. \* represents a plasma  $\beta$ -HBA value/change that was significantly different from the fasting plasma  $\beta$ -HBA at time = 0 h (p < 0.05). Abbreviations:  $\beta$ -HBA,  $\beta$ -hydroxybutyrate.



Figure 6.9 A Plasma AcAc as a Function of Time

#### Figure 6.9 B Changes in Plasma AcAc Over Time

Points and error bars represent means and SEM; n = 6/point. Dashed lines indicate the times at which a ketogenic meal was consumed. \* represents a Plasma AcAc value/change that was significantly different from the fasting plasma AcAc at time = 0 h (p < 0.05). Abbreviations: AcAc, acetoacetate.

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end of the 12-h study, plasma AcAc increased significantly to 0.38 mM  $\pm$  0.12 mM (p < 0.05). Unlike changes in plasma  $\beta$ -HBA, changes in plasma AcAc were described by a much smoother curve without defined peaks and troughs. As with blood glucose and plasma  $\beta$ -HBA, there did not appear to be a plateauing of plasma AcAc by the end of the 12-h study period.

Figure 6.10 illustrates breath acetone at each time point and changes in breath acetone over time. Similar to plasma  $\beta$ -HBA and AcAc, breath acetone progressively increased with the consumption of each ketogenic meal. The fasting breath acetone of the six subjects was 37 nM  $\pm$  48 nM. By the end of the 12-h study, breath acetone increased significantly to 99 nM  $\pm$  35 nM (p < 0.001). Changes in breath acetone are similar to those observed with plasma AcAc in that the changes were smooth; they did not fluctuate in the same manner that plasma  $\beta$ -HBA, and plasma AcAc, breath acetone failed to plateau within the 12-h period studied.

Urinary AcAc as a function of time is depicted in Figure 6.11. Urinary AcAc concentrations in Figure 6.11 A were determined using Ketostix dipsticks (Bayer, Etobicoke, Ontario) while urinary AcAc concentrations in Figure 6.11 B were determined using the enzymatic assay that was used to determine plasma AcAc. Regardless of the analytical method used, urinary AcAc increased progressively during the consumption of a KD. Initially, urinary AcAc was undetectable, when measured by Ketostix dipsticks, and 0.07 mM  $\pm$  0.06 mM, when measured enzymatically. By the end of the 12-h study period, urinary AcAc rose to 1.6 mM  $\pm$  1.5 mM when measured by Ketostix dipsticks (p = 0.6489), and to 1.02 mM  $\pm$  1.13 mM when measured enzymatically (p = 0.2392). The increase in urinary AcAc did not occur smoothly; rather the concentration of AcAc in the urine fluctuated in a manner similar to that of plasma  $\beta$ -



Figure 6.10 A Breath Acetone as a Function of Time

#### Figure 6.10 B Changes in Breath Acetone Over Time

Points and error bars represent means and SEM; n = 6/point. Dashed lines indicate the times at which a ketogenic meal was consumed.  $\bullet$  represents a breath acetone value/change that was significantly different from the fasting breath acetone at time = 0 h (p < 0.05).

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#### Figure 6.11 B Urinary AcAc as Determined Enzymatically

Points and error bars represent means and SEM; n = 6/point. Dashed lines indicate the times at which a ketogenic meal was consumed.  $\bullet$  represents a urinary AcAc value that was significantly different from the fasting urinary AcAc at time = 0 h (p < 0.05). Abbreviations: AcAc, acetoacetate.

HBA. Again, as with blood glucose, plasma AcAc, plasma  $\beta$ -HBA, and breath acetone, urinary AcAc failed to stabilize in the 12-h period studied.

In the home setting, parents rely on Ketostix dipsticks to monitor their child's ketone levels. In this study, urinary AcAc was measured in two ways: i.) Using Ketostix dipsticks; and ii.) Enzymatically. A linear regression analysis between urinary AcAc obtained with the dipsticks and urinary AcAc obtained enzymatically for each subject is depicted in Figure 6.12. Figure 6.13 shows that there was a significant correlation between Ketostix urinary AcAc and urinary AcAc determined enzymatically ( $r^2 = 0.94$ ; p < 0.0001); however, the ketostix dipsticks consistently overestimated the actual urinary AcAc concentration. Thus, the Ketostix urinary AcAc values were corrected using the equation found in Figure 6.13.

One of the primary objectives of this study was to determine whether breath acetone was a significant predictor of plasma  $\beta$ -HBA and plasma AcAc. If breath acetone was found to be a significant predictor of the plasma ketones, then a second objective was to determine whether breath acetone was a better predictor of the plasma ketones than urinary AcAc. In order to address these issues, several linear regression analyses had to be conducted. First, the ability of breath acetone to predict plasma AcAc was determined. Next, the ability of breath acetone to predict plasma AcAc was determined. Next, the ability of breath acetone to predict plasma  $\beta$ -HBA was assessed. The ability of corrected Ketostix urinary AcAc to predict the concentration of each of the plasma ketones (AcAc and  $\beta$ -HBA) was finally determined. The raw data points depicting the relationships for each individual are shown in Figures 6.14 – 6.17. Figures 6.18 and 6.19 show the relationship between breath acetone and each of the plasma ketones.



#### Figure 6.12 The Relationship Between Urinary AcAc Measured Enzymatically and Urinary AcAc Measured with Ketostix Dipsticks

n = 12 - 13 points/linear regression analysis. In general, Ketostix dipsticks overestimated the actual urinary AcAc concentration determined enzymatically. Abbreviations: AcAc, acetoacetate.



# Figure 6.13 The Relationship Between Urinary AcAc Determined Enzymatically and Ketostix Urinary AcAc

Points and error bars represent means  $\pm$  SEM at each time point; n = 6/point. Dashed lines represent 95% confidence intervals. Equation of the line: y = 0.3824(Ketostix, mM) + 0.1426. Abbreviations: AcAc, acetoacetate.



# Figure 6.14 The Relationship Between Plasma AcAc and Breath Acetone For Each Subject

n = 11 - 13 points/linear regression analysis. Abbreviations: AcAc, acetoacetate.



# Figure 6.15 The Relationship Between Plasma $\beta$ -HBA and Breath Acetone for Each Subject

n = 12 - 13 points/linear regression analysis. Abbreviations:  $\beta$ -HBA,  $\beta$ -hydroxybutyrate.



#### Figure 6.16 The Relationship Between Plasma AcAc and Corrected Ketostix Urinary AcAc For Each Subject

n = 11 - 13 points/linear regression analysis. Ketostix urinary AcAc values were corrected using the equation y = (0.3284)(Ketostix, mM) + 0.1426. Abbreviations: AcAc, acetoacetate.



# Figure 6.17 The Relationship Between Plasma $\beta$ -HBA and Corrected Ketostix Urinary AcAc for Each Subject

n = 12 - 13 points/linear regression analysis. Ketostix urinary AcAc values were corrected using the equation y = (0.3284)(Ketostix, mM) + 0.1426. Abbreviations: AcAc, acetoacetate;  $\beta$ -HBA,  $\beta$ -hydroxybutyrate.



Figure 6.18 The Relationship Between Plasma AcAc and Breath Acetone

Points and error bars represent means  $\pm$  SEM at each time point; n = 6/point. Dashed lines represent the 95% confidence intervals. Equation of the line: y = 0.0043(breath acetone, nM) + 0.0050. Abbreviations: AcAc, acetoacetate.



# Figure 6.19 The Relationship Between Plasma $\beta$ -HBA and Breath Acetone

Points and error bars represent means  $\pm$  SEM at each time point; n = 6/point. Dashed lines represent the 95% confidence intervals. Equation of the line: y = (0.0062)(breath acetone, nM) + 0.0709. Abbreviations:  $\beta$ -HBA,  $\beta$ -hydroxybutyrate.



## Figure 6.20 The Relationship Between Plasma AcAc and Corrected Ketostix Urinary AcAc

Points and error bars represent means  $\pm$  SEM at each time point; n = 6/point. Dashed lines represent the 95% confidence intervals. Ketostix Urinary AcAc values were corrected using the equation y = (0.3284)(Ketostix, mM) + 0.1426. Equation of the line: y = (0.2192)(Corrected Ketostix, mM) + 0.1411. Abbreviations: AcAc, acetoacetate.



# Figure 6.21 The Relationship Between Plasma $\beta$ -HBA and Corrected Ketostix Urinary AcAc

Points and error bars represent means  $\pm$  SEM at each time point; n = 6/point. Dashed lines represent the 95% confidence intervals. Ketostix urinary AcAc values were corrected using the equation y = (0.3284)(Ketostix, mM) + 0.1426. Equation of the line: y = (0.3026)(Corrected Ketostix, mM) + 0.2718. Abbreviations: AcAc, acetoacetate;  $\beta$ -HBA,  $\beta$ -hydroxybutyrate. Breath acetone was a significant predictor of both plasma AcAc ( $r^2 = 0.88$ ; p < 0.0001; Figure 6.18) and plasma  $\beta$ -HBA ( $r^2 = 0.66$ ; p < 0.0001; Figure 6.19). Corrected Ketostix urinary AcAc was also a significant predictor of plasma AcAc ( $r^2 = 0.72$ ; p = 0.0003; Figure 6.20), and of plasma  $\beta$ -HBA ( $r^2 = 0.52$ ; p = 0.0057; Figure 6.21). For a summary of the equations of each of the lines of best fit as well as other statistical results, refer to Table 6.3.

#### 6.2.3 Discussion of Experiment 2

During the consumption of a KD, breath acetone and plasma AcAc changed more smoothly than blood glucose, plasma  $\beta$ -HBA, and urinary AcAc (Figures 6.7 – 6.11). Urinary AcAc determined with Ketostix dipsticks was significantly correlated with urinary AcAc determined enzymatically (Figure 6.13); however, the Ketostix dipsticks generally overestimated the actual urinary AcAc concentration and a correction factor had to be applied to Ketostix urinary AcAc values (Figure 6.13). Breath acetone was significantly correlated with both plasma AcAc (Figure 6.18) and plasma  $\beta$ -HBA (Figure 6.19). As well, corrected Ketostix urinary AcAc was significantly correlated with both plasma AcAc (Figure 6.20) and plasma  $\beta$ -HBA (Figure 6.21).

#### Table 6.3

RELATIONSHIP 7 **EQUATION OF THE LINE OF BEST** SIGNIFICANCE FIT Ketostix Urinary y = 0.3824(Ketostix, mM) + 0.1426 0.94 p < 0.0001 AcAc & Urinary AcAc Determined Enzymatically Breath Acetone & p < 0.0001y = 0.0043 (breath acetone, nM) + 0.0050 0.88 Plasma AcAc y = 0.0062 (breath acetone, nM) + 0.0709 Breath Acetone & 0.66 p < 0.0001 Plasma B-HBA Corrected<sup>1</sup> y = 0.0715 (Ketostix, mM) + 0.1696 0.72 p = 0.0003Ketostix Urinary AcAc & Plasma AcAc Corrected<sup>1</sup> y = 0.1002 (Ketostix, mM) + 0.3090 0.52 p = 0.0057Ketostix Urinary AcAc and Plasma β-ΗΒΑ

Results of Linear Regression Analyses

<sup>1</sup>Because the Ketostix dipsticks generally overestimated actual urinary AcAc concentrations determined enzymatically, Ketostix values were corrected for using the equation y = 0.3824(Ketostix, mM) + 0.1426.

Abbreviations: AcAc, acetoacetate;  $\beta$ -HBA,  $\beta$ -hydroxybutyrate.

#### CHAPTER 7.0 DISCUSSION

#### 7.1 CHANGES IN METABOLITES

#### 7.1.1 Rats

In Experiment 1, when rats in chronic ketosis were studied, it was observed that, in general, between 37 d – 41 d of age, plasma  $\beta$ -HBA (Figure 6.4) and breath acetone (Figure 6.5) reached a maximum, while blood glucose reached a minimum (Figure 6.3). After this age range, blood glucose, plasma  $\beta$ -HBA and breath acetone began to plateau, but were still significantly different from control values (p < 0.05). Interestingly, rats reach puberty in this age range and since pre-pubescent rats are capable of attaining higher ketone levels (Swink et al., 1997), this may explain why the ketones began to decline and blood glucose began to increase. However, in an unpublished study (Likhodii et al., 1999), the same trends in blood glucose and plasma  $\beta$ -HBA were observed in adult rats. Thus, puberty may not be the reason why ketone levels suddenly decrease. An alternative explanation may be that at the molecular level, there is an increase in the transcription and translation of key proteins and enzymes necessary for the uptake and utilization of ketones (Likhodii et al., 1999). Thus, an increased uptake/utilization of ketones by certain organs (i.e. brain, heart, skeletal muscle, kidney cortex) could result in reduced plasma ketones.

#### 7.1.2 Humans

When acute ketosis in the adult volunteers was studied, it was observed that, in general, plasma  $\beta$ -HBA, plasma AcAc, and breath acetone increased while blood glucose decreased over time. The increase in plasma AcAc (Figure 6.9) and breath acetone (Figure 6.10), however, seemed much more consistent and far less sensitive to the actual consumption of a ketogenic meal compared to plasma  $\beta$ -HBA (Figure 6.8), which appeared to increase in a series of steps rather than smoothly. During acute ketosis, plasma  $\beta$ -HBA fluctuated in a manner similar to that of blood glucose in that the time of last meal consumption had a significant effect. Upon the consumption of a ketogenic meal, blood glucose would sharply decrease (Figure 6.7) while plasma  $\beta$ -HBA would sharply increase (Figure 6.8). Breath acetone and plasma AcAc were not so sensitive to the consumption of a ketogenic meal. This needs further study and may have clinical implications. If plasma  $\beta$ -HBA is the ketone being measured, then when it is measured in relation to the consumption of a ketogenic meal needs consideration. Furthermore, perhaps plasma  $\beta$ -HBA is not the ketone (or at least not the only ketone) that should be monitored.

### 7.2 BREATH ACETONE AND URINARY ACAC AS PREDICTORS OF PLASMA KETONES

Although the mechanism of the KD is unknown, its efficacy seems to be dependent upon the maintenance of blood levels of  $\beta$ -HBA above 2 mM and of AcAc above 0.6 mM (Huttenlocher, 1976). The KD is initiated in a hospital setting where blood  $\beta$ -HBA is often monitored. When parents take their child home and continue with the KD, the only noninvasive analytical tool they have to assess their child's ketone levels is the Ketostix dipstick urinary test for ketosis. A few studies have found the Ketostix urinary test for ketosis to be a poor indicator of blood ketones (Livingston, 1972 and Schwartz et al., 1989).

One of the objectives of this study was to determine whether the urinary test for ketosis is reliable and whether it is a significant predictor of plasma  $\beta$ -HBA and/or plasma AcAc. Urinary AcAc determined with Ketostix dipsticks correlated strongly with urinary AcAc determined enzymatically ( $r^2 = 0.94$ , p < 0.0001; Figure 6.13); however, urinary AcAc values obtained with the Ketostix dipsticks were usually more exaggerated than the actual urinary AcAc determined enzymatically, and a correction factor had to be applied to Ketostix urinary AcAc values. It was found that corrected Ketostix urinary AcAc was significantly correlated with both plasma AcAc ( $r^2 = 0.72$ , p = 0.0003; Figure 6.20) and plasma  $\beta$ -HBA ( $r^2 = 0.52$ , p = 0.0057; Figure 6.21).

A second objective of this study was to determine whether significant correlations existed between breath acetone and each of the plasma ketones. In adults in acute ketosis, breath acetone was significantly correlated with both plasma AcAc ( $r^2 = 0.88$ , p < 0.0001; Figure 6.18) and plasma  $\beta$ -HBA ( $r^2 = 0.66$ , p < 0.0001; Figure 6.19). In rats consuming a 3.5:1 flaxseed oil-based KD for over one month, breath acetone was significantly correlated with plasma  $\beta$ -HBA ( $r^2 = 0.73$ , p < 0.0001; Figure 6.6). All of the correlations aforementioned were linear except the relationship between breath acetone and plasma  $\beta$ -HBA in the rat model of the KD. This relationship was curvilinear and was best described by a one phase exponential equation. Unfortunately, because plasma AcAc was not analyzed immediately, plasma AcAc values were not obtained and correlations between breath acetone and plasma AcAc could not be examined in our rat model of the KD.

It was hypothesized that in rats and humans consuming a KD, a significant, positive correlation would be observed between breath acetone and plasma AcAc and  $\beta$ -HBA, and that breath acetone would be a more significant indicator of these plasma ketones than the urinary test for ketosis. As was predicted, a significant, positive correlation was observed between breath acetone and each of the plasma ketones. Results of the correlation analyses revealed that both breath acetone and urinary AcAc were significantly correlated with each of the plasma ketones; however, the correlations involving breath acetone had higher  $r^2$ values. It is premature to state with absolute certainty that breath acetone is a more reliable indicator of the plasma ketones compared to the urinary ketone dipstick test because the studies conducted thus far have limitations. Although chronic ketosis was examined in the rat study, neither plasma nor urinary AcAc were measured; thus, correlations dealing with these parameters could not be assessed. In the human study, the sample size studied was small (n =6) and the dietary study period assessed was relatively short (i.e. urinary AcAc may be a very reliable indicator of plasma ketones in subjects in chronic, as opposed to acute, ketosis). When chronic ketosis in a larger sample size is examined, a multiple linear regression analysis that controls for inter-subject variability (i.e. gender, body mass index, smoking status, age, etc.) can be conducted and mathematical models can be generated that actually predict a plasma ketone concentration from a breath acetone/urinary AcAc concentration. The generation of such mathematical models at this stage is premature.

Overall, the preliminary results of Experiments 1 and 2 are encouraging and suggest that although both breath acetone and Ketostix urinary AcAc are significantly correlated with the plasma ketones, breath acetone may, in fact, be a better indicator of plasma ketones than the urinary dipstick ketone test.

#### 7.3 IMPLICATIONS

AcAc is the only ketone that is actually involved in energy production and lipogenesis (Mitchell et al., 1995). B-HBA is important only in the sense that it has the potential to be converted to AcAc via the enzyme,  $\beta$ -HBDH; otherwise, it is considered a "dead-end metabolite" (Mitchell et al., 1995). While it has been observed that a minimum blood concentration of about 2 mM of  $\beta$ -HBA is necessary for the KD to be effective (Huttenlocher, 1976), it may be that plasma  $\beta$ -HBA concentrations in great excess of 2 mM are unnecessary and perhaps even potentially harmful (Mitchell et al., 1995). As stated in the literature review, a high ratio of plasma B-HBA/plasma AcAc is indicative of a reduced mitochondrial matrix (i.e. the ratio of NADH/NAD<sup>+</sup> is high). In the mitochondria, many reactions occur which require the transfer of electrons. NADH and NAD<sup>+</sup> are involved in most of these reactions since they are capable of donating and accepting electrons, respectively. When there is more NADH than NAD<sup>+</sup> in the mitochondria, the reactions requiring electrons from NADH (such as the production of ATP in the electron transport chain) are favored over those that require NAD<sup>+</sup> to accept electrons. It has been hypothesized that the efficacy of the KD is dependent on a reduced mitochondrial matrix in which the synthesis of ATP is more efficient (Kashiwaya et al., 1997). However, if the ratio of NADH:  $NAD^+$  is too high (i.e. the ratio of plasma  $\beta$ -HBA:plasma AcAc is too high), a case of "overkill" can arise with respect to the reducing environment of the mitochondria, since

insufficient amounts of AcAc would be available to the TCA cycle for energy production (Kashiwaya et al., 1997).

Whether the mechanism of the KD is more dependent on plasma AcAc or on the ratio of plasma  $\beta$ -HBA:plasma AcAc, the importance of plasma AcAc should not be neglected. Plasma AcAc is difficult to measure because AcAc is a volatile compound and must be analyzed immediately (Mitchell et al., 1995). Breath acetone is produced directly from the spontaneous decarboxylation of AcAc, and it is significantly correlated with plasma AcAc, as observed in this thesis. Furthermore, breath acetone is also significantly correlated with plasma  $\beta$ -HBA. Because breath acetone analysis is non-invasive and because it can be performed as frequently as necessary (unlike urinary AcAc analysis), it has the potential to become an important tool for parents with a child on the KD.

#### 7.4 ACHIEVEMENT OF OBJECTIVES

Several objectives were outlined in Chapter 3. The following is a review of these objectives as well as a Y (for yes) or N (for no) indicating whether or not each objective was achieved

- Develop a system for breath acetone collection and analysis in a rat model of the KD. Y
- Examine correlations between breath acetone and plasma AcAc and  $\beta$ -HBA in a rat model of the KD. Y/N

Because AcAc is a volatile compound, it must be analyzed immediately. Since the rat plasma samples were frozen prior to AcAc analysis, all of the AcAc within the plasma samples was lost and reliable AcAc data could not be obtained. Thus, correlations between breath acetone and plasma AcAc could not be examined in the rat model of ketosis.

• Develop a system of breath acetone collection and analysis for humans. Y/N

Although a system of breath acetone collection and analysis for humans was developed, this system had limitations. Breath samples could not be stored in tubes for longer than 6 min without losing a significant amount of the acetone. Thus, subjects had to be present in the laboratory during breath collection so that breath samples could be injected directly into the gas chromatograph. This is not very convenient, especially if the subjects we wish to work with in the future are hospitalized epileptic patients being initiated on the KD. Thus, the system of breath collection and analysis for humans must be modified in order to accommodate future off-site studies.

- Examine correlations between breath acetone and plasma AcAc and β-HBA in adult volunteers in acute/short-term ketosis. Y
- Examine correlations between urinary AcAc and plasma AcAc and β-HBA in adult volunteers in acute/short-term ketosis. Y

#### 7.5 FUTURE STUDIES

These findings regarding the reliability of breath acetone as an indicator of ketosis are promising; however, more research needs to be conducted. Rats in a state of long-term ketosis have been studied, but adults in such a state have not. Furthermore, the relationship between breath acetone and each of the plasma ketones needs to be examined in children both in an acute and long-term state of ketosis. In order to address these issues, several methodological problems must be addressed. As the situation stands, breath acetone samples cannot be stored in evacuated glass tubes for longer than 6 minutes (see section 5.4.2). Thus, either a method of breath sample storage must be developed or a portable breath acetone analyzer must be created. Also, children with epilepsy are often developmentally and mentally delayed and getting a breath sample from them may be quite challenging. Thus, the method of breath collection must be modified. When these issues are resolved, it will be more practical to study children on the KD in clinical/home settings.

#### 7.6 POTENTIAL CLINICAL APPLICATIONS OF BREATH ACETONE ANALYSIS

Although this thesis has focused on the therapeutic effects of the KD in treating intractable epilepsy, breath acetone analysis is not limited to this area. Other populations may benefit from breath acetone analysis. Breath acetone has already been found to correlate with metabolic states (ie. weight loss, weight gain, diabetes mellitus) both in newborn babies and in children (Nelson et al., 1998). Diabetics, for whom ketoacidosis and diabetic coma are life-threatening, can benefit greatly from breath acetone analysis.

The KD is being used to treat conditions other than intractable epilepsy. The KD has been used for weight loss purposes (Ross et al., 1991; Saunders et al., 1993), and breath acetone analysis may be an important tool for monitoring progress in KD-based weight loss programs. Also, the KD is being studied for its therapeutic use in the treatment of certain cancers. Associated with cancer is cancer cachexia, a catabolic state in which there is accelerated weight loss (Beck et al., 1989). The weight loss arises primarily from the increased energy needs of tumor cells. Tumor cells are known to have a high rate of glucose metabolism and to be susceptible to carbohydrate deprivation (Fearon et al., 1988). Furthermore, many tumors are obligate glucose metabolizers since they lack certain key mitochondrial enzymes necessary for fat/ketone body metabolism (Fearon et al., 1988). Thus, a rationale for implementing the KD in certain cancer patients is that the tumor might be deprived of energy while an adequate energy supply would be maintained to the host (Fearon et al., 1988). An MCT-based KD has already been shown to induce significant weight gain in severely cachectic patients (Fearon et al., 1988) and to prevent weight loss and reduce the tumor burden in a mouse model of cachexia, (Tisdale et al., 1987; Beck et al., 1989). In the future, breath acetone analysis may become an important, non-invasive indicator of ketosis for cancer patients consuming a KD.

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### 9.0 APPENDICES

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#### APPENDIX A

### EXPERIMENT 3: THE EFFECT OF A FLAXSEED OIL KETOGENIC DIET ON SEIZURE PROTECTION IN A RAT MODEL OF KETOSIS

#### Objective

• To determine the effects of a flaxseed oil KD on seizure protection in a rat model of ketosis.

#### Hypothesis

• Rats consuming a flaxseed oil KD will exhibit seizure protection when stimulated by a convulsant.

#### Materials and Methods

The control and ketotic rats that were seizure tested were the same rats used in Experiment 1. For an overview of the experiment (i.e. when the rats were weaned, length of time on the diet, etc.), refer to section 6.1.1. For differences in blood glucose, plasma  $\beta$ -HBA, breath acetone, and food intake between control and ketotic rats, refer to section 6.1.2.

A PTZ seizure test was conducted when the rats of Experiment 1 were 53 d old, using a dosage of 80 mg/kg, injected i.p. (This particular dosage and route of administration were used because in a study by Yehuda et al. (1994), rats comparable in age and strain to the rats of this experiment and receiving an essential fatty acid preparation, were challenged with 100 mg/kg of PTZ, i.p.) The seizure test was videotaped and several parameters were measured including incidence of generalized tonic-clonic seizures, latency of seizure onset, and incidence of mortality. The timeframe assessed was the fifteen minutes post injection.

#### Results

Neither control nor ketotic rats exhibited seizure protection during the PTZ seizure test (see Table A-1).

#### Discussion

Although seizure protection in the ketotic rats was not observed, it is very likely that the dosage of PTZ used to induce the seizures was too strong and way above the threshold of the KD.
## Table A-1

Results of Seizure Test<sup>1</sup>

VARIABLE ASSESSED	CONTROL GROUP	KETOGENIC GROUP		
Incidence of Seizures <sup>2</sup>	5/6	6/6		
Latency to seizures <sup>3</sup>	<b>48.3</b> ± 15.0	<b>46.7 ± 8.7</b>		
Incidence of Deaths	6/7	7/7		
Incidence of Deaths	6/7	7/7		

<sup>1</sup> Seizures were induced by injecting rats with the convulsant, pentylenetetrazol, at a dosage of 80 mg/kg, i.p; results indicate events which occurred in the fifteen minutes post-injection.

<sup>2</sup> Number of rats having a generalized tonic-clonic seizure.

<sup>3</sup> Latency to the first generalized tonic-clonic seizure was measured in seconds.

### **APPENDIX B**

# EXPERIMENT 4: PENTYLENETETRAZOL (PTZ) DOSE RESPONSE STUDY

#### Objective

• To determine the dose of PTZ required to elicit sub-maximal seizures (generalized tonicclonic, without hind limb extension) in the rats of Experiment 5 (see Appendix C).

### Hypothesis

• The dose of PTZ required to elicit sub-maximal seizures in the rats of Experiment 5 is less than 80 mk/kg and should be injected s.c. rather than i.p.

#### Materials and Methods

The rats in Experiment 5 (see Appendix C) varied greatly in their body weights (i.e. MCT rats weighed approximately half as much as control rats); thus, it was necessary to decide whether to conduct the PTZ dose-response study on rats that were similar in weight to the heavier (control) rats or the lighter (MCT) rats. A study conducted by Grecksch et al., (1997) indicated that younger (lighter) rats have a higher seizure threshold compared to older rats. Thus, rats similar in weight to the control rats of Experiment 5 were used for the PTZ dose-response study.

Forty-eight male albino Wistar rats weighing between 251 g and 275 g were ordered for this study. Upon arrival, the rats were given four days to acclimitize to the environment and were allowed unlimited access to the AIN 93G diet (for details on this diet, refer to section 5.2.1). Rats were then randomly assigned to one of four groups (n = 12 per group), and the PTZ dose-response test was conducted.

#### Results

The first group of twelve rats was stimulated with PTZ at a dosage of 60 mg/kg, injected s.c., and observed for thirty minutes. Many of these rats had several seizures each, and although these seizures were not maximal (generalized tonic-clonic with hind limb extension) they were prolonged. As well, some of the rats died. Thus, the next group of twelve rats was injected with 50 mg/kg PTZ, injected s.c. Although none of these rats died, all of them experienced mild seizures (generalized tonic-clonic seizures without hind limb extension).

Six of the twelve rats in the next group were stimulated with PTZ at a dosage of 40 mg/kg, injected s.c. Although the seizures induced by this dosage were mild, not all of the rats had seizures. Thus, the other six rats were stimulated with PTZ at a dosage of 45 mg/kg, injected s.c. Again, the seizures induced were mild, but not all six rats experienced seizures. Thus, it was decided that the optimal dosage of PTZ that should be used when seizure testing the rats of Experiment 5 was 50 mg/kg, injected s.c. (Table B-1). The final group of twelve rats were seizure tested the day the rats of Experiment 5 were seizure tested to ensure the effects observed

were reproducible.

B-ii

### Table B-1

DOSAGE <sup>T</sup>	SAMPLE SIZE	INCIDENCE OF SEIZURES <sup>2</sup>	MJs	FFC	FLE	HLE	DEATHS
60	12	12	0	0	12	0	4
50	12	12	0	0	12	0	0
40	6	3	3	2	1	0	0
45	6	5	1	1	4	0	0

# Results of Pentylenetetrazol (PTZ) Dose-Response Study

<sup>1</sup> Dosages are expressed in mg/kg; all injections were given subcutaneously.

<sup>2</sup> Rats exhibiting only myoclonic jerks were not included.

Abbreviations: MJs - myoclonic jerks; FFC - face and forelimb clonus; FLE - forelimb extension; HLE - hindlimb extension.

## Discussion

The dosage of PTZ that was found to elicit 100% incidence of mild seizures (generalized tonic-clonic seizures without hind limb extension) in rats comparable in weight to the control rats of Experiment 5 was 50 mg/kg, s.c. (Table B-1).

•

## **APPENDIX C**

# EXPERIMENT 5: THE EFFECT OF FAT SOURCE ON PLASMA KETONES, BREATH ACETONE, AND SEIZURE PROTECTION IN A RAT MODEL OF THE KETOGENIC DIET

### **Objectives**

- To determine the effect of fat source on plasma β-HBA levels, breath acetone levels, and seizure protection in a rat model of the KD.
- To determine the effect of fat source on the correlation between breath acetone and plasma β-HBA.

#### Hypotheses

- As long as rats attain a plasma β-HBA concentration of at least 2 mM at the time of seizure-testing, there will be no effect of fat source on seizure protection.
- Rats in the MCT oil KD group will attain the highest breath acetone and plasma  $\beta$ -HBA.

### Materials and Methods

Six litters of 17 d old male albino Wistar rats (ten rats per litter) arrived at the facility with their dams. The pups were given three days to acclimatize to their new environment and at 20 d of age, the rats were weaned and randomly divided into one of five groups: control, butter-based KD, flaxseed oil-based KD, MCT oil-based KD, or fat mixture (consisting of equal proportions of butter, flaxseed oil, and MCT oil) KD. (Refer to section 5.1.1 for details on control and ketogenic diet compositions; the information presented on the flaxseed oil KD in section 5.1.1 is applicable to the other KDs as well.) Rats were housed in plastic cages

with wood chip bedding and were placed on a 12 h light/12 h dark cycle (lights were turned on at 7:00 AM). For the first eight days after being weaned, the rats were housed two to a cage in order to minimize the stresses associated with being weaned. From 28 d of age until the end of the experiment (68 d of age) rats were housed individually.

At 20 d of age, control rats were given the AIN 93 G diet *ad lib*. In order to assist the rats in the ketogenic diet groups to adjust to the 3.5:1 KDs, they were gradually weaned onto the diets; that is, at 20 d of age, these rats were given unlimited access to a 1:1 KD, and then at 24 d of age, they were given unlimited access to a 2:1 KD. *Ad lib* feeding of the 3.5:1 KD did not occur until the rats were 28 d old. The added fat source in the 1:1, 2:1, and 3.5:1 KDs was consistent (i.e. the butter-based KD group received a 1:1 butter-based KD for four days, a 2:1 butter-based KD for the next four days, and then the actual 3.5:1 butter-based KD from 28 d of age until the remainder of the experiment). All rats were weighed at 20 d of age and approximately every four days thereafter until the rats were 68 days of age. All the rats from each group underwent periodic blood and breath sampling. Also, these same rats underwent seizure testing when they were 68 d old, using the convulsant, PTZ.

The dosage of PTZ that would elicit only mild seizures in the experimental rats was determined in Experiment 4 (see Appendix B). The appropriate dosage was found to be 50 mg/kg of PTZ, injected s.c.

#### Results

Several unexpected problems with acetone analysis were encountered during the course of this experiment. The gas chromatograph began to malfunction early in the experiment; thus, breath acetone analyses could not be performed until later on in the

experiment (rats were 59 d old). Although breath acetone was analyzed when the gas chromatograph was repaired, the measurements were very inaccurate because the gas-tight syringe that was being used to inject the breath samples into the gas chromatograph had lost its seal. Thus, changes in breath acetone over time as well as correlations dealing with breath acetone could not be analyzed. Changes in weight, weight gain, blood glucose, and plasma  $\beta$ -HBA were monitored throughout the experiment. Because groups of rats were blood sampled on different days, across-group comparisons of final concentrations of blood glucose and plasma  $\beta$ -HBA were difficult to make. In order to estimate these differences, the averages of the last two measures of blood glucose and plasma  $\beta$ -HBA were calculated for each rat and these figures were used to determine differences between the five diet groups.

By the end of the experiment, rats in the control, butter, flaxseed, and mixture groups did not differ significantly in weight or weight gain; however, rats in the MCT group weighed significantly less and gained significantly less weight than all the other rats (p < 0.05; see Figures C-1 and Figure C-2).

Changes in blood glucose and plasma  $\beta$ -HBA over time are depicted in Figures C-3 and C-4, respectively. Final blood glucose and plasma  $\beta$ -HBA concentrations are presented in Table C-1. By the end of the experiment, control rats had the highest blood glucose and the lowest plasma  $\beta$ -HBA while MCT rats had the lowest blood glucose and the highest plasma  $\beta$ -HBA. Rats in the butter, flaxseed, and mixture diet groups had a very similar blood glucose and plasma  $\beta$ -HBA. For significant differences between the groups, refer to Table C-1.

Results of the PTZ seizure test (Table C-2) indicated there were no significant differences in the latency of seizures, severity of seizures, and incidence of mortality across



Figure C-1 Weights of Control and Ketotic Rats

Points and error bars represent means  $\pm$  SEM; n = 11 - 12 rats/group. Different letters (a&b) denote weights which were significantly different at 66 d of age (p < 0.05). From age 20 d to 23 d, ketotic rats were fed a 1:1 KD and from age 24 d to 27 d, ketotic rats were fed a 2:1 KD. A 3.5:1 KD was fed to ketotic rats from age 28 d until the termination of the experiment. Control rats were fed the AIN 93G diet throughout the entire experiment. Food was offered to all rats *ad lib*. Abbreviations: KD, ketogenic diet; MCT, medium chain triglyceride.



Figure C-2 Weight Gain of Control and Ketotic Rats

Points and error bars represent means  $\pm$  SEM; n = 11 - 12 rats/group. Different letters (a&b) denote weight gains which were significantly different 46 d after being weaned (p < 0.05). From age 20 d to 23 d, ketotic rats were fed a 1:1 KD and from age 24 d to 27 d, ketotic rats were fed a 2:1 KD. A 3.5:1 KD was fed to ketotic rats from age 28 d until the termination of the experiment. Control rats were fed the AIN 93G diet throughout the entire experiment. Food was offered to all rats ad lib. Abbreviations: KD, ketogenic diet; MCT, medium chain triglyceride.



Figure C-3 Blood Glucose in Control and Ketotic Rats

Points and error bars represent means  $\pm$  SEM; n = 11 - 12 rats/group. From age 20 d to 23 d, ketotic rats were fed a 1:1 KD and from age 24 d to 27 d, ketotic rats were fed a 2:1 KD. A 3.5:1 KD was fed to ketotic rats from age 28 d until the termination of the experiment. Control rats were fed the AIN 93G diet throughout the entire experiment. Food was offered to all rats ad *lib*. Abbreviations: KD, ketogenic diet; MCT, medium chain triglyceride.



Figure C-4 Plasma  $\beta$ -HBA in Control and Ketotic Rats

Points and error bars represent means  $\pm$  SEM; n = 11 - 12 rats/group. From age 20 d to 23 d, ketotic rats were fed a 1:1 KD and from age 24 d to 27 d, ketotic rats were fed a 2:1 KD. A 3.5:1 KD was fed to ketotic rats from age 28 d until the termination of the experiment. Control rats were fed the AIN 93G diet throughout the entire experiment. Food was offered to all rats ad lib. Abbreviations:  $\beta$  -HBA,  $\beta$ -hydroxybutyrate; KD, ketogenic diet; MCT, medium chain triglyceride.

## **Table C-1**

EXPERIMENTAL GROUP	<b>BLOOD GLUCOSE</b>	PLASMA β-HBA
Control	7.0 ± 0.7 *	0.11 ± 0.03 *
Butter	5.6 ± 0.3 <sup>b, c</sup>	0.95 ± 0.20 <sup>b</sup>
Flaxseed	6.2 ± 0.3 • b	0.77 ± 0.28 <sup>b</sup>
Mixture	6.1 ± 0.4 • •	1.06 ± 0.21 <sup>b, c</sup>
МСТ	3.5 ± 0.9 °	4.59 ± 2.01 °

Final Blood Glucose and Plasma β-HBA Concentrations of Control and Ketotic Rats<sup>1</sup>

<sup>1</sup> Final blood glucose and plasma  $\beta$ -HBA concentrations (in Mm) are averages of the last two measures taken for each rat.

A pair of letters that are the same within columns denote results which were not significantly different (p > 0.05). (i.e. For blood glucose, because butter and flaxseed share a "b", the blood glucose values were not significantly different.)

# Table C-2

Results of Seizure Test<sup>1</sup>

EXPERIMENTAL GROUP	INCIDENCE OF SEIZURES <sup>2</sup>	LATENCY <sup>3</sup>	SEVERITY *	INCIDENCE OF DEATHS <sup>5</sup>
Control	12/12 •	1041 ± 490 •	1.25 ± 0.45 •	0/12 *
Butter	8/12 <sup>b</sup>	1045 ± 458 *	0.75 ± 0.75	0/12 *
Flaxseed	6/12 <sup>b</sup>	897 ± 354 *	0.75 ± 0.75 •	0/12 -
МСТ	8/12 <sup>b</sup>	909 ± 433 *	0.75 ± 0.75 *	0/12 *
Mixture	9/11 **	950 ± 488 ª	1.18 ± 0.75 •	0/11 •

<sup>1</sup> Seizures were induced by injecting rats with the convulsant, pentylenetetrazol, at a dosage of 50 mg/kg, injected subcutaneously (s.c.); results indicate events which occurred within the thirty minutes post-injection.

<sup>2</sup> Rats exhibiting only myoclonic jerks were not included.

<sup>3</sup> Latency to the most severe seizure was measured in seconds.

<sup>4</sup> Severity was assessed using the following scale:

- 0 No motor seizure or myoclonic jerks only
- 1 Minimal seizure (face and forelimb clonus, tail erection, chewing)
- 2 Whole body clonus with forelimb extension
- 3 Whole body clonus with forelimb and hind limb extension

<sup>5</sup> Only deaths occurring within the thirty minutes post-injection were counted.

Different letters within columns indicate results which were significantly different (p < 0.05).

the five diet groups; however, overall, rats in the flaxseed, butter, and MCT diet groups had significantly lower incidences of seizures compared to rats in the control and mixture diet groups (p < 0.05).

#### Discussion

Although there were no significant differences in weight (Figure C-1) and weight gain (Figure C-2) between control, butter, flaxseed, and mixture rats, there were significant differences in weight and weight gain between MCT rats and all the other rats. MCT rats weighed significantly less and gained significantly less weight compared to all the other rats. Although food intakes were not measured, it was obvious from the food that remained in the food dishes that MCT rats were not eating as much as the other rats, in terms of both amount of food and energy intake. It is very likely that due to the high amount of MCT oil in the diet and the gastrointestinal side effects that are associated with MCT oil, the diet was unpalatable. The MCT rats were probably unintentionally semi-starved, and this may explain their significantly lower blood glucose (Table C-1) and significantly higher plasma  $\beta$ -HBA (Table C-1) compared to all the other rats. Blood glucose and plasma  $\beta$ -HBA were not significantly different in the butter, flaxseed, and mixture rats, but their blood glucose was generally lower than control rats and higher than MCT rats while their plasma  $\beta$ -HBA was generally higher than control rats and lower than MCT rats (Table C-1). When examining changes in blood glucose and plasma  $\beta$ -HBA over time, the same trends as those observed in Experiment 1 can be seen (see Section 6.1.3 and Figures C-3 and C-4).

Seizure test results indicated that flaxseed, butter, and MCT rats had a significantly lower incidence of seizures compared to control rats but that mixture rats were not significantly different neither from the control nor the other ketotic rats (Table C-2). Why the mixture rats did not exhibit seizure protection is unclear. Interestingly, the mixture rats were the last group to be seizure tested, and they were seizure tested early in the afternoon as opposed to during the morning. Thus, a change in circadian rhythms may have had something to do with the effects observed.

It was hypothesized that compared to control rats, rats consuming a KD would exhibit seizure protection when stimulated with a convulsant, so long as plasma  $\beta$ -HBA was at least 2 mM. Although the MCT group had the highest plasma  $\beta$ -HBA (4.59 mM ± 2.01 mM) at the time of the seizure test, they were not afforded the greatest seizure protection; rather, the flaxseed oil group (whose plasma  $\beta$ -HBA was only 0.77 mM ± 0.28 mM), showed the greatest degree of seizure protection. Interestingly, Bough (1999) also found no significant correlation between  $\beta$ -HBA and seizure protection in his rat model of the KD.

It was also hypothesized that the MCT group would attain the highest plasma  $\beta$ -HBA, and this was the case. Because MCFAs can be readily absorbed from the stomach into the portal venous system and cross directly into hepatic mitochondria independently of the carnitine acyltransferase system, they are more ketogenic (Swink et al., 1997). Thus, it was expected that the MCT KD group would attain the highest plasma  $\beta$ -HBA.

# **APPENDIX D**

# CONSENT FORM FOR PARTICIPATION IN THE KETOGENIC DIET STUDY

I, \_\_\_\_\_\_, understand that to participate in this study, I must undergo (Print Name) a 12-hour overnight fast. I also understand that I will be given four identical meals during a period of twelve hours, one meal every three hours, and that the meals will be composed of 35% Beatrice whipping cream and ProMod protein powder. I also understand that during the 12-hour study, I will be required to give hourly blood, breath, and urine samples and that blood will be collected from the fingertips. I am aware that if I feel ill during the study or I wish not to continue, I may withdraw at any time. I am also aware that I will be awarded \$50 for my participation.

(Signature)

(Date)

(Witness)

(Date)