THE DIFFERENTIAL EFFECTS OF DIETARY FAT IN A RAT MODEL OF THE KETOGENIC DIET

By

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ABSTRACT

The purpose of this study was to examine rat tissue fatty acid profiles induced by the ketogenic diet to determine whether significant relationships exist between plasma free long chain polyunsaturated fatty acids (LC-PUFA) and the seizure protection afforded by different fats in the ketogenic diet. The effects of butter, medium chain triglyceride oil (MCT), or flaxseed oil were examined in rats for 48 d. Correlations were performed between plasma n-3 PUFA and the seizure test results obtained by Musa (1999) in a parallel experiment from different rats fed the same diets. Flaxseed oil induced significantly higher levels of plasma free n-3 PUFA than the other fats ($p<0.05$). Flaxseed oil also induced lower plasma and liver triacylglycerols (TG). All of the diet groups had similar levels of docosahexaenoic acid (DHA) in the brain, despite large differences in the amount of α-linolenic acid (ALA) in the diets. A pentylenetetrazol seizure (PTZ) threshold test indicated that up to 50% of rats on the ketogenic diets were protected from seizures ($p<0.05$). No significant association was found between the level of free n-3 PUFA in plasma or liver and the seizure parameters measured from the PTZ test.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid (20:4n-6)
AcAc	Acetoacetate
AED	Anti-epileptic drugs
β-OHB	Beta hydroxybutyrate
DHA	Docosahexaenoic acid (22:6n-3)
EPA	Eicosapentaenoic acid (20:5n-3)
FAME	Fatty acid methyl esters
FFA	Free fatty acid
G(L)C	Gas - liquid chromatography
IS	Internal standard
LA	Linoleic acid (18:2n-6)
ALA	α- Linolenic acid (18:3n-3)
LCFA	Long chain fatty acid
LDL	Low density lipoprotein
MCFA	Medium chain fatty acid
MCT	Medium chain triglyceride
MUFA	Monounsaturated fatty acid
PTZ	Pentylenetetrazol
PL	Phospholipid
PUFA	Polyunsaturated fatty acids
SCFA	Short chain fatty acid
TBARS	Thiobarbituric acid-reactive substances
TG	Triglyceride

TLC	Thin layer chromatography
VLDL	Very low density lipoprotein

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CHAPTER 1

INTRODUCTION

CHAPTER 1. INTRODUCTION

Epidemiologic studies show that approximately 20% of epilepsy cases cannot achieve seizure control with existing anti-epileptic drugs (AEDs) (Shorvon, 1996). The ketogenic diet is a very high fat, low carbohydrate diet that emerged over 50 years ago and is used as a therapy for drug-resistant epilepsy (Swink et al. 1997). The mechanism of action of the ketogenic diet is currently unknown.

Several animal studies have implicated the rise in ketone levels experienced on the diet, specifically β- hydroxybutyrate (β-OHB), as the primary factor in providing the anticonvulsant protection (Uhlemann and Neims 1972, Appleton and DeVivo 1974, Hori et al. 1997, Bough and Eagles 1999). These studies, however, are not consistent in the diets, seizure tests, or age and strain of the animals used. The absence of literature reproducing these studies raises further doubt that anticonvulsant protection experienced on the ketogenic diet is a direct function of the level of ketonemia.

In the clinical setting there are three main types of ketogenic diets used. The "classic" ketogenic diet comprised mainly of dairy fat provides 75% of the recommended daily allowance (RDA) of energy for a child's ideal weight and height. It is calculated on a 4:1 weight ratio of fat to carbohydrate and protein (Freeman et al 1996). The MCT oil - based diet, with energy at 100% of RDA levels, is comprised of 60% of energy from MCT, 11% of energy from long chain saturated fat, 10% of energy from protein, and 19% of energy from carbohydrate (Schwartz et al 1989). A modified version of the MCT diet is also used which changes the fat content to only 30% of energy from MCT and 40% of energy from long chain saturated fat.

Recent studies have demonstrated an increase in the anticonvulsant threshold of rats after the administration of n-6 and n-3 PUFA. Yehuda et al. (1994) found

anticonvulsant protection using 4 different seizure models after rats were orally administered α- linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6) in a 1:4 ratio. Voskuyl et al. (1998) found that i.p. injection of docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) in rats exerted an anticonvulsant effect in vivo using the cortical stimulation model. Their studies have implicated a possible anticonvulsant role for the PUFA DHA, EPA, ALA, and LA in seizure protection. They are unique in that the fatty acids were administered either orally or injected i.p. while the rats consumed regular laboratory rat chow. Although no ketone body data were reported, their work suggests that dietary enrichment using specific fatty acids may also have a more important role to play in anticonvulsant protection. It also suggests that plasma concentrations of fatty acids may be potential indicators in predicting the extent of control of epileptic seizures.

It is unclear whether PUFA play a role in the ketogenic diet given that the classical and MCT versions contain primarily saturated fatty acids. No published studies to date have investigated the effects of adding PUFA to the ketogenic diet on tissue lipid profiles and seizure protection. Analysis of tissue lipid profiles will allow the determination of the relative proportion, as well as the concentration of fatty acids suggested in the literature to be anticonvulsant. It was the objective of my research and this thesis to establish the possible role of raised plasma free n-3 PUFA derived from the diet or from adipose tissue on ketosis and seizure protection. This was done through the comparison of the classic and MCT diets with a ketogenic diet comprised of flaxseed oil and a modified diet comprised of a mixture of MCT, butter, and flaxseed oil. The effects of these diets on organ fatty acid profiles were examined.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2. LITERATURE REVIEW

2.1 EPILEPSY AND THE KETOGENIC DIET

2.1.1 Overview

Epilepsy is a chronic condition characterized by a predisposition to recurrent, usually spontaneous epileptic seizures. An epileptic seizure is defined as an abnormal and excessive discharge of brain neurons accompanied by some behavioral change (Guberman, and Bruni, 1997). Seizures are classified as to whether or not their onset is partial or generalized. Partial seizures are subcategorized into simple and complex, while generalized seizures include tonic clonic and absence (Fisher, 1989). Partial seizures are referable to abnormal electrical activity in a relatively localized part of the brain, whereas generalized seizures lack apparent focality (Fisher, 1989).

The prevalence of epilepsy in the Western population is approximately 1% (Shorvon, 1996). The incidence of epilepsy varies greatly with age with rates greatest in early childhood, falling to low levels in early adult life, and rising again in the elderly (Shorvon, 1996). AED therapy is the most commonly prescribed treatment for epilepsy, providing success in about 60% of all cases. Patients with chronic epilepsy that is partially responsive to AEDs but with a tendency to relapse (20% of cases), or patients in whom epilepsy is unresponsive to AED treatment (refractory, or intractable epilepsy- about 20% of all cases) require more innovative treatment to control their seizures (Shorvon, 1996).

A resurgence of interest in the ketogenic diet in the last five years arose as an attempt to successfully treat and reduce the number of refractory epilepsy cases. The diet is viewed as a less invasive treatment compared to surgery, and less toxic than the lifetime use of combinations of AEDs. The ketogenic diet was first introduced by Wilder

(1921) in an attempt to mimic the positive effects of fasting on seizure control while providing patients with enough calories to maintain their nutritional state. The diet is initiated in the hospital where the patient undergoes a preliminary fasting period. During this period nothing is received by mouth until the examination of urine reveals high (6-12 mmol/L) levels of ketones (Freeman, 1996). Although the mechanism of action of the diet is unknown, there are known factors that influence its effectiveness. These factors include age of the patient, type of epilepsy, and compliance of both the patient and the parents (Freeman et al., 1996).

2.1.2 Clinical Use of the Ketogenic Diet

In practice, the classical ketogenic diet is a weighed diet in which the amount of fat (in grams) is at least three times the amount (in grams) of carbohydrate and protein. Calculating the diet also takes into consideration the age, height, and weight of the child, and the daily energy required by a child of that age and size required to maintain growth (Livingston, 1972). In order to maintain the appropriate ketogenic/anti-ketogenic ratio a child is supplied with 75% of the daily required energy for an ideal child of that same weight and age (Freeman et al, 1996). Heavy cream and butter are the fat sources primarily used on this diet.

In an attempt to make the ketogenic diet more palatable and to encourage more families to try it as an effective form of therapy, Dr. Peter Huttenlocher devised the MCT Oil- based diet. MCTs are made up of a mixture of 6:0 (1-2%),8:0 (65-75%), 10:0 (25-35%) and 12:0 (1-2%) obtained by the hydrolysis of coconut oil followed by the fractionation of fatty acids and are the primary fat source on this diet (Bach et al., 1982). They provide 60% of the total energy on this diet. An MCT diet with energy at 100% of RDA levels and with more carbohydrates and protein will produce the same

ketosis as the classical diet with energy at 75% of RDA levels (Huttenlocher, 1976). A modified MCT diet developed at the John Radcliffe Hospital is also used clinically. The modified diet is similar to the MCT diet in that the energy is based on 100% of RDA levels. It differs from the MCT diet in that only 30% of energy comes from MCT oil, and 40 % of energy comes from long chain saturated fatty acids (Schwartz et al 1989a).

From a therapeutic perspective for the ketogenic diet, it would appear that MCTs may be a more desirable fat to sustain ketosis and prevent seizures. There appears, however, to be no significant increase in the effectiveness of the MCT or modified MCT diet when compared to the classical ketogenic diet (Freeman et al., 1996, Schwartz et al., 1989b). Gastrointestinal complications and chronic diarrhea are frequently reported on diets containing MCT oil and are the main deterrents for children and families (Freeman et al 1996). Regardless of which diet is used, it will completely control epilepsy in one-third of children whose seizures are otherwise uncontrollable. In half the remaining children, the diet will either markedly decrease the frequency of seizures or enable a reduction in AEDs (Freeman et al. 1996).

2.1.3 Epilepsy and the Use of Seizure Models

Little research currently exists that has attempted to elucidate the mechanism of action of the ketogenic diet on the control of epilepsy in children (Swink et al, 1997). Epileptic research has become dependent upon the use of animal models to investigate the effectiveness and mechanism of action of any new treatment (diet or drug). Animal models allow control of the timing, duration, frequency, and characteristics of seizures in response to a given treatment. There are multiple types of epilepsies to model, and no one animal model is fully trustworthy as an imitation of clinical epilepsy. Currently there are several models of seizures, and researchers tend

to employ the model of greatest familiarity and convenience (Fisher, 1989). An analysis of standardized seizure tests exceeds the scope of this thesis, however there are two relevant models requiring some explication. They are the cortical stimulation and pentylenetetrazol (PTZ) models.

The cortical stimulation model consists of the stimulation of the cortex via chronically implanted electrodes with a current pulse train, which continuously increases in intensity (Hoogerkamp et al, 1993). This type of stimulation induces a consistent and progressive pattern of convulsive activity that eventually involves the whole body. Within this pattern, the current intensity required to elicit forelimb clonus has been defined as the threshold for localized seizure activity (Voskuyl et al 1998). When stimulation is stopped at this point, seizure activity is aborted and immediately the animal will resume its previous activities (Hoogerkamp et al, 1993). The current at which self-sustained seizure activity occurs has been defined as the threshold for generalized seizure activity (Voskuyl et al 1998). Animals are stimulated twice daily for ten days to stabilize baseline values before beginning treatment with the compound of interest (Hoogerkamp et al 1993).

The measure of anticonvulsant efficacy is an elevation in seizure threshold in an individual animal compared to that same animal's baseline response (Hoogerkamp et al, 1993). This model has tested drugs commonly used as reference compounds when comparing different seizure models (eg. carbamazepine, phenytoin, phenobarbital, and valproate) and found anticonvulsant effects (Hoogerkamp, 1993). Recently this model has been used to compare the anticonvulsant effects of polyunsaturated fatty acids (discussed in a later section), and for this reason it is of interest to this thesis. It is also of interest to this thesis because of this model's ability to quantify a relationship

between plasma concentrations of the compound of interest and anticonvulsant efficacy.

The drug PTZ is commonly used in epilepsy research and is accepted as a standardized model of generalized absence seizures (Fisher, 1989). PTZ initially produces myoclonic jerks which then become sustained, and may lead to a generalized tonic-clonic seizure (Fisher, 1989). In general PTZ results in clonic seizures in 97% of animals tested with dosages of 85mg/kg in mice and 70mg/kg in the rat (Fisher, 1989). Anticonvulsant protection is generally measured by the presence or absence of forelimb clonus (threshold), or hindlimb extension greater than 90° (maximal). Other parameters that are frequently measured are latency to the first myoclonic jerk, as well as duration of seizures (Krall et al, 1978). Seizure scoring systems are also frequently used to quantify results and determine anticonvulsant effectiveness (Foote and Gale, 1984). Several studies in the literature have employed PTZ to test the ketogenic diet, with conflicting results (Uhelmann and Neims, 1972, Appleton and DeVivo 1974, Otani et al, 1984, Hori et al 1997, Bough and Eagles, 1998, Thavendiranathan et al 1999, Likhodii et al 1999).

Several mechanisms have been proposed for the success of the ketogenic diet and current research is divided into four prominent theories. These theories include 1) acidosis, 2) water balance and dehydration, 3) ketosis and cerebral metabolism, and 4) lipid metabolism. The evidence in support of these theories is largely circumstantial observations, and no one theory has been able to clearly isolate the single most important determinant for the mechanism of action of the ketogenic diet. In many cases observations are reported with no attempt to propose an actual mechanism. A description these theories, and where possible, the anecdotal evidence in support of and against these theories is presented in the following section.

2.2 THEORIES ON THE MECHANISM OF ACTION OF THE KETOGENIC DIET

2.2.1 Acidosis

The accumulation of ketoacids (β- hydroxybutyrate, acetoacetate, and acetone) in plasma is caused by the shift of the body's metabolism from glycogenolysis to ketosis because of the lack of glucose as a substrate. Ketosis is maintained on the ketogenic diet because it is low in both carbohydrate and protein. Ketoacidosis causes a rise in blood pH and a decrease in plasma bicarbonate (HCO_3-) concentrations producing metabolic acidosis (Swink et al., 1997). It was therefore postulated that this increased acidosis seen in patients on the ketogenic diet was the mechanism of the diet's anticonvulsant action (Swink et al., 1997).

Several studies have indicated that this may not be the case. Withrow (1980) demonstrated that, in rats maintained on a high-fat ketogenic diet, there was an initial decrease in pH which, after several days on the diet, was completely compensated by hyper-ventilation and a lowering of the partial pressure of carbon dioxide (PCO_2), after which the blood pH remained constant. Al-Mudallal et al. (1996) measured intracellular brain pH in rats and found no change between rats fed high-fat diets for five weeks compared with rats fed regular chow.

Consistent with these animal model results are those obtained by Huttenlocher (1976) who measured the venous pH of children treated with the MCT ketogenic diet for a year or more and found that all children had normal pH values but reduced values of both PCO_2 and HCO_3-. These studies have led to the conclusion that the

anticonvulsant action of the ketogenic diet is unlikely to be mediated directly through cerebral acidosis (Swink et al., 1997).

2.2.2 Water Balance and Dehydration

Very few studies exist which have examined the effects of water, electrolyte balance, and dehydration on anticonvulsant thresholds in animals or humans on a ketogenic diet. Most of the literature available on these topics involved studies performed between 30 and 60 years ago. It was thought then that fasting and the ketogenic diet lead to the partial dehydration of tissues and thus to the prevention of seizures (Livingston, 1972). Millichap and Jones (1964) examined seizure susceptibility in mice and children on ketogenic diets and concluded that the mechanism of action was a result of a negative balance of sodium and potassium due to water loss of tissues and dehydration. They failed however, to actually define a mechanism by which the change in potassium and sodium would affect the anticonvulsant threshold of the brain.

In 1974, Appleton and DeVivo found no significant differences between whole brain electrolytes and water content in rats fed a high-fat diet compared with those fed regular chow. Similarly in children, Schwartz et al. (1989) observed no changes in plasma concentrations in sodium, potassium, chloride, and bicarbonate. The carbohydrate restriction on the ketogenic diet is known to cause a saline diuresis (Swink et al., 1997). In the clinical situation, children placed on the ketogenic diet are maintained on moderate fluid restriction (65mL per kg body weight) as part of the treatment (Freeman et al., 1996). In terms of formulating a mechanism of action for the ketogenic diet, the anticonvulsant action of these measures is currently unknown.

2.2.3 Ketosis and Cerebral Metabolism

While on a ketogenic diet, the liver switches from an organ of carbohydrate utilization and fatty acid synthesis to one of fatty acid oxidation and ketone body production. The term ketone "bodies" refers to the 3 and 4 carbon molecules acetone, acetoacetate (AcAc) and β-OHB which are produced in the liver and exported to extrahepatic tissues (Mitchell et al. 1995). Acetone is produced by the spontaneous decarboxylation of AcAc and is exhaled (Mitchell et al., 1995). In extrahepatic tissues, AcAc and β-OHB are reconverted to acetyl-CoA and oxidized via the citric acid cycle. Ketone body formation can be considered an overflow pathway for the acetyl-CoA produced during the oxidation of fatty acids, providing another way for the liver to distribute fuel to peripheral tissues (McGarry and Foster, 1980). This theory hypothesizes that the anticonvulsant action of the diet comes from the utilization of β-OHB as the major fuel for the brain. In the clinical situation, the level of ketosis in children on the diet is frequently monitored through the use of urinary measurements. A level of 6-12mmol/L of acetoacetate as detected in urine is seen as the optimal level of ketosis needed for epileptic control (Livingston et al 1972, Freeman et al., 1996). The parallel observations that the brain can use ketone bodies for fuel, and a ketogenic diet increases the circulating levels of ketones still does not lay a strong enough foundation to base the mechanism of action of the ketogenic diet.

Despite the successful experiences and reports describing the anticonvulsant properties of chronic ketosis, little was known about the effects of the ketogenic diet on seizure threshold and cerebral metabolism until the 1970's (Swink et al., 1997). Uhlemann and Neims (1972) led the way in a study performed on mice. They fed 16 day old (pre-pubertal) mice pups a ketogenic diet for 10 days while monitoring the

degree of ketosis. They performed a battery of electroconvulsive tests on the mice and found that the "ketotic" mice on the ketogenic diet were protected against maximal electroshock, hydration threshold electroshock and bicuculline-induced seizures (Uhlemann et al., 1972). They also demonstrated that initiating the ketogenic diet on adult mice reduced the degree of ketosis that was achieved compared to the younger mice, and subsequently these older animals showed no protective effects of the diet. This is one of the few studies that has looked at age-related differences in the anticonvulsant action of the diet and found a difference between age, and anticonvulsant protection.

This study is very similar to what is seen in the human clinical situation. When glucose is given to previously ketotic children, a dramatic and immediate lowering of seizure threshold, and the recurrence of seizures is witnessed (Swink et al., 1997, Huttenlocher, 1976). It is also thought that adults are not able to sustain, or develop ketosis to the same degree, or as rapidly as children. However, this topic has not been studied in depth (Freeman et al., 1996).

Many other investigators have also suggested that ketosis is the principal anticonvulsant determinant of the ketogenic diet (Appleton and DeVivo, 1974, Hori et al, 1997, Bough and Eagles, 1999). However, these studies vary widely in diet composition, seizure tests, strain and age of rats, length of time on the diet and the ketone levels achieved. It is well known that the classic and MCT ketogenic diets are able to maintain ketosis in children, and a strong correlation is seen with seizure protection and the degree of ketosis (Freeman et al., 1996, Swink et al., 1997, Livingston, 1972). This correlation has not yet been strongly established and replicated in other animal models.

2.2.4 Lipid Metabolism

Currently, there is a lot of controversy over the role of fatty acids in metabolism and the subsequent health benefits from fat. Very few comprehensive biochemical studies have been performed on the effects of the composition of various tissue lipids of subjects on a ketogenic diet. The importance of dietary fat in a ketogenic diet may be linked to seizure protection either through its ability to be oxidized into ketone bodies, or through a functional role such as membrane stability and repair.

Dekaban et al (1966) pioneered studies in lipid metabolism and the ketogenic diet in an investigation of 11 children with epilepsy. When compared to the pre-treatment values, increases were seen after 7 to 90 days on the high-fat diet in plasma free fatty acids (FFA), total fatty acids, total cholesterol, and triacylglycerols (TG) (Dekaban et al, 1966). The increases they observed in the levels of plasma lipids occurred concomitantly with a decline in seizure activity. Dekaban et al (1966) also noted that, blood and urine ketones reached their highest levels early in the initiation stages of the diet, whereas the plasma lipid levels took 2-3 weeks to reach their final high plateaus. The rise observed in plasma lipids corresponded to the length of time it took for optimal improvement in seizure activity.

Several fatty acids have unique properties, which have the potential to alter the susceptibility of the brain to seizures. Oleic acid (18:1n-9) is one of the principal fatty acids in brain phospholipid (PL), and is a precursor to nervonic acid (24:1). Both of these are major fatty acids in mature myelin (Leyton et al 1987). The PUFA, DHA (22:6n-3) and arachidonic acid (AA; 20:4n-6), are also important components of neuronal membranes. The dietary deficiency of these PUFA or their precursors has

been identified in term and pre-term infants (Farquharson et al. 1995). DHA and AA have been associated with the normal function of neuronal tissue growth and repair, as well as the development of learning patterns (Agostoni et al. 1995).

Recent studies have begun to suggest an anticonvulsant role for the n-3 and n-6 PUFA (Voskuyl et al 1998, Yehuda 1994). It has previously been demonstrated that PUFA have an antiarrhythmic effect on cardiac cells both in vivo and in vitro (Kang and Leaf 1994, and 1996). These authors found that PUFA decreased the excitability of cardiomyocytes by hyperpolarizing the diastolic membrane potential, increasing the threshold for fast Na^+ action potentials, prolonging the relative refractory period, and decreasing the spontaneous contraction rate (Kang and Leaf 1996). The antiarrhythmic effect of PUFA appears to be only active when it is in the FFA form. PUFA that are incorporated into membrane PL are not active (Kang and Leaf 1996).

Very similar actions on Na^+ channels have been observed for antiepileptic drugs such as phenytoin, which led the Voskuyl group to hypothesize a plausible anticonvulsant role for PUFA (Van den Berg et al 1993). Using the cortical stimulation model Voskuyl et al (1998) demonstrated that the n-3 PUFA, DHA and EPA exerted an anticonvulsant effect in rats. They reported that, for DHA and EPA, the thresholds for localized and generalized seizure activity increase at the end of infusion, and remain elevated during the entire period in which the threshold was measured (Voskuyl et al 1998). The results of Voskuyl et al (1998) are presented in figure 2.1 and illustrate that the increase in threshold for generalized seizure activity for EPA and DHA was significantly greater than that from LA or 18:1n-9 (Voskuyl et al 1998).

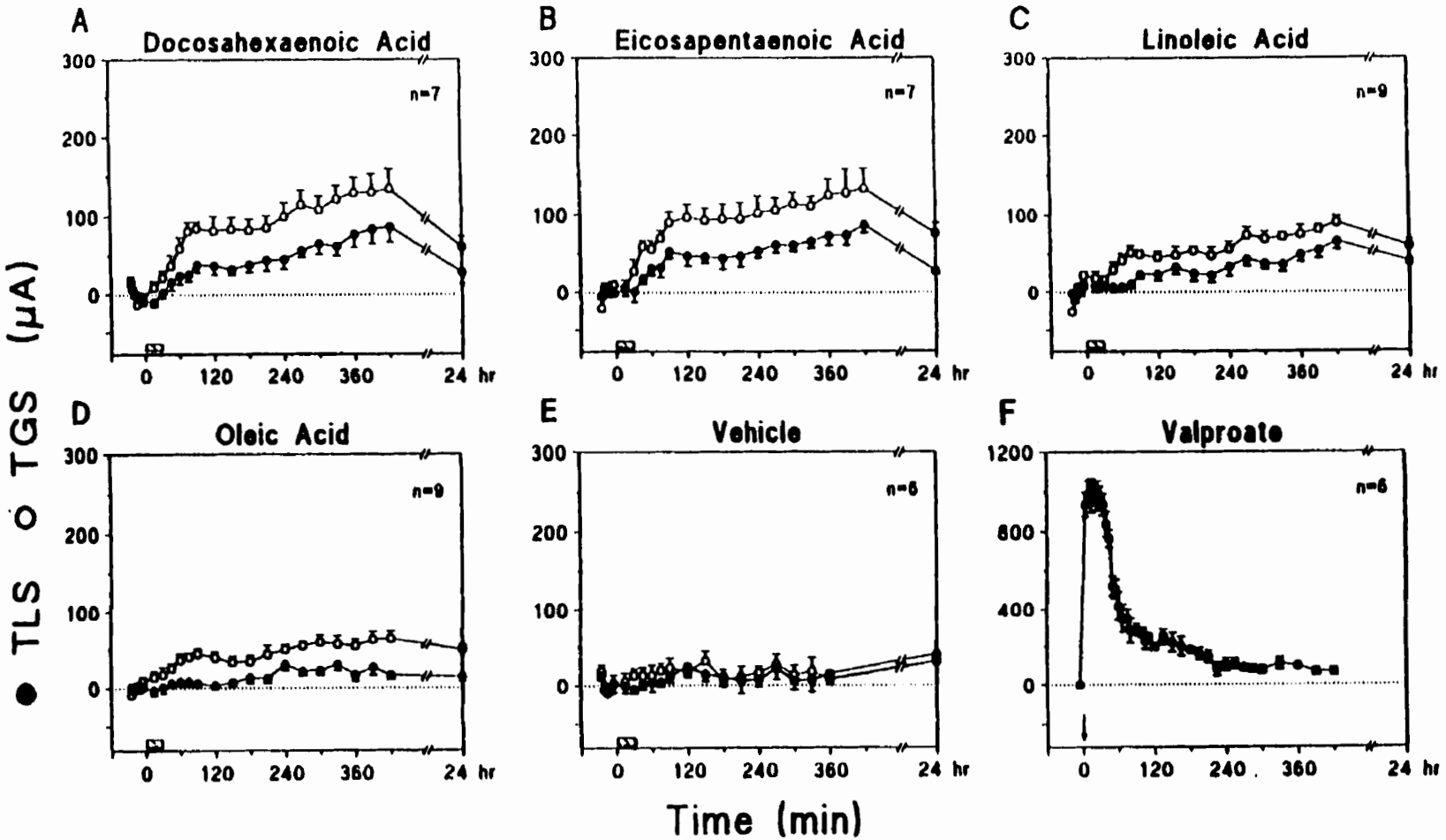

Figure 2.1 Time course of the effects of different fatty acids, their vehicle and valproate on the threshold for localized seizure activity (TLS, •) and for generalized seizure activity (TGS; o). [1]

[1] from Voskuyl et al,199

Cholesterol is another important lipid constituent of membranes and is a significant portion of myelin lipid. Cholesterol has been implicated in improved neurological function such as memory, and may be linked to anticonvulsant protection in the brain (Jurgelski et al 1973, Bierkamper and Cenedella 1978, Colannino and Lipke 1983, Vining et al. 1996). Statistically significant increases in plasma cholesterol in children on a ketogenic diet have been reported, but their connection to seizure protection remains unknown (Vining et al. 1996). It is known that exogenous cholesterol does not enter the brain (even in a hypercholesterolemic condition), and the brain biosynthesizes de novo all of the cholesterol it requires (Colannino and Lipke 1983, Edmond et al 1991). Previous studies have demonstrated that ketone bodies (β-OHB, and AcAc) serve as precursors for fatty acid and cholesterol synthesis in the brain (Edmond, 1974, Patel and Owen, 1977). Thus the ketogenic diet may be providing important substrates (in the form of ketone bodies) for energy metabolism and lipid biosynthesis to protect the brain from seizures.

2.3 DIGESTION AND TRANSPORT OF FATTY ACIDS

Dietary triacylglycerols (TG) are the major component of energy intake on the ketogenic diet. The fatty acid composition of the TG varies depending on the type of ketogenic diet used. It is known that fatty acids are not utilized on an equal basis and studies have shown that cellular uptake and oxidation of long chain fatty acids (LCFA) varies with degree of unsaturation (Leyton et al 1987). Differences have been reported in the intestinal absorption of saturated and unsaturated fatty acids and their level of incorporation into chylomicrons, as well as their ability to be oxidized (Bach et al 1982).

Thus the rate of digestion and oxidation of fatty acids may affect their ketogenic capacity.

Dietary TG are absorbed in the small intestine. In the intestinal mucosa LCFA are converted into acyl-CoAs, which are incorporated into TG and packaged with cholesterol into chylomicrons (Bach et al 1982). Chylomicrons then move through the lymphatic system from which they enter the blood and are dispersed to the liver and other tissues for oxidation. Short and medium chain fatty acids (SCFA, and MCFA) are released by the action of intramucosal lipase and are transported directly to the liver via the portal- venous system and are subsequently oxidized. Therefore, a shorter period of time elapses before they become available for oxidation (Figure 2.2). LCFA move via extrahepatic tissues, where they may be retained, and thus, it is thought that SCFA and MCFA reach the liver in greater abundance than exogenous LCFA (Bach et al 1987).

2.4 HEPATIC METABOLISM OF FATTY ACIDS

2.4.1 Oxidation of Saturated Fatty Acids

In order to be metabolized, long chain fatty acyl- CoA esters entering the outer mitochondrial membrane of the hepatocyte do not cross the inner mitochondrial membrane intact. Instead, the long chain fatty acyl-CoA group is transiently attached to carnitine and carried across the inner mitochondrial membrane catalyzed by the enzyme carnitine palmityl transferase (CPT) – I (Bach et al 1987, McGarry and Foster, 1980). Once inside the mitochondrial matrix, the fatty acyl group is liberated from carnitine (through the action of CPT II) and is ready for oxidation. Long chain fatty acyl-CoAs may also be stored in tissue TG and phospholipids (PL) for later use.

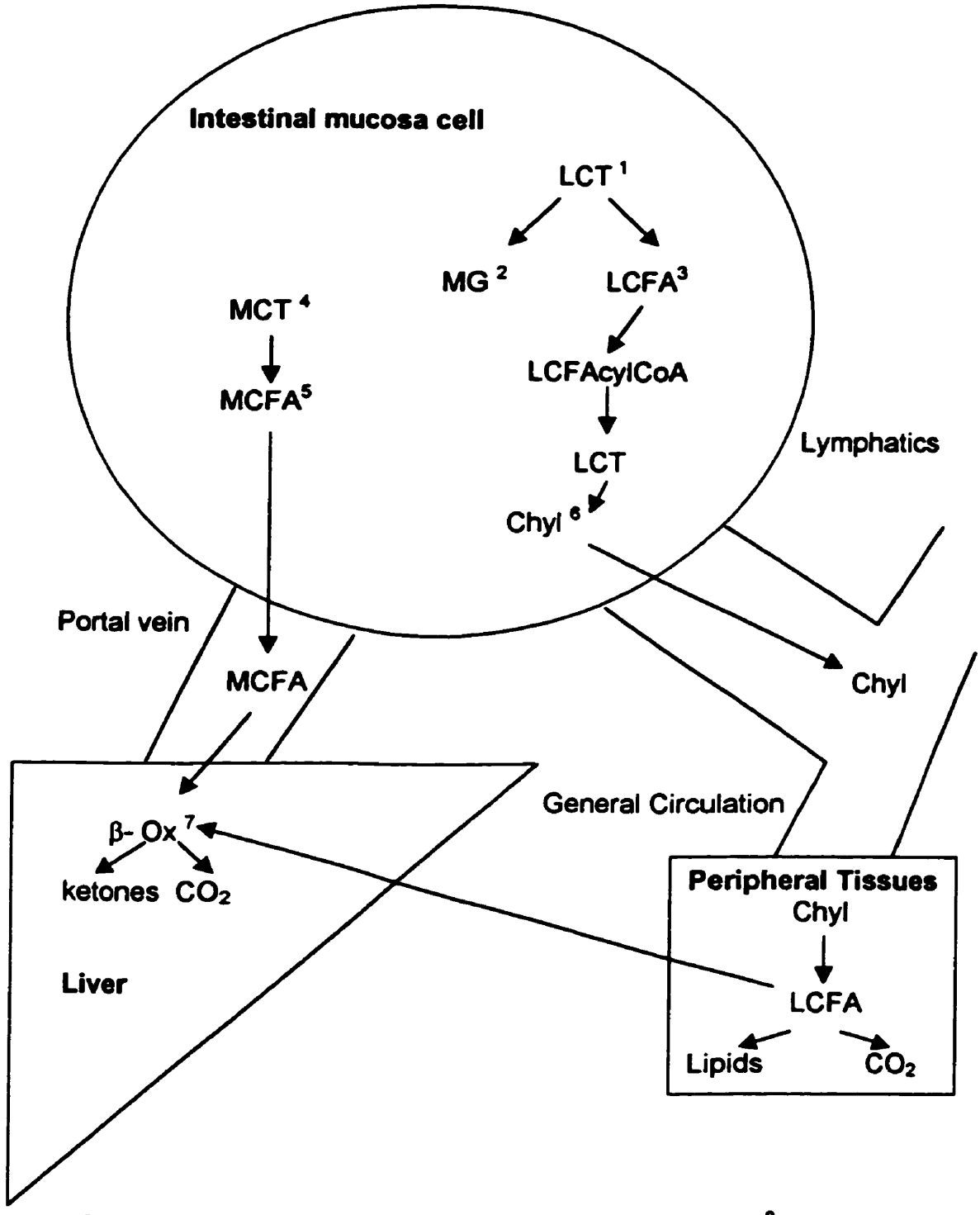

Figure 2.2 Digestion, Absorption and Transport of Fats[8]

[1] Long chain triglyceride, [2] Monoacylglycerol, [3] Long chain fatty acid, [4] Medium chain triglyceride, [5] Medium chain fatty acid, [6] chylomicrons, [7] β- oxidation

[8] Adapted from Bach et al. 1982.

In the fasted state, or as a result of high fat feeding, there is an increase in activity of CPT I resulting in increased β-oxidation. The acetyl-CoA produced in this state exceeds the oxidative capacity of the citric acid cycle and, as a result, an increase in ketone body production occurs (McGarry and Foster, 1980).

SCFA and MCFA on the other hand cross the double mitochondrial membrane very rapidly and do not require the presence of carnitine. Therefore, they reach the liver quickly, and are oxidized rapidly, leading to greater ketone body production. It is believed that, because of the slower rate of absorption and metabolism of LCFA, they are less ketogenic than SCFA and MCFA (Bach et al 1982, Leyton et al 1987). This appears to be the case for long chain saturated fatty acids (greater than 12 carbons) but does not correspond to published data on the oxidation of 18 carbon unsaturated fatty acids.

2.4.2 Oxidation of Unsaturated Fatty Acids

Previous studies using isotope (stable and radio-) techniques have shown that long chain fatty acids are not utilized on an equal basis for oxidation (Jones et al, 1985). With respect to PUFA of the n-6 family, increasing desaturation and chain elongation resulted in a gradual reduction in the rate of fatty acid oxidation (i.e. LA> GLA> DGLA> AA). Hence LA is more easily oxidized than its respective long chain PUFA. Further more, both ALA and 18:1n-9 have been shown to be preferentially oxidized over palmitate and stearate and LA in neonatal and adult rats (Kohout 1971, Leyton et al 1987, Emmisson et al 1995). The results of these studies demonstrate that both ALA and LA are oxidized as fast as, or faster than medium chain saturated fatty acids (which are known sources of available energy)(Leyton et al., 1987). The Cunnane group has also contributed to the accumulating evidence of the high oxidation rates of

n-3 PUFA, and have demonstrated that at least 80% of ALA intake in young animals seems to disappear via β- oxidation (Cunnane and Anderson, 1997). Thus, even PUFA may potentially play an important role in the overall energy expenditure of the animal and may enhance their ability to produce ketones. The potential for PUFA to be as ketogenic as MCT may have therapeutic benefits in a ketogenic diet.

2.4.3 β- Oxidation: Production and Fates of Acetyl-CoA

Acetyl Co A is an end product of the β- oxidation of fatty acids. Under conditions of high fat feeding (as seen on the ketogenic diet), the excess acetyl CoA has several fates. In the mitochondria, it can take part in the citric acid cycle, ketogenesis, or the elongation and desaturation of fatty acids (Bach et al 1982). In the cytosol, it can take part in the de novo synthesis of fatty acids and cholesterol (Dupont, 1966, Cenedella and Allen 1969). As mentioned previously, the role of dietary PUFA in the partitioning of fatty acids between oxidation, de novo synthesis of fatty acids and cholesterol, or elongation into LC-PUFA on a ketogenic diet is largely unknown.

Figure 2.3 illustrates the proposed role that dietary ALA may have in supporting brain development, and has been adopted here to illustrate the proposed role it may play in seizure protection (Cunnane et al, 1999). High fat feeding on a ketogenic diet results in increased fat oxidation in the liver and subsequently increased ketone body production. The resulting ketones are released into the plasma where they may be transported to the brain. In the brain ketones may serve as substrates for energy, as well as precursors to the synthesis of the majority of brain lipids (Edmond 1974, Patel and Owen, 1977, Cunnane, 1999).

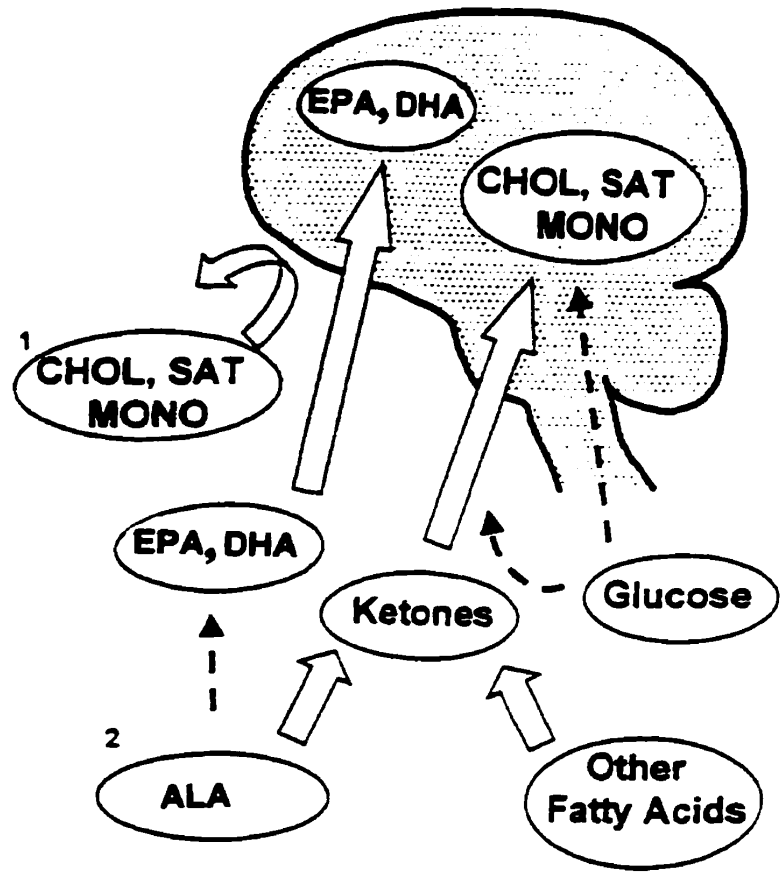

Figure 2.3 Outline of the proposed role of ALA in supplying brain lipids for the provision of seizure protection.[3]

[1]Cholesterol, monunsaturated fatty acids, saturated fatty acids
[2] α-linolenic acid
[3] from Cunnane et al. 1999

The brain uptake of fatty acids is a highly controversial topic in lipid nutrition, with no definitive mechanisms (Dhopeshwarkar and Mead 1973, Spector 1988, Edmond et al,1998, Cunnane et al ,1999). It is known that cholesterol and long chain saturated and monounsaturated fatty acids do not appear to access the brain post-natally, and that the accumulation of these fatty acids is mainly from their synthesis de novo (Edmond et al, 1998). Thus, regardless of the fat source used, the supply of ketones on a ketogenic diet may be providing important substrates for the brain for energy, or for the synthesis of fatty acids needed to stabilize or repair membranes, which may result in increased seizure protection.

ALA is unique to saturated and monounsaturated fatty acids traditionally used on a ketogenic diet in that it and its n-3 LC-PUFA precursors are capable of crossing the blood brain barrier (Dhopeshwarkar and Mead, 1973, Edmond et al, 1998). The brain is also capable of oxidizing fat, and a ketogenic diet with increased ALA content may be directly providing a relatively ketogenic fat source to the brain. A ketogenic diet comprised with a high source of ALA may also result in an increase in the n-3 LC-PUFA precursors, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)(Figure 2.3). Although the brain is capable of synthesizing DHA and EPA, they appear to be primarily supplied exogenously, and are also able to cross the blood brain barrier. The importance of these LC-PUFA in membrane stability, repair, and anticonvulsant properties have been discussed in the previous section. This dual role of ALA as an energy source via ketone bodies, as well as a structural and possibly anticonvulsant role as an intact fatty acid (or the precursor to intact EPA and DHA) makes it especially attractive in the context of a ketogenic diet. Although MCT are good energy substrates, they are poor precursors for cell membrane lipids.

This thesis attempts to provide some further insight into the metabolic effects of a ketogenic diet comprised of various lipid sources on tissue lipids, fatty acid profiles, and its implications for seizure protection.

CHAPTER 3

RATIONALE, OBJECTIVES AND HYPOTHESIS

CHAPTER 3. RATIONALE, OBJECTIVES, AND HYPOTHESIS

3.1 RATIONALE

In an attempt to understand the metabolic effects of a ketogenic diet, fatty acid analysis was performed on the adipose, liver, brain, and plasma. Adipose tissue was examined because of its role as the reservoir of dietary fat and supplier of free fatty acids. The fatty acid analysis of the brain was performed to demonstrate whether or not high fat feeding effects the accumulation of fatty acids in the mature brain, and whether or not this results in an increase in fatty acids proposed to be anticonvulsant. The liver and plasma analysis increased our understanding of the metabolic effects of high fat feeding of different fat sources and the partitioning of fat between PL, TG, and FFA. The concentration of free n-3 PUFA in these tissues was used to determine whether or not a relationship exists between their concentration and seizure protection.

3.2 GENERAL OBJECTIVES

To establish the possible role of raised plasma free PUFA with ketosis and seizure control in animals fed ketogenic diets comprised of different fat sources.

3.3 SPECIFIC OBJECTIVES

To compare the lipid profiles of rats fed the classic ketogenic diet comprised primarily of dairy fat, and the MCT ketogenic diet to a ketogenic diet comprised primarily of flaxseed oil, and a modified ketogenic diet comprised of dairy fat, MCT, and flaxseed oil in a 1:1:1 ratio by weight.

To establish whether or not a relationship exists between seizure protection (seizure incidence, % of rats protected, and seizure score), and plasma free n-3 PUFA.

3.4 HYPOTHESIS

Rats which consume greater amounts of fatty acids that preferentially are partitioned towards β-oxidation (MCTs, high n-3 PUFA) and, subsequently, ketogenesis will have a higher state of ketosis than those rats consuming greater amounts of long chain saturated fatty acids.

In comparing diets differing in fat composition, the diet producing the greater amount of ketosis or the greatest amount of plasma free DHA will have the strongest seizure protective effect.

CHAPTER 4

MATERIALS AND METHODS

CHAPTER 4. MATERIALS AND METHODS

4.1 STUDY DESIGN

The study was designed and some analysis performed by Kathy Musa, a concurrent MSc student. Kathy Musa carried out the plasma β- OHB analysis and weight data from these animals. The study began with 60 animals that were divided into 5 different experimental diet groups. Kathy Musa performed ketone and breath acetone measurements on 30 of these animals as well as seizure testing. I performed a quantitative total lipid and individual class lipid analysis on the remaining 30 animals using gas liquid chromatography. Plasma ketone data and seizure data performed by Kathy Musa will also be presented in this thesis to be used in correlations with the lipid analysis.

4.2 ANIMALS AND DIETS

Twenty-one day old rats (n=60) of the Wistar strain (Charles River Canada Inc., St Constant, PQ, Canada) were used. Rats at weaning were randomly divided into one of 5 diet groups. The ketogenic diets were prepared in our laboratory by mixing the specific fat/oils with a custom formulated powdered diet premix containing nutrients, fiber, minerals, and vitamins (Table 4.1). The premixes for the ketogenic diet and the ready-to-use AIN-93G control diet were ordered from Dyets (Bethlehem, PA, U.S.A). Butter, flaxseed oil, and MCT oil, or a mixture of the three were used as separate fat sources for the ketogenic diets and were obtained locally. MCT Oil was a gift of Mead Johnson Nutritionals (Evansville, IN, U.S.A). The premixed powder for the ketogenic diets contained minimal necessary amounts of n-3 and n-6 PUFA, but no attempt was made to balance the total amounts of n-3 and n-6 PUFA between diet groups.

In order to acclimatize to a high fat diet (and to more accurately model the clinical situation), during the first three days the animals divided into the ketogenic diet groups received a diet containing a 1:1 ratio of fat to carbohydrate and protein. After the third day, animals were then given a diet consisting of a 2:1 ratio of fat to carbohydrate and protein for five days (Tables 4.1 and 4.2). Finally, the rats were placed on the 3.5:1 diet and maintained on this diet for 40 days.

Thirty animals (6 from each diet group) were sacrificed and the brains, livers, plasma, and the perirenal adipose tissue were immediately removed and stored at -20°C until further analysis. Animals were not fasted for more than 6 hours before they were sacrificed. Although the lack of control of the last meal effect makes distinguishing the contribution of fatty acids from the diet from fatty acids released from adipose difficult, it was not part of my objective to distinguish where the contribution of plasma fatty acids were derived. My responsibility was the quantitative analysis of the lipids extracted from the various tissues for fatty acid composition, individual lipid classes and individual fatty acids.

4.3 ANALYSES

4.3.1 Plasma and Organ Collection

Blood samples were collected by cardiac puncture. Cardiac puncture involved anesthetizing the rats with pentabarbitol (0.3mL / 100g of rat). A 1 mL syringe was used and blood was collected. The blood was transferred from the syringe to heparinized microfuge tubes and placed on ice. The tubes were centrifuged at 2500 rpm for 10 minutes. Serum was removed and transferred to a newly labeled tube and stored at -20°C until analysis. Brain, liver and perirenal adipose tissue were removed from the

Table 4.1
Composition of the Diets

Ingredient	Control		1:1 KD [1]		2:1 KD [2]		3.5 :1 KD [3]	
	g/kg [4]	mg/kcal [5]	g/kg	mg/kcal	g/kg	mg/kcal	g/kg	mg/kcal
Protein:								
Casein	206.6	51.3	212.0	50.0	192.4	35.4	160.1	26.1
Carbohydrate:								
Corn starch	404.5	100.3	0	0	0	0	0	0
Dextrose	134.3	33.3	114.6	27.0	54.8	10.1	12.6	2.1
Sucrose	101.8	25.3	0	0	0	0	0	0
Non- Nutritive fiber:								
Cellulose	51.0	12.7	289.9	68.3	199.9	36.8	165.1	26.9
Vitamins	0.3	0.1	0.3	0.1	0.3	0.1	0.3	0.1
Minerals	27.7	6.9	28.5	6.7	29.3	5.4	23.0	3.8
Fat:								
Soybean Oil	71.3	17.7	73.1	17.2	75.1	13.8	58.7	9.6
Other [6]	0	0	253.3	59.7	419.3	77.1	545.3	89.0
TBHQ [7]	0.01	2.5*	0.01	2.4*	0.01	1.8*	0.01	1.6*
Choline bitartrate	2.5	0.6	2.6	0.6	2.6	0.5	2.0	0.3
Sucaryl	0	0	25.7	6.1	26.3	4.8	32.9	5.4

[1] Ketogenic diet with a 1:1 ratio of fat : carbohydrate + protein
[2] Ketogenic diet with a 2:1 ratio of fat: carbohydrate + protein
[3] Ketogenic diet with a 3.5:1 ratio of fat: carbohydrate + protein
[4,5] Refers to the amount of ingredient in grams per kg of diet, and the amount of ingredient in mg per kcal of diet
[6] Refers to the different fats used in the ketogenic diets: MCT oil, flaxseed oil, butter, and a mixture group containing a 1:1:1 ratio of MCT oil, flaxseed oil, and butter.
[7] t- butylhydroquinone, values with asterisks (*) indicate units are µg/kcal

Table 4.2
Percent Composition of Fatty Acids in the 3.5:1 Ketogenic Diets

Fatty Acids	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
6:0	0	1.5	0	1.6	1.3
8:0	0	57.5	0	1.7	31.6
10:0	0	32.8	0	3.8	19.7
12:0	0	0.6	0	4.4	1.5
14:0	0.4	0	0.1	13.1	3.6
16:0	11.7	0.9	5.7	31.7	9.8
18:0	4.5	0.2	3.1	9.7	3.3
Total Saturates	16.6	93.5	8.9	66.0	70.8
14:1n-5	0	0	0	1.3	0.3
16:1n-7	0.2	0	0	1.7	0.5
18:1n-7	1.7	0.1	0.8	0.8	0.4
18:1n-9	20.7	1.6	21.5	22.3	10.6
Total Monounsaturates	22.6	1.7	22.3	26.1	11.8
18:2n-6	52.8	4.3	20.6	6.5	6.9
18:3n-3	8.0	0.5	48.2	1.4	10.5
Total Polyunsaturates	60.8	4.8	68.8	7.9	17.4
n-6/n-3 ratio	6.6	8.6	0.4	4.6	0.7

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups

[2] Flax denotes the flaxseed oil ketogenic diet groups

[3] Butter denotes the butter ketogenic diet group

[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flax seed oil and butter diet group in a 1:1:1 ratio by weight

animals for tissue lipid analysis. Great care was taken to remove the entire organ so that accurate weight data could be recorded.

4.3.2 Lipid Extraction Procedure

Tissue samples of liver, brain, and perirenal adipose tissue were stored at –20°C prior to analysis. At the time of analysis, the samples were removed from the freezer and allowed to thaw. Approximately 1 g of tissue (or 1 mL plasma) samples were weighed and put into large amber vials. An internal standard solution in chloroform was prepared using heptadecanoate, L- α- phosphatidylcholine diheptadecanoyl and triheptadecanoin (Sigma Chemical Co., St. Louis, MO, USA) added to the equivalent of 10-20% of the fatty acids for quantification of free fatty acids (FFA), phospholipids (PL) and triglycerides (TG), respectively. The volume of the internal standard solution was calculated so that each sample received 250μL of solution. Ten mL of chloroform: methanol (2:1) and 2.0 mL of saline was added to each sample, followed by homogenization until they were completely dispersed using the Brinkman Polytron. Total lipid extracts were prepared using the Folch extraction method (Folch et al, 1957).

The Folch extraction is the standard method employed in lipid analysis. Given that two of my experimental diets contained MCT, there was a potential concern that recovery of the shorter chain 6 and 8 carbon fatty acids might not be effectively recovered from tissues by this method. Short chain fatty acids (generally considered between 2 and 6 carbons) are easily esterified but quantitative recovery of methyl esters from the reaction medium is difficult because of their high volatility and solubility in water (Christie, 1982). Fatty acid analysis of the diets (Table 4.2) by a procedure analogous to that of the body organs revealed proportions of MCT within the same

range given by the manufacturer. Thus, if there were fatty acids between 6 and 8 carbons in length in the various organs and tissues analyzed, it can be said with confidence that they would have been detected if they were present in appreciable amounts.

In the case of the plasma and liver, lipid classes of the total lipid extract were fractionated by neutral thin layer chromatography (TLC). Approximately 5mg of extracted lipid was applied to a scored TLC plate (20 X 20 cm Whatman LK6D plates precoated with 250μm of silica gel 60Å; Chromatographic Specialties, Brockville, ON, Canada) containing 3 separate tracks. The lipid samples of interest were applied to the two outside tracks while the middle track contained a mixture of lipid standards of known composition. The plates were transferred to a covered TLC tank containing the mobile phase solvent system (petroleum ether: diethyl ether: acetic acid, 80:20:1 v/v/v) and allowed to develop for 30 minutes. The plates were then allowed to dry for 10 minutes in the fumehood and then stained with 2',7' dichlorofluorescein (0.02% methanol solution) under N_2 gas. The individual lipid classes were identified by co-chromatography with authentic standards and marked under ultraviolet light. Subsequently, the individual bands were scraped into test tubes.

4.3.2.1 Saponification

This procedure was performed on total lipid extracts, and the PL class in the case of plasma and liver. Known aliquots of the total lipid extracts were placed in clean dry test tubes and dried completely under N_2 gas. Three mL of methanolic KOH (60g/L) was added to the samples, which were then capped under a gentle stream of N_2 gas and placed in a dry heating block at 90°C for 60 minutes. After cooling, 2.0 mL of saline and 5.0 mL of hexane were added. The samples were shaken vigorously and centrifuged at 1500 rpm for 4 minutes. The hexane (top) layer, which contained the

non-saponifiable material was removed and discarded. The bottom layer was then acidified with 300µL of concentrated HCl, another 5.0 mL of hexane was added and the mixture was shaken vigorously and centrifuged at 1500 rpm for 4min. The hexane (top) layer was then transferred to a clean dry tube and the solvent was completely dried under a gentle stream of N_2 gas in a 45°C water bath.

4.3.2.2 Methylation

All total lipid extracts (following saponification) and all individual classes of the liver and plasma were transmethylated using 3.0 mL of 14% boron trifluoride in methanol (Sigma Chemical Co., St. Louis, MO, USA) for 30 minutes in a 90°C dry heating block. Once cooled, 2.0 mL saline and 5.0 mL hexane were added to the samples, which were shaken vigorously and centrifuged at 1500 rpm for 4 min. The hexane (top) layer containing the fatty acid methyl esters (FAME) was transferred into a separate test tube, dried under N_2 gas and made up to a known concentration with hexane. Aliquots of a known concentration (0.3- 0.5 mg/mL) were transferred to GLC vials which were capped with aluminum seals.

4.3.2.3 FAME Analysis

The FAME were analyzed by GLC (Hewlett Packard, Palo Alto, CA, USA) on a chromatograph equipped with a fused silica capillary Column (J&W Scientific, Folsom, CA, USA) and a flame ionizer detector (see Appendix 1 for a list of GLC parameters). Fatty acid peaks were identified using standards of known composition and comparing retention times (NuChek 68, NuChek 96; NuChek Prep Inc., Elysian, MN, USA and Supelco PUFA2, Sigma-Aldrich Canada Ltd., Mississauga, ON, Canada). Peak areas were normalized according to the amount of internal standard present and fatty acid data were expressed in relative (% composition) and absolute (mg fatty acid/ g tissue) as per the following calculations:

$$\% \text{ COMPOSITION} = \frac{100 \text{ X FA area \%}}{\Sigma \text{ FA Area \% (excluding 17:0)}}$$

$$\text{mg FA/ g TISSUE} = \frac{\text{mg IS}}{\text{IS area \%}} \text{ X } \frac{\text{FA area \%}}{\text{g SAMPLE}}$$

Where FA= fatty acid; IS= internal standard; TL= total lipid

4.3.3 Plasma and Brain Cholesterol Measurements

4.3.3.1 Plasma Cholesterol

Plasma cholesterol measurements were obtained using an enzymatic assay (Diagnostic Chemicals Ltd., Charlottetown PEI, Canada). The biochemical principle of the assay is based on the cleavage of cholesterol esters to free cholesterol and fatty acids by the presence of a cholesterol esterase. The free cholesterol in the sample is then oxidized by a cholesterol oxidase to cholest-4-ene-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide produced couples with 4-aminoantipyrine and p-hydroxybenzoate (present in the reagent) in the presence of peroxidase, to yield a chromagen with maximum absorbance at 505nm. The intensity of the colour produced by the assay is directly proportional to the concentration of total cholesterol in the sample.

The procedure required pipetting into separate cuvettes 25μL of deionized water, calibrator, and serum to be analysed. Following this was the addition of 2.5mL of reagent and the samples were capped and inverted to mix. The samples were incubated for exactly 20 minutes. After incubation the absorbance of the calibrator (A_c) and each unknown (A) were measured using a UV/visible spectrophotometer (Ultrosec 2000, Pharmacia Biotech Ltd. Cambridge, England) at 505nm using the deionized water sample as the reagent blank. Total serum was calculated by the equation:

$$\text{Cholesterol (mmol/L)} = \frac{A}{A_c} \times \text{concentration of the calibrator}$$

Where A = the absorbance of the unknown; A_c = absorbance of the calibrator

4.3.3.2 Brain cholesterol

Total brain cholesterol was determined using GC. Samples were extracted as previously described (Chapter 4, 4.3.2). The internal standard 5- alpha cholestane (Sigma Chemical Co., St.Louis MO, USA) was used and added in a 1:1 ratio with cholesterol. Samples were saponified as described in section 4.3.2.1, with the initial top hexane layer containing the non-saponifiable material (sterols) preserved and placed into another culture tube where it was evaporated under N_2. The chemical t-BDMS (t-butyldimethylsilylchloride) was used to make sterol derivatives. For each 10mL of reagent, 151mg of t-BDMS was mixed with 136.2mg imidazole and 10mL of N,N-dimethylformamide (all chemicals are from Sigma Chemical Co., St.Louis MO, USA). One mL of this solution was then added to each sample. Samples were capped under N_2 and heated at 60°C for 30min. Samples were quickly cooled by placing them in the

freezer for 5 min. Four mL of hexane, and 1 mL of saline were added to each sample, after which they were capped and shaken vigorously. Samples were centrifuged at 1000 rpm for 4 minutes and the hexane (top) layer was transferred to a new tube. Samples were then prepared for the GC at a concentration of 0.05mg/mL (see Appendix 1 for GC parameters).

4.4 SEIZURE TESTING

The PTZ seizure threshold test was performed by Kathy Musa and was administered according to a modification of the procedure of Krall et al. (1978). Twelve rats from each diet group were randomly chosen for seizure testing. PTZ (Sigma, St. Louis, MO, U.S.A) was injected at a dose of 50mg/kg body weight subcutaneously into a loose fold of skin on the back of the neck. Seizures in the PTZ test were scored using the following scale: 0 – no motor seizure; 1- myoclonic jerks; 2- minimal seizure (brief face and forelimb clonus); 3- whole body clonus with forelimb tonic extension; 4- whole body clonus with forelimb and hind limb tonic extension. Seizure protection was defined as the absence of whole body clonus, i.e. at the seizure scores $\leq$2, within 30 minutes after PTZ injection.

4.5 STATISTICAL ANALYSIS

Statistical analysis was performed using the Sigma Stat software package (Release 2.0, Jandel Corporation, 1998). A one-way analysis of variance (ANOVA) was performed to detect any difference between groups and the Tukey's test was used as the post-hoc test to determine which of the ketogenic diet groups were significantly different. All data are expressed as mean $\pm$ SD and all comparisons significant at $p<0.05$ are identified in tables and figures. Correlations were performed using the

The Spearman rank order correlation test was used to measure the strength of association between specified plasma and liver FFA and the seizure parameters. For the correlations of the seizure score and plasma and liver free n-3 PUFA, β-OHB values from the animals tested by Musa (1999) were paired with β-OHB values from animals that were used for lipid analysis. Using this pairing system, correlations were then performed between an individual rat's seizure score, and the corresponding concentration of free plasma n-3 PUFA.

CHAPTER 5

RESULTS

CHAPTER 5. RESULTS

5.1 BODY AND ORGAN WEIGHTS

Final body weights of the control and 3F rats were significantly higher than the flax, MCT and butter rats ($p<0.05$). However, there was no significant difference in body weight between the control and 3F rats. The body weights of the butter, flax, and MCT rats (listed in order of highest to lowest) were all significantly different from one another ($p<0.05$). All of the diet groups had significantly higher liver weights than the MCT rats ($p<0.05$). The liver weights of the control rats were also significantly higher than the flax, and butter rats ($p<0.05$). The liver weight of the 3F rats was not significantly different from the control or flax rats, but was significantly higher than the butter rats ($p<0.05$). There was no significant difference in the liver weights of the flax and butter rats. When liver weights were expressed as a percentage of body weight they were similar among all groups (Table 5.1). The brain weights were similar among all groups, but when expressed as a percentage of body weight the MCT rats had a significantly greater percentage compared to the other rats ($p<0.05$).

5.2 FATTY ACID PROFILES

5.2.1 Liver Phospholipids

Relative (Table 5.2) and absolute (Table 5.3) amounts of myristic acid (14:0) and palmitate (16:0) were significantly higher in the butter groups as compared to all other groups ($p<0.05$). The total absolute amount of fatty acids in phospholipid was approximately 1.5 fold higher in all of the ketogenic diet groups as compared to the control group ($p<0.05$; Table 5.3).

Table 5.1

Final Body, Liver and Brain Weights

	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
Body (g)	351.1 ± 15.0 [5a]	204.9 ± 14.0 [b]	289.5 ± 20.7 [c]	308.5 ± 18.7 [d]	333.4 ± 14.9 [a]
Liver (g)	15.2 ± 1.5 [a]	8.8 ± 0.9 [b]	12.5 ± 1.9 [cd]	11.5 ± 0.8 [d]	13.9 ± 1.2 [ac]
(%BW [6])	4.3 ± 0.3	4.3 ± 0.2	4.0 ± 0.4	4.0 ± 0.2	4.2 ± 0.4
Brain (g)	1.8 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.2	1.8 ± 0.1
(%BW)	0.5 ± 0.04 [a]	0.8 ± 0.04 [b]	0.6 ± 0.03 [a]	0.6 ± 0.04 [a]	0.6 ± 0.02 [a]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] values are mean ± SD, n= 6 rats/ group. Values in each row with different letter superscripts are significantly different, $p<0.05$
[6] BW – body weight

Table 5.2

Percent Composition of Long Chain Fatty Acids in Liver Phospholipids

Fatty Acid	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	0.1 ± 0.0 [5a]	0.4 ± 0.1 [b]	0.06 ± 0.0 [c]	1.4 ± 0.3 [d]	0.2 ± 0.0 [e]
16:0	19.6 ± 0.4 [a]	15.4 ± 0.7 [b]	10.2 ± 0.6 [c]	21.1 ± 1.4 [d]	13.3 ± 0.9 [e]
18:0	22.8 ± 1.2 [a]	26.2 ± 2.2 [b]	31.4 ± 0.6 [c]	24.0 ± 2.1 [a]	28.9 ± 0.8 [d]
18:1n-9	4.1 ± 0.5 [a]	6.6 ± 0.7 [b]	4.9 ± 0.2 [c]	9.5 ± 1.5 [d]	4.7 ± 0.4 [c]
18:2n-6	14.6 ± 0.7 [a]	10.7 ± 1.4 [b]	20.3 ± 1.5 [c]	13.9 ± 0.6 [a]	15.7 ± 1.2 [a]
20:4n-6	23.3 ± 0.9 [a]	25.4 ± 0.9 [b]	13.9 ± 0.9 [c]	16.7 ± 1.4 [d]	14.3 ± 1.3 [c]
18:3n-3	0.1 ± 0.0 [ac]	0.2 ± 0.0 [a]	2.1± 0.3 [b]	0.2 ± 0.1 [abc]	1.0 ± 0.3 [bc]
20:5n-3	0.3 ± 0.1 [a]	0.3 ± 0.1 [a]	5.5 ± 0.6 [b]	0.5 ± 0.1 [c]	4.3 ± 0.4 [d]
22:6n-3	8.8 ± 0.6 [a]	6.9 ± 0.7 [b]	6.9 ± 0.8 [b]	5.9 ± 0.4 [b]	10.3 ± 1.2 [c]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] values are mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different, $p<0.05$. Fatty acids listed are the main representatives of the total fatty acids identified. For fatty acids less than 14 carbons, only 12:0 was detected in the Butter and MCT rats (<0.2%).

Table 5.3

Concentration of Long Chain Fatty Acids in Liver Phospholipids

Fatty Acid	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	*26.6 ± 2.3 [5a]	*119.5 ± 18.9 [b]	*21.2 ± 3.3 [c]	0.4 ± 0.1 [d]	*64.4 ± 8.9 [e]
16:0	3.5 ± 0.2 [a]	4.3 ± 0.2 [b]	3.3 ± 0.4 [a]	6.4 ± 0.7 [c]	3.8 ± 0.3 [ab]
18:0	4.1 ± 0.2 [a]	7.3 ± 0.6 [b]	10.2 ± 1.4 [c]	7.3 ± 0.5 [b]	8.3 ± 0.5 [b]
18:1n-9	0.7 ± 0.1 [a]	1.8 ± 0.2 [b]	1.6 ± 0.2 [b]	2.9 ± 0.5 [c]	1.3 ± 0.1 [d]
18:2n-6	2.6 ± 0.2 [a]	3.0 ± 0.5 [a]	6.6 ± 0.7 [b]	4.3 ± 0.5 [c]	4.5 ± 0.5 [c]
20:4n-6	4.2 ± 0.3 [ac]	7.1 ± 0.3 [b]	4.6 ± 0.6 [ac]	5.1 ± 0.8 [a]	4.1 ± 0.4 [c]
18:3n-3	*26.2 ± 3.8 [a]	*42.2 ± 13.2 [ac]	0.7 ± 0.1 [b]	*83.1 ± 13.6 [abc]	0.3 ± 0.1 [bc]
20:5n-3	*62.0 ± 12.6 [a]	*73.8 ± 20.1 [a]	1.8 ± 0.2 [b]	*160.3 ± 38.2 [c]	1.2 ± 0.1 [d]
22:6n-3	1.6 ± 0.6 [a]	1.9 ± 0.3 [ab]	2.3 ± 0.5 [b]	1.8 ± 0.4 [ab]	2.9 ± 0.2 [c]
TOTAL	18.4 ± 1.0 [a]	28.9 ± 1.3 [b]	33.1 ± 3.9 [c]	31.9 ± 3.1 [bc]	29.8 ± 1.4 [bc]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] All values are in mg/g, values with asterisks (*) the units are in μg/g; mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different, $p<0.05$. Fatty acids listed are the main representatives of the total fatty acids identified. For fatty acids less than 14 carbons, only 12:0 was detected in the Butter and MCT rats (<17μg/g).

The relative amount of palmitate (16:0) in the flax groups was significantly lower than all other diet groups, but in absolute amount did not differ from the control or 3F group (Table 5.2). The relative and absolute amounts of 18:0 and 18:2n-6 were significantly higher in the flax group than all other diet groups ($p<0.05$). Although less than the flax group, the remaining ketogenic diet groups had significantly higher 18:0 and 18:2n-6 (relative and absolute) than controls ($p<0.05$). The butter group had significantly higher 18:1n-9 (relative and absolute) than all other diet groups, and all of the KD groups had significantly greater 18:1n-9 than controls ($p<0.05$). The relative and absolute amounts of ALA in the flax and 3F groups were significantly more than 10 fold higher than the control and MCT groups but did not differ from butter. The flax groups had a 11 fold higher proportion of EPA than the control, MCT, and butter groups (5.5% vs. 0.3, 0.3 and 0.5% respectively; $p<0.05$) and a 1.3 fold higher proportion than the 3F group (5.5% vs. 4.3%; $p<0.05$). The relative amounts of DHA were significantly higher in the 3F group than all other groups, while the MCT, butter, and flax groups had significantly lower DHA than the control group ($p<0.05$). The absolute amount (Table 5.3) of DHA was significantly higher than all other groups, with no differences between control, MCT and butter groups.

5.2.2 Liver Triglycerides

The total amount of fatty acids in TG was similar between the control, MCT, and flax groups. The total amount of fatty acids in TG was also similar between the 3F and butter group which were both 10.5 fold higher than the other groups ($p<0.05$). Relative (Table 5.4) and absolute (Table 5.5) amounts of 14:0 and

Table 5.4

Percent Composition of Long Chain Fatty Acids in Liver Triglycerides

Fatty Acid	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	0.8 ± 0.2 [5a]	3.5 ± 1.1 [b]	0.2 ± 0.1 [c]	5.3 ± 0.7 [d]	1.9 ± 0.3 [e]
16:0	32.9 ± 4.5 [a]	28.5 ± 9.8 [a]	9.5 ± 2.0 [b]	28.2 ± 1.7 [a]	14.9 ± 0.8 [c]
18:0	2.9 ± 0.9 [a]	13.1 ± 3.2 [b]	6.9 ± 1.2 [c]	7.7 ± 0.6 [c]	4.9 ± 0.2 [d]
18:1n-9	22.9 ± 2.4 [a]	17.4 ± 2.9 [b]	21.9 ± 2.6 [a]	29.5 ± 0.7 [c]	21.0 ± 0.9 [a]
18:2n-6	26.9 ± 5.4 [a]	20.2 ± 8.7 [c]	24.3 ± 1.1 [ac]	14.5 ± 1.1 [b]	19.3 ± 0.3 [c]
20:4n-6	3.1 ± 1.2 [a]	5.8 ± 3.9 [a]	1.0 ± 0.2 [b]	1.7 ± 0.2 [a]	0.9 ± 0.1 [b]
18:3n-3	2.5 ± 0.5 [a]	2.1 ± 1.7 [a]	24.3 ± 4.5 [b]	1.5 ± 0.2 [a]	17.8 ± 1.2 [c]
20:5n-3	0.3 ± 0.1 [a]	0.6 ± 0.6 [a]	2.6 ± 0.7 [b]	0.7 ± 0.2 [c]	3.7 ± 0.2 [d]
22:6n-3	0.6 ± 0.2 [a]	1.2 ± 0.7 [a]	3.1 ± 1.1 [b]	1.7 ± 0.3 [c]	4.9 ± 0.4 [d]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] values are mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different, $p<0.05$. Fatty acids listed are the main representatives of the total fatty acids identified. For fatty acids less than 14 carbons, only 12:0 was detected in the Butter and MCT group (<1%).

Table 5.5

Concentration of Long Chain Fatty Acids in Liver Triglycerides

Fatty Acid	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	*55.9 ± 44.7 [5a]	0.3 ± 0.3 [b]	*16.4 ± 6.7 [c]	3.7 ± 1.1 [d]	1.3 ± 0.4 [e]
16:0	2.3 ± 1.6 [a]	2.1 ± 2.1 [a]	0.9 ± 0.6 [a]	19.3 ± 4.5 [b]	10.2 ± 2.5 [c]
18:0	0.2 ± 0.1 [a]	0.9 ± 0.8 [b]	0.7 ± 0.4 [b]	5.3 ± 1.3 [c]	3.4 ± 0.7 [d]
18:1n-9	1.6 ± 0.9 [a]	1.2 ± 1.2 [a]	1.7 ± 0.6 [a]	20.4 ± 4.8 [b]	14.2 ± 3.0 [b]
18:2n-6	1.7 ± 0.6 [a]	1.0 ± 0.6 [b]	1.8 ± 0.6 [a]	10.0 ± 2.6 [c]	19.3 ± 0.3 [c]
20:4n-6	*177.1 ± 34.5 [a]	0.2 ± 0.1 [b]	*95.2 ± 50.8 [c]	1.2 ± 0.3 [d]	0.6 ± 0.1 [e]
18:3n-3	*152.9 ± 52.9 [a]	*92.8 ± 45.1 [a]	1.7 ± 0.6 [b]	1.0 ± 0.3 [c]	12.0 ± 2.6 [d]
20:5n-3	*14.3 ± 1.3 [a]	*22.4 ± 8.3 [b]	0.2 ± 0.1 [c]	0.5 ± 0.2 [d]	2.5 ± 0.4 [e]
22:6n-3	*33.3 ± 4.8 [a]	*48.3 ± 10.3 [b]	0.3 ± 0.1 [c]	1.1 ± 0.3 [d]	3.3 ± 0.6 [e]
TOTAL	6.9 ± 3.7 [a]	7.3 ± 5.7 [a]	7.8 ± 2.4 [a]	73.2 ± 17.3 [b]	68.8 ± 13.8 [b]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups, [2] Flax denotes the flax seedoil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group, [4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight, [5] All values are mg/g, values with asterisks (*) the units are in μg/g); mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different, $p<0.05$. Fatty acids listed are the main representatives of the total fatty acids identified. For fatty acids less than 14 carbons, only 12:0 was detected in the Butter and MCT group (<0.7mg/g)

16:0 were significantly higher in the butter group than in all other groups ($p<0.05$). In relative terms, the amount of 14:0 and 16:0 was significantly lower for the flax group as compared to all other groups, and the 3F groups was significantly lower than the control, and MCT ($p<0.05$). However, there was no difference in the absolute amount of 16:0 in the control, MCT or flax groups. The absolute amount of 16:0 in the 3F group was significantly greater than that of the control, MCT and flax groups ($p<0.05$).

The relative amount of 18:0 was significantly higher in the MCT group as compared to all other groups, while the butter, flax, and 3F groups were significantly higher than the control group ($p<0.05$). The absolute amount of 18:0 was highest in the butter group as compared to all other groups and the remaining KD groups were still significantly higher than controls ($p<0.05$). The butter group had a significantly higher relative amount of 18:1n-9 as compared to all other groups ($p<0.05$). The absolute amounts of 18:1n-9 in the 3F and butter groups were similar and significantly higher than MCT, flax and control. The relative amount of 18:2n-6 was significantly lower in the butter diet group as compared to all other groups ($p<0.05$). There was no difference in the relative amount of 18:2n-6 between the MCT, flax, and 3F groups. The absolute amounts of 18:2n-6 in the butter and 3F groups (although not different from each other) were significantly higher than that of the Flax, MCT and control ($p<0.05$). There was no difference in the relative amount of 18:2n-6 between the flax, control, butter, or 3F groups.

There was no difference in the relative amount of 20:4n-6 between the control, butter, and MCT groups, but these groups were significantly higher than the flax and 3F groups ($p<0.05$). The relative amount of 20:4n-6 were significantly different between all groups with the highest amount in the butter group and the lowest amount in the flax

group ($p<0.05$). The relative amount of 18:3n-3 was significantly higher in the flax group as compared to all other groups. Although the differences were significant, the flax group was only 1.3 fold higher than the 3F group as opposed to more than 9.7 fold higher than the other groups. The control, MCT and butter groups were similar but significantly lower than the relative amount of 18:3n-3 in the 3F group ($p<0.05$).

The absolute amount (Table 5.5) of 18:3n-3 was highest in the 3F group compared to all other groups and was approximately 7 fold higher than the flax group (12.0mg/g vs. 1.7mg/g; $p<0.05$). The control and MCT groups contain similar absolute amounts of 18:3n-3 and were significantly lower than all other groups ($p<0.05$). The relative and absolute amounts of EPA in the 3F group were significantly higher than all other groups. The relative and absolute amounts of EPA in the control group were significantly lower than the other KD groups. The relative and absolute amounts of DHA in the 3F group were significantly higher than all other groups. The relative amount (Table 5.4) of DHA in the flax group was significantly higher than the control, butter, and MCT groups ($p<0.05$). The absolute amount of DHA in the control group was significantly lower than all other groups ($p<0.05$).

5.2.3 Liver Free Fatty Acids

The total absolute amount of free fatty acids was similar between the flax and 3F groups, which were significantly higher than the control, MCT, and butter diet groups ($p<0.05$; Table 5.7). The ketogenic diets resulted in an overall increase in total free fatty acids between 1.3 and 2.7 fold higher than that of the control group. Relative (Table 5.6) and absolute amounts of 14:0 were significantly higher in the butter group and lower in the flax group than all other groups ($p<0.05$). Butter and control groups were

not significantly different from each other in the relative amounts of 16:0 but they were significantly higher than all other groups ($p<0.05$). The absolute amounts of 16:0 were similar among all groups. The relative amounts of 18:0 were highest in the MCT group over all other groups. Control and butter groups were similar in their relative amounts of 18:0 but were higher than the flax and 3F groups ($p<0.05$). MCT, flax, butter, and 3F groups were all similar in their absolute amounts of 18:0, and all were significantly higher than the control group ($p<0.05$).

The relative amount of 18:1n-9 was higher in the butter group than all other groups. The control, flax, and 3F groups were all similar in relative amounts of 18:1n-9 and were all significantly higher than the MCT group ($p<0.05$). On the other hand, the absolute amount of 18:1n-9 was highest for the flax group as compared to all other groups. The butter and 3F group were similar to each other but contained higher absolute amounts of 18:1n-9 than the control and MCT groups (which were also similar to one another) ($p<0.05$).

There was a significantly lower relative amount (Table 5.6) of 18:2n-6 in the ketogenic diet groups than in the control group. Within the ketogenic diet groups, the flax group had a higher relative amount of 18:2n-6 than the MCT, butter, and 3F groups ($p<0.05$). The relative and absolute amount of 20:4n-6 was significantly higher in the MCT group than all other groups. The flax group and 3F group were similar to each other in both the relative and absolute amounts of 20:4n-6 and both groups were significantly greater than the control and butter group (which were also similar to one another)($p<0.05$).

The relative and absolute amounts of 18:3n-3 were highest in the flax group when compared to all other groups ($p<0.05$). The relative and absolute amounts of

Table 5.6

Percent Composition of Long Chain Fatty Acids in Liver Free Fatty Acids

Fatty Acid	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	0.7 ± 0.1 [5a]	1.3 ± 0.3 [b]	0.1 ± 0.0 [c]	3.8 ± 0.8 [d]	1.4 ± 0.2 [b]
16:0	22.8 ± 1.5 [a]	15.9 ± 2.7 [b]	8.6 ± 0.6 [c]	20.6 ± 1.2 [a]	12.9 ± 1.1 [d]
18:0	8.7 ± 0.8 [a]	13.5 ± 2.2 [b]	6.4 ± 0.6 [c]	9.1 ± 0.5 [a]	6.9 ± 1.0 [c]
18:1n-9	17.4 ± 1.4 [a]	20.6 ± 3.4 [b]	16.3 ± 1.0 [a]	25.6 ± 0.5 [c]	16.9 ± 0.5 [a]
18:2n-6	27.1 ± 2.5 [a]	20.5 ± 3.8 [b]	24.1 ± 1.0 [c]	17.7 ± 0.9 [d]	18.9 ± 0.4 [b]
20:4n-6	9.3 ± 0.7 [a]	11.6 ± 1.8 [b]	2.4 ± 0.4 [c]	6.5 ± 0.6 [d]	2.8 ± 0.6 [c]
18:3n-3	2.1 ± 0.2 [a]	1.3 ± 0.5 [b]	27.0 ± 3.0 [c]	1.8 ± 0.2 [b]	17.1 ± 1.6 [d]
20:5n-3	0.4 ± 0.1 [a]	0.7 ± 0.2 [b]	5.4 ± 0.4 [c]	1.8 ± 0.2 [d]	6.8 ± 0.5 [e]
22:6n-3	3.1 ± 0.7 [a]	3.8 ± 0.7 [a]	2.9 ± 0.6 [a]	3.1 ± 0.5 [a]	4.9 ± 0.3 [b]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] values are mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different, $p<0.05$. Fatty acids listed are the main representatives of the total fatty acids identified. For fatty acids less than 14 carbons, only 12:0 was detected in the Butter and MCT group (<0.2%).

Table 5.7

Concentration of Long Chain Fatty Acids in Liver Free Fatty Acids

Fatty Acid	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	*114.6 ± 39.8 [5a]	0.3 ± 0.1 [b]	*61.8 ± 14.4 [c]	1.1 ± 0.6 [d]	0.5 ± 0.1 [e]
16:0	3.9 ± 1.2	3.3 ± 0.5	4.1 ± 1.1	5.5 ± 2.1	4.5 ± 1.5
18:0	1.5 ± 0.3 [a]	2.8 ± 0.7 [b]	3.1 ± 0.7 [b]	2.4 ± 0.9 [b]	2.4 ± 0.9 [b]
18:1n-9	3.0 ± 1.0 [a]	4.4 ± 1.1 [b]	7.7 ± 1.7 [c]	6.8 ± 2.4 [d]	5.9 ± 1.8 [d]
18:2n-6	4.8 ± 1.8 [a]	4.4 ± 1.6 [a]	11.4 ± 2.4 [b]	4.7 ± 1.7 [a]	6.6 ± 1.9 [a]
20:4n-6	1.6 ± 0.4 [a]	2.4 ± 0.6 [c]	1.1 ± 0.2 [b]	1.7 ± 0.5 [a]	1.0 ± 0.5 [b]
18:3n-3	0.4 ± 0.1 [a]	0.3 ± 0.2 [a]	12.8 ± 3.4 [b]	0.5 ± 0.2 [a]	5.9 ± 1.6 [c]
20:5n-3	*76.4 ± 28.2 [a]	*145.8 ± 53.4 [b]	2.5 ± 0.6 [c]	0.5 ± 0.1 [d]	2.4 ± 0.7 [c]
22:6n-3	0.5 ± 0.1 [a]	0.8 ± 0.3 [a]	1.4 ± 0.3 [b]	0.8 ± 0.2 [a]	1.7 ± 0.4 [b]
TOTAL	17.7 ± 5.4 [a]	22.3 ± 4.7 [b]	48.2 ± 10.9 [c]	28.6 ± 10.6 [b]	45.5 ± 13.6 [c]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] All values are mg/g, values with asterisks (*) the units are in μg/g); mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different, $p<0.05$.Fatty acids listed are the main representatives of the total fatty acids identified. For fatty acids less than 14 carbons, only 12:0 was detected in the Butter and MCT group (<0.1mg/g)

18:3n-3 were higher in the 3F group than the control, MCT, and butter groups ($p<0.05$). The relative amounts of EPA in all groups were significantly different from one another (3F > Flax > Butter > MCT > Control) ($p<0.05$). The absolute amounts (Table 5.7) of EPA were similar between the flax and 3F group and these groups were significantly higher than the control, MCT and butter groups ($p<0.05$). The relative amount of DHA was highest in the 3F group over all other groups (<0.05). The relative amount of DHA was similar between the control, MCT, butter, and flax groups. The absolute amount of DHA was similar between the flax and 3F group, however these groups were significantly higher than the control, MCT and butter groups ($p<0.05$).

In summary, the butter group had the highest proportion of saturates (14:0, 16:0, and 18:0) and MUFA (18:1n-9) in liver PL, TG, and FFA compared to all other groups. The flax group had the highest proportion of LA in PL and FFA. The proportion of LA in TG was similar between the MCT, flax, and 3F groups. The MCT group had the highest proportion of AA in liver PL, TG, and FFA. The proportion of ALA was highest in PL, FFA, and TG in the flax group compared to all other groups. The proportion of EPA was highest in PL in the flax group, however the proportion of EPA was higher in TG and FFA in the 3F group. The proportion of DHA in liver PL, TG, and FFA was highest in the 3F group. Finally the total concentration of fatty acids in PL in the ketogenic diet groups was significantly greater than the control group. The total concentration of fatty acids in TG was similar in the 3F and butter groups, which were greater than the control, MCT, and flax diet groups. The total concentrations of fatty acids in FFA in the ketogenic diet groups were all higher than the control group. The flax and 3F group were similar, and had a greater total concentration of free fatty acids than the butter and MCT ketogenic diet groups.

Table 5.8

Percent Composition of Long Chain Fatty Acids in Perirenal Adipose Tissue Total Lipids

Fatty Acids	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
10:0	0.1 ± 0.02 [5a]	14.4 ± 2.8 [b]	0.1 ± 0.01 [a]	1.3 ± 0.1 [c]	4.2 ± 0.4 [d]
12:0	0.2 ± 0.04 [a]	2.3 ± 0.3 [b]	0.1 ± 0.03 [d]	3.4 ± 0.2 [c]	1.6 ± 0.1 [e]
14:0	1.7 ± 0.1 [a]	2.8 ± 0.4 [b]	0.4 ± 0.1 [d]	11.1 ± 0.3 [c]	5.0 ± 0.2 [e]
16:0	24.7 ± 1.2 [a]	23.8 ± 4.5 [a]	7.9 ± 0.6 [c]	29.5 ± 0.4 [b]	18.0 ± 1.8 [d]
18:0	3.5 ± 0.5 [a]	4.5 ± 0.5 [b]	3.4 ± 0.3 [a]	7.0 ± 0.5 [c]	5.0 ± 0.5 [d]
18:1n-9	28.3 ± 1.3 [a]	17.3 ± 1.1 [b]	25.6 ± 0.4 [d]	30.8 ± 0.3 [c]	25.0 ± 0.4 [d]
18:2n-6	28.6 ± 2.4 [a]	25.9 ± 2.9 [a]	21.7 ± 0.6 [c]	10.6 ± 0.1 [b]	17.8 ± 0.9 [d]
20:4n-6	0.3 ± 0.1 [a]	1.1 ± 0.2 [b]	0.1 ± 0.02 [c]	0.3 ± 0.03 [a]	0.2 ± 0.02 [d]
18:3n-3	2.9 ± 0.3 [a]	3.2 ± 0.5 [a]	38.9 ± 0.5 [c]	1.7 ± 0.1 [b]	19.6 ± 1.3 [d]
22:6n-3	0.05 ± 0.06 [a]	0.2 ± 0.05 [b]	0.1 ± 0.06 [a]	0.02 ± 0.03 [a]	0.3 ± 0.04 [b]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] values are mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different, $p<0.05$. Fatty acids listed are the main representatives of the total fatty acids identified.

5.2.4 Perirenal Adipose Tissue Lipids

The relative amount of 10:0 was highest in the MCT group compared to all other groups (Table 5.8). The amount of 10:0 was 144 fold higher than the control group, 11 fold higher than butter, 144 fold higher than flax, and 3.4 higher than the 3F groups (0.1%, 1.3%, 0.1%, and 4.2% respectively) ($p<0.05$). The relative amounts of 12:0 and 14:0 were significantly different among all groups but were highest in the butter group and lowest in the flax group. The relative amount of 16:0, 18:0 and 18:1n-9 were significantly higher in the butter group as compared to all other groups. The amount of 16:0 was similar between the control and MCT groups and was significantly higher than the flax and 3F groups ($p<0.05$). The amount of 18:0 was similar between the control and flax groups but these groups were significantly lower than all other groups. The flax and 3F groups were similar in their amounts of 18:1n-9 and were significantly higher than the control and MCT groups ($p<0.05$).

The MCT and control groups were similar in their relative amount of LA, which was higher than all other groups. The MCT group had the highest proportion of AA, which was 3.7 fold higher than control, 11 higher than flax, 3.7 fold higher than butter and 5.5 fold higher than the 3F group (1.1% vs. 0.3%, 0.1%, 0.3%, and 0.2% respectively; $p<0.05$).The proportion of ALA in the flax group was 13.4 higher than control, 122 fold higher than MCT, 22.9 fold higher than butter, and 2 fold higher than 3F (38.9% vs. 2.9%, 3.2%, 1.7%, and 19.6% respectively). Relatively, trace amounts of DHA were detected in all groups, and similarities in the relative amounts were seen between the control, flax and butter groups. Similarities in DHA were also seen between the 3F and MCT groups and were significantly higher than the control, flax and butter groups ($p<0.05$).

5.2.5 Brain Lipids

The total absolute concentrations of brain fatty acids were similar in all diet groups (Table 5.10). The relative and absolute amounts of DHA and total fatty acids were similar between all groups (Table 5.9 and 5.10). The relative and absolute amounts of 14:0 were similar between the control, MCT and flax groups. They were also similar between the butter and 3F, which were both significantly higher than the control, MCT, and flax groups ($p<0.05$). The relative amount of 16:0 in the MCT group was significantly higher than the control and 3F groups ($p<0.05$). The absolute amount of 16:0 was similar in all groups. The relative and absolute amounts of 18:0 were similar among all groups.

The relative amount of 18:1n-9 was significantly lower in the MCT group compared to all other groups. The absolute amount of 18:1n-9 was similar between the control, MCT, flax and butter groups and these groups were significantly lower than the 3F group ($p<0.05$). The relative and absolute amounts of LA were significantly higher in the flax group and lower in the MCT group compared to all other groups ($p<0.05$). The proportion of LA in the flax group was approximately 2 fold higher than controls, MCT and butter, and 1.3 fold higher than 3F (2.9% vs. 1.5%, 1.1%, 1.4%, and 2.1% respectively; $p<0.05$). ALA and EPA were not detected in the brains of the control, MCT, or butter groups but were detected in trace amounts in the flax and 3F groups. Comparisons of the relative and absolute amounts of ALA and EPA in these groups were significantly higher for the flax group ($p<0.05$).

Table 5.9

Percent Composition of Long Chain Fatty Acids in Brain Total Lipids

Fatty Acids	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	0.2 ± 0.01 [5a]	0.2 ± 0.01 [a]	0.2 ± 0.02 [a]	0.4 ± 0.04 [b]	0.3 ± 0.03 [c]
16:0	21.6 ± 0.3 [a]	22.0 ± 0.1 [b]	21.7 ± 0.2 [ab]	21.8 ± 0.2 [ab]	21.4 ± 0.4 [a]
18:0	12.4 ± 0.2	12.9 ± 1.0	12.3 ± 0.4	12.9± 1.4	12.3 ± 0.6
18:1n-9	21.2 ± 0.6 [a]	20.6 ± 0.4 [b]	21.8 ± 0.4 [ac]	21.5 ± 0.2 [a]	22.2 ± 0.3 [c]
18:2n-6	1.5 ± 0.1 [a]	1.1 ± 0.1 [b]	2.9 ± 0.1 [c]	1.4 ± 0.2 [a]	2.1 ± 0.2 [d]
20:4n-6	13.4 ± 0.8 [a]	14.0 ± 0.6 [b]	11.8 ± 0.2 [c]	13.1 ± 0.7 [a]	12.3 ± 0.5 [d]
18:3n-3	ND	ND	0.3 ± 0.04 [a]	ND	0.2 ± 0.01 [b]
20:5n-3	ND	ND	0.3 ± 0.04 [a]	ND	0.2 ± 0.01 [b]
22:6n-3	16.4 ± 0.6	16.1 ± 0.8	16.0 ± 0.8	16.1 ± 0.8	16.4 ± 1.1

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] values are mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different ($p<0.05$). Fatty acids listed are the main representatives of the total fatty acids identified. For fatty acids less than 14 carbons, only 10:0 was detected (<0.1%).

ND – not detected

Table 5.10

Concentration of Long Chain Fatty Acids in Brain Total Lipids

Fatty Acids	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	*92.0 ± 3.3[5a]	*94.6 ± 8.3[a]	*91.1 ± 8.3[a]	0.2 ± 0.02[b]	0.1 ± 0.01[c]
16:0	8.9 ± 0.1	9.0 ± 0.4	8.8 ± 0.2	8.7 ± 0.6	8.9 ± 0.3
18:0	5.2 ± 0.1	5.3 ± 0.3	5.0 ± 0.2	5.1± 0.3	5.1 ± 0.1
18:1n-9	8.8 ± 0.3[a]	8.4 ± 0.4[a]	8.9 ± 0.1[a]	8.6 ± 0.6[a]	9.3 ± 0.3[b]
18:2n-6	0.6 ± 0.03[a]	0.5 ± 0.1[a]	1.2 ± 0.04[b]	0.6 ± 0.1[a]	0.9 ± 0.1[c]
20:4n-6	5.6 ± 0.4[a]	5.7 ± 0.5[a]	4.8 ± 0.2[b]	5.3 ± 0.7[a]	5.2 ± 0.3[a]
18:3n-3	ND	ND	*138.3 ± 14.0[a]	ND	*72.7 ± 32.8[b]
20:5n-3	ND	ND	*119.1 ± 17.4[a]	ND	*78.7 ± 6.4[b]
22:6n-3	6.8 ± 0.2	6.7 ± 0.6	6.5 ± 0.4	6.5 ± 0.7	6.9 ± 0.7
TOTAL	41.7 ± 0.6	40.9 ± 1.8	40.6 ± 0.8	40.1 ± 2.6	41.8 ± 1.6

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] All values are mg/g; values with asterisk (*) units are μg/g; mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different ($p<0.05$). Fatty acids listed are the main representatives of the total fatty acids identified. For fatty acids less than 14 carbons, only 10:0 was detected (<0.1%).

ND – not detected

5.2.6 Plasma and Brain Cholesterol

There was no effect of the various ketogenic diets on the concentration of cholesterol in plasma (Table 5.11). Brain cholesterol levels were similar between the MCT, butter and 3F groups and these groups had significantly higher levels than the control and flax groups ($p<0.05$). There were no differences in brain cholesterol between the flax and control group.

5.2.7 Plasma Phospholipids

The total absolute amount of plasma PL in the butter group was significantly greater in the butter group compared to all other groups ($p<0.05$). The relative and absolute amounts (Tables 5.12 and 5.13) of myristic, and oleic acids, in plasma PL, were significantly higher in the butter group as compared to all other groups ($p<0.05$). The relative and absolute amounts of 14:0, 16:0 and 18:0 were generally lower in the flax group as compared to all other groups. The relative amount of AA was highest in the MCT group, while the absolute amount of AA was similar for both the butter and MCT groups. The relative amount of EPA was highest in the flax group as compared to the MCT and control groups (1.3 vs. 0.3 and 0.2% respectively) ($p<0.05$). In general, all groups had similar relative and absolute amounts of DHA in plasma PL. The relative amount of DHA was higher in the butter group than the 3F group, while the absolute amount was lower in the flax group compared to the 3F and MCT groups.

5.2.8 Plasma Triglycerides

The total absolute amount of fatty acids in plasma TG in the butter group was 3.5 fold higher than the MCT group, while all other groups were similar (Table 5.15). The relative and absolute amounts of 16:0 were significantly higher in the butter group as compared to all other groups (Tables 5.14 and 5.15) ($p<0.05$). The absolute

Table 5.11

Total Plasma and Brain Cholesterol

	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
Plasma[5]	2.5 ± 0.2	2.8 ± 0.4	2.5 ± 0.2	3.0 ± 0.4	2.7 ± 0.4
Brain[6]	17.5 ± 1.3 [a]	19.4 ± 0.7 [b]	17.5 ± 1.9 [a]	20.3 ± 3.1 [b]	20.7 ± 0.8 [b]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5,6] Values for plasma cholesterol data are in mmol/L, brain cholesterol data are in mg/g; mean ± SD, n=6 rats/group

Table 5.12

Percent Composition of Long Chain Fatty Acids in Plasma Phospholipids

	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	0.4 ± 0.1^{5ab}	0.5 ± 0.03^{a}	0.2 ± 0.1^{b}	0.8 ± 0.1^{c}	0.5 ± 0.1^{a}
16:0	26.7 ± 0.8^{a}	19.9 ± 0.7^{b}	15.3 ± 0.7^{c}	20.9 ± 1.4^{b}	14.2 ± 1.5^{b}
18:0	20.0 ± 0.9^{ac}	24.0 ± 1.4^{abc}	34.2 ± 10.2^{b}	23.9 ± 0.2^{c}	26.5 ± 1.3^{bc}
18:1n-9	5.7 ± 0.4^{a}	5.8 ± 0.6^{a}	5.8 ± 0.5^{a}	7.5 ± 0.3^{b}	5.6 ± 0.1^{a}
18:2n-6	25.3 ± 0.7^{ab}	23.5 ± 1.7^{a}	30.8 ± 5.9^{b}	29.0 ± 0.9^{ab}	29.8 ± 1.2^{ab}
20:4n-6	13.3 ± 0.7^{a}	17.2 ± 0.8^{b}	5.8 ± 1.9^{c}	11.1 ± 0.9^{d}	7.8 ± 0.6^{c}
18:3n-3	0.2 ± 0.03^{ab}	0.2 ± 0.03^{ab}	2.3 ± 0.9^{a}	0.3 ± 0.1^{b}	1.3 ± 0.2^{a}
20:5n-3	0.3 ± 0.1^{a}	0.2 ± 0.1^{a}	1.3 ± 0.1^{b}	0.4 ± 0.1^{ab}	1.5 ± 0.2^{b}
22:6n-3	3.1 ± 0.5^{ab}	2.9 ± 0.5^{ab}	1.8 ± 0.8^{ab}	1.7 ± 0.1^{a}	3.1 ± 0.3^{b}

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] Values are mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different ($p<0.05$). Fatty acids listed are the main representatives of the total fatty acids identified.

Table 5.13

Concentration of Long Chain Fatty Acids in Plasma Phospholipids

	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
*14:0	2.7 ± 0.8^{5ac}	4.1 ± 0.7^{ab}	1.5 ± 0.4^{c}	9.7 ± 1.1^{d}	5.0 ± 0.6^{b}
16:0	2.0 ± 0.3^{ab}	1.8 ± 0.4^{a}	1.1 ± 0.2^{c}	2.5 ± 0.2^{b}	1.8 ± 0.3^{a}
18:0	1.5 ± 0.3^{a}	2.2 ± 0.4^{ab}	2.5 ± 0.5^{bc}	2.9 ± 0.2^{c}	2.5 ± 0.3^{bc}
18:1n-9	0.4 ± 0.1^{a}	0.5 ± 0.1^{a}	0.4 ± 0.1^{a}	0.9 ± 0.1^{b}	0.5 ± 0.1^{a}
18:2n-6	1.9 ± 0.3^{a}	2.1 ± 0.4^{ab}	2.3 ± 0.6^{ab}	3.5 ± 0.3^{c}	2.8 ± 0.4^{bc}
20:4n-6	1.0 ± 0.2^{a}	1.6 ± 0.3^{b}	0.4 ± 0.2^{c}	1.3 ± 0.2^{ab}	0.7 ± 0.2^{ac}
*18:3n-3	1.7 ± 0.3^{a}	2.1 ± 0.4^{ab}	16.9 ± 7.6^{c}	2.6 ± 0.5^{ac}	12.5 ± 2.8^{bc}
*20:5n-3	2.2 ± 1.1^{ab}	1.3 ± 0.3^{a}	9.6 ± 0.9^{bc}	5.1 ± 0.9^{abc}	14.3 ± 3.1^{c}
22:6n-3	0.3 ± 0.1^{ab}	0.3 ± 0.1^{a}	0.1 ± 0.01^{b}	0.2 ± 0.01^{ab}	0.3 ± 0.1^{a}
TOTAL	7.9 ± 1.4^{a}	10.4 ± 2.1^{a}	7.4 ± 0.9^{a}	12.3 ± 1.0^{b}	9.7 ± 1.3^{a}

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] All values are mg/dL, values with asterisk (*) the units are in μg/g); mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different ($p<0.05$). Fatty acids listed are the main representatives of the total fatty acids identified.

amounts of 18:0 and18:1n-9 were generally similar among all groups with only the MCT group containing less of these two fatty acids than the butter group. The control group contained a greater absolute amount of LA in plasma TG compared to the MCT group, while all other groups were generally similar. The flax and 3F groups contained the highest relative amount of EPA as compared to all other groups ($p<0.05$). The relative amount of DHA in the butter and 3F groups was greater than that in the control and MCT groups ($p<0.05$). All of the groups were similar in the absolute amount of DHA in plasma triglyceride.

5.2.9 Plasma Free Fatty Acids

The total absolute amount of FFA was similar between the flax and 3F groups while all other groups were similar to one another (Table 5.17). The relative amounts of free 14:0, 16:0 and 18:0 were highest in the butter groups as compared to all other groups (Table 5.16) ($p<0.05$). The relative and absolute amounts of free 18:1n-9 were higher in the 3F groups as compared to the control group, while all other groups were generally similar. The relative amounts of LA were similar between the control, MCT, Flax, and 3F groups, which were all greater than butter ($p<0.05$). The relative amount of free ALA was greater than the control, MCT, and butter groups ($p<0.05$), but similar to the 3F group. No EPA was detected in the control, MCT or butter groups but it was detected in the flax and 3F groups. The flax and 3F group had similar absolute and relative amounts of free EPA. The relative amount of free DHA was similar between the flax and 3F groups. The 3F group had higher DHA than the control, MCT, and butter groups ($p<0.05$).

In summary, the proportion of saturates (14:0, 16;0 and 18:0) in the plasma were generally higher in plasma PL, TG, and FFA in the butter group as compared to the

Table 5.14

Percent Composition of Long Chain Fatty Acids In Plasma Triglyceride

	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	0.9 ± 0.2 [5ac]	1.7 ± 0.3 [abc]	0.4 ± 0.2 [a]	9.8 ± 0.6 [bc]	3.8 ± 0.2 [c]
16:0	23.5 ± 3.7 [a]	17.4 ± 4.2 [b]	8.4 ± 1.2 [c]	31.6 ± 0.5 [d]	18.8 ± 1.3 [ab]
18:0	3.8 ± 0.2 [a]	7.9 ± 1.3 [b]	5.4 ± 0.9 [a]	10.7 ± 0.7 [c]	8.3 ± 0.8 [b]
18:1n-9	23.4 ± 1.7	24.9 ± 3.1	25.9 ± 1.7	26.6 ± 0.6	25.3 ± 1.3
18:2 n-6	34.4 ± 5.3 [a]	26.5 ± 3.4 [ab]	20.9 ± 0.4 [abc]	9.9 ± 0.4 [c]	16.9 ± 0.7 [bc]
20:4 n-6	1.3 ± 0.3 [ab]	4.1 ± 1.6 [b]	0.5 ± 0.1 [a]	0.9 ± 0.2 [ab]	0.7 ± 0.3 [a]
18:3 n-3	4.1 ± 1.0 [abc]	2.3 ± 0.6 [ac]	33.5 ± 4.7 [b]	1.6 ± 0.2 [c]	17.0 ± 3.5 [ab]
20:5n-3	0.2 ± 0.1 [a]	0.9 ± 0.4 [b]	1.9 ± 0.5 [c]	0.5 ± 0.1 [ab]	1.9 ± 0.3 [c]
22:6 n-3	0.5 ± 0.1 [a]	1.9 ± 0.9 [a]	0.8 ± 0.4 [ab]	0.5 ± 0.1 [b]	2.0 ± 0.9 [b]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] Values are mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different ($p<0.05$). Fatty acids listed are the main representatives of the total fatty acids identified. For fatty acids less than 14 carbons, only 12:0 was detected in the Butter and MCT group (<2%).

Table 5.15

Concentration of Long Chain Fatty Acids in Plasma Triglycerides

	Control	MCT	Flax	Butter	3F
*14:0	5.2 ± 1.3 [abc]	4.2 ± 1.4 [ac]	1.5 ± 0.6 [a]	85.5 ± 30.9 [b]	12.8 ± 8.0 [bc]
16:0	1.4 ± 0.3 [a]	0.4 ± 0.2 [c]	0.4 ± 0.3 [ac]	2.8 ± 0.9 [b]	0.9 ± 0.6 [ac]
18:0	0.2 ± 0.1 [ab]	0.2 ± 0.05 [a]	0.3 ± 0.2 [ab]	0.9 ± 0.3 [b]	0.4 ± 0.3 [ab]
18:1n-9	1.4 ± 0.3 [ab]	0.6 ± 0.2 [a]	1.4 ± 1.1 [ab]	2.3 ± 0.8 [b]	1.2 ± 0.9 [ab]
18:2 n-6	2.1 ± 0.7 [a]	0.7 ± 0.3 [b]	1.1 ± 0.9 [ab]	0.9 ± 0.3 [ab]	0.8 ± 0.6 [ab]
*20:4 n-6	7.8 ± 2.0 [a]	7.5 ± 2.7 [a]	2.2 ± 1.4 [b]	7.7 ± 1.3 [a]	3.0 ± 1.7 [b]
18:3 n-3	0.3 ± 0.1 [ab]	0.1 ± 0.04 [b]	1.9 ± 1.9 [a]	0.1 ± 0.05 [c]	0.9 ± 0.9 [ac]
*20:5 n-3	1.3 ± 0.5 [a]	2.4 ± 1.9 [ab]	8.6 ± 4.5 [ab]	3.9 ± 0.7 [b]	10.4 ± 5.9 [b]
*22:6 n-3	3.4 ± 1.5	2.9 ± 1.0	3.6 ± 2.1	3.8 ± 0.8	7.9 ± 4.2
TOTAL	6.1 ± 1.5 [ab]	2.5 ± 1.1 [a]	4.8 ± 4.3 [ab]	8.7 ± 3.0 [b]	4.8 ± 3.7 [ab]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] All values are mg/dL; Values with asterisks (*) the units are in μg/mL; mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different ($p<0.05$).
Fatty acids listed are only representatives of the total. For fatty acids less than 14 carbons, only 12:0 was detected in the Butter and MCT group (<0.2mg/dL).

Table 5.16

Percent Composition of Long Chain Fatty Acids in Plasma Free Fatty Acids

	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	1.9 ± 1.0 [5a]	2.9 ± 0.5 [a]	1.0 ± 0.5 [a]	5.4 ± 1.9 [b]	2.7 ± 0.5 [a]
16:0	32.5 ± 5.3 [ac]	27.5 ± 5.8 [ad]	13.4 ± 2.8 [b]	34.7 ± 2.6 [c]	23.1 ± 1.7 [d]
18:0	11.3 ± 0.4 [ab]	12.5 ± 1.9 [a]	9.6 ± 1.6 [b]	16.4 ± 1.3 [c]	13.1 ± 1.3 [a]
18:1n-9	15.9 ± 1.9 [a]	16.6 ± 1.7 [a]	17.9 ± 1.0 [ac]	20.9 ± 2.5 [bc]	20.8 ± 1.8 [c]
18:2n-6	20.9 ± 5.0 [a]	21.7 ± 5.3 [a]	18.5 ± 1.1 [a]	12.0 ± 1.0 [b]	15.5 ± 0.3 [ab]
20:4n-6	5.4 ± 1.0 [a]	4.6 ± 0.8 [ac]	1.9 ± 0.6 [b]	3.5 ± 0.5 [c]	2.2 ± 0.5 [b]
18:3n-3	2.5 ± 0.8 [ab]	2.6 ± 0.9 [ab]	31.2 ± 4.4 [c]	1.5 ± 0.3 [a]	15.6 ± 1.9 [bc]
20:5n-3	ND	ND	1.4 ± 0.4	ND	1.2 ± 0.3
22:6n-3	0.5 ± 0.4 [a]	0.6 ± 0.3 [a]	0.7 ± 0.2 [ab]	0.5 ± 0.1 [a]	1.1 ± 0.4 [b]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] Values are mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different ($p<0.05$). Fatty acids listed the main representatives of the total fatty acids identified.For fatty acids less than 14 carbons, only 10:0 and 12:0 were detected in the MCT group (<5%).

ND – not detected

Table 5.17

Concentration of Long Chain Fatty Acids in Plasma Free Fatty Acids

	Control	MCT [1]	Flax [2]	Butter [3]	3F [4]
14:0	0.9 ± 0.5 [5a]	2.0 ± 0.6 [ab]	0.9 ± 0.3 [b]	3.9 ± 1.9 [b]	2.4 ± 0.9 [ab]
16:0	15.8 ± 2.3 [ab]	19.2 ± 6.6 [ab]	14.7 ± 6.6 [b]	24.3 ± 6.3 [a]	19.3 ± 4.8 [ab]
18:0	5.5 ± 0.5 [a]	8.5 ± 0.9 [ab]	10.8 ± 5.5 [ab]	11.4 ± 2.2 [b]	10.9 ± 3.2 [b]
18:1n-9	7.8 ± 1.4 [a]	11.7 ± 3.6 [ab]	15.1 ± 5.2 [ab]	14.9 ± 5.4 [ab]	17.6 ± 5.2 [b]
18:2n-6	10.4 ± 3.1	15.4 ± 6.4	15.5 ± 5.4	8.5 ± 2.7	13.0 ± 3.5
20:4n-6	2.6 ± 0.7 [ab]	3.2 ± 0.9 [a]	2.1 ± 1.1 [ab]	2.4 ± 0.5 [ab]	1.8 ± 0.4 [b]
18:3n-3	1.2 ± 0.5 [ac]	1.9 ± 0.9 [abc]	25.8 ± 10.0 [b]	1.0 ± 0.4 [c]	13.4 ± 4.8 [ab]
20:5n-3	ND	ND	1.0 ± 0.3	ND	0.9 ± 0.2
22:6n-3	0.4 ± 0.1 [a]	0.4 ± 0.1 [ab]	0.8 ± 0.4 [ab]	0.3 ± 0.1 [a]	0.9 ± 0.2 [b]
TOTAL	53.8 ± 5.3 [a]	79.2 ± 19.7 [ab]	91.8 ± 26.5 [b]	78.6 ± 20.5 [ab]	89.9 ± 23.9 [b]

[1] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[2] Flax denotes the flaxseed oil ketogenic diet groups
[3] Butter denotes the butter ketogenic diet group
[4] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight
[5] Values are μg/mL; mean ± SD, n= 6 rats/ group. Values in each row are with different letter superscripts are significantly different ($p<0.05$). Fatty acids listed are only representatives of the total. For fatty acids less than 14 carbons, only 10:0 and 12:0 were detected in the MCT group (<2.0mg/g).

ND – not detected

other diet groups. The proportion of AA in plasma TG in the MCT diet group was greater than all other diet groups. The proportion of AA in plasma FFA was similar between control, MCT, and butter, which were all greater than the flax and 3F diet groups.

The proportion of ALA in plasma TG and FFA of the flax group was greater than the MCT and butter group, but similar among all other groups. The proportion of EPA in plasma TG was similar between the flax and 3F diet groups, which were greater than all other diet groups. Plasma FFA EPA was only detected in the flax and 3F diet groups. The proportion of DHA in plasma TG and FFA was similar between the flax, butter, and 3F diet groups, which were all greater than the control and MCT diet groups. Thus there was a statistically significant increase in the levels of n-3 PUFA in the plasma of the flax and 3F groups.

Finally, the total concentration of fatty acids in plasma PL was highest in the butter group compared to all other groups. The total concentration of fatty acids in plasma TG in the MCT group were higher than the butter group, otherwise all other diet groups were similar. The total concentration of fatty acids in plasma FFA in the flax and 3F groups were greater than the control group, whereas all other groups were similar.

5.3 KETONE LEVELS AND SEIZURE TEST DATA

As mentioned previously, seizure data and ketone levels were measured by a previous MSc student, Kathy Musa (1999) in a group of animals fed the same test diets in a parallel study with the animals used for lipid analysis. The ketone levels and seizure data are summarized in Table 5.18. They are presented to illustrate that the level of fat oxidation was higher in the ketogenic diet groups when compared to

Table 5.18

Pentylenetetrazole (PTZ) Threshold Seizure Test Results and Ketone Levels [1]

Diet Group	β- OHB [2] (mmol/L)	% of Rats Protected from Seizures	Seizure Incidence	Seizure [3] Score
Control	0.1 ± 0.03[a]	0[a]	25/25[a]	3.2 ± 0.4[a]
MCT [4]	5.2 ± 2.0[b]	33[b]	8/12[b]	2.1 ± 1.6[a,b]
Flax [5]	0.7 ± 0.2[c]	50[b]	6/12[b]	1.7 +1.8[b]
Butter [6]	0.9 ± 0.3[c]	42[b]	7/12[b]	1.8 ± 1.5[b]
3F [7]	0.8 ± 0.3[c]	18[a,b]	9/11[a,b]	2.5 ± 1.2[a,b]

[1] Data obtained from Musa (1999)
[2] β- OHB -- β- hydroxybutyrate. Values are mean ± SD, n=12 rats/ group
[3] values are mean ± SD, n=12 rats/ group
[4] MCT denotes the medium chain triglyceride oil ketogenic diet groups
[5] Flax denotes the flaxseed oil ketogenic diet groups
[6] Butter denotes the butter ketogenic diet group
[7] 3F denotes the mixture ketogenic diet group of medium chain triglyceride oil, flaxseed oil and butter diet group in a 1:1:1 ratio by weight

controls. They also demonstrate that the level of ketosis between the different ketogenic diet groups was not the same. The level of β-OHB was significantly higher in the MCT group, and lower in the control group compared to all other diet groups ($p<0.05$). The level of β-OHB was similar between the flax, and butter and 3F diet groups.

The seizure data are summarized as they were used to determine whether or not a relationship exists between these data and the level of n-3 PUFA FFA in plasma. Depending on the fat source used, the ketogenic diets used in this study protected against PTZ – induced seizures in 18-50% of rats, while no rats on the control diet were protected (Table 5.18). This anticonvulsant effect was statistically significant for the butter, flaxseed, and MCT KD diet groups, but was not significant for the 3F diet group ($p<0.05$). The severity of seizures as defined by the seizure score was significantly lower for the flax and MCT groups, while all other groups were similar ($p<0.05$). The latency and duration of seizures were similar in all groups (see Musa, 1999).

5.4 RELATIONSHIPS BETWEEN DIET, LIPID PROFILES AND SEIZURE PROTECTION

5.4.1 Diet and Tissue Profiles of LA and ALA

The data from Figure 5.1 have been extracted from the appropriate tables in the Results section to illustrate some interesting relationships between dietary and tissue fatty acid profiles of the ketogenic diet groups. LA and ALA have been chosen for this purpose because of their relatively high oxidatizability and therefore ketogenic potential, their hypothesized links to anticonvulsant protection, as well as their dietary role as precursors to LC-PUFA (Leyton et al 1987, Cunnane and Anderson 1997, Voskuyl et al 1998).

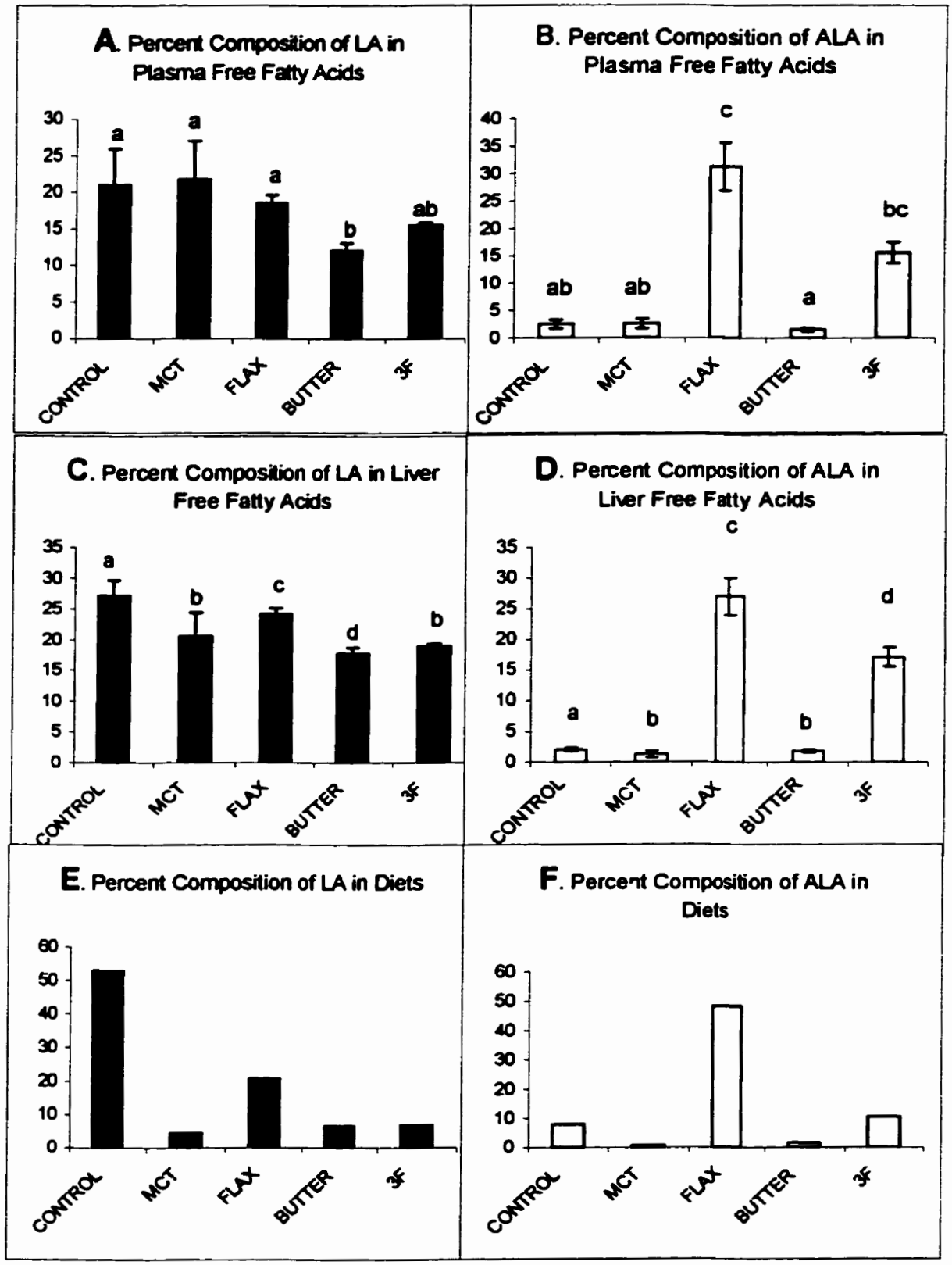

Figure 5.1 Overview of the Percent Composition of LA and ALA in Tissues and Diet
Values with different letters are significantly different ($p<0.05$)

In the control group, the proportion of LA in the diet was approximately 2 fold higher than the proportions of LA found in the liver and plasma (see figure 5.1A,C,E). In the control group, the proportion of ALA was 3 fold higher in the diet than liver and plasma FFA. The flax and butter diet groups had proportionately the same amount of LA in their respective diets as the amount found in the liver and plasma FFA. The flax group had over 1.5 fold higher ALA in the diet than what was found in the plasma and liver FFA.

The MCT group on the other hand had proportionately 5 times higher liver and plasma free LA than the proportion given in the diet. The MCT groups also had 5 fold higher ALA in plasma FFA and 2.7 fold higher ALA in liver FFA as compared to the proportion given in the diet. The proportion of LA in the liver and plasma FFA of the MCT group were very similar. The butter group had 2 fold higher LA in plasma FFA, and 4 fold higher LA in liver FFA than the proportion given in the diet. However, the amount of ALA in the butter group in plasma and liver was proportionate to what was given in the diet. The 3F group had proportionately 2 fold higher LA and 1.5 fold higher ALA in plasma and liver FFA than the proportion given in the diet.

5.4.2 Comparison of the Tissue Compositions of DHA, ALA, and EPA to Ketone Levels and Seizure Protection

No relationship was evident between the levels of free DHA in tissues and the plasma ketone levels or seizure protection. The 3F group had significantly higher plasma free DHA levels than the MCT, butter and control groups, and significantly higher DHA in the liver compared to all diet groups (Figure 5.2A,C,E). Despite these differences, all of the groups contained similar proportions of DHA in brain total lipids. The seizure responses were also similar among the ketogenic diet groups, which were

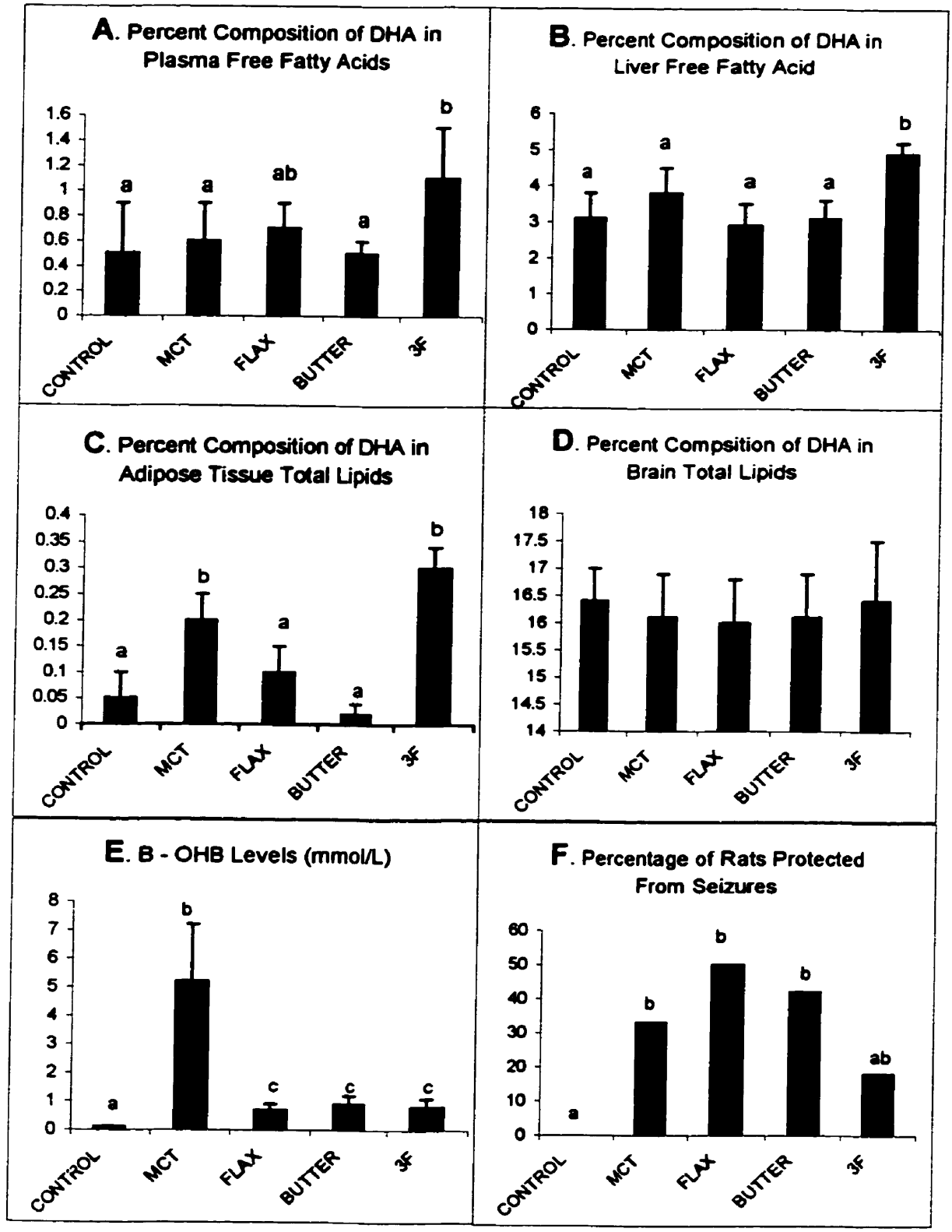

Figure 5.2. Overview of DHA % Composition in Tissues, Ketone Levels, and Seizure Protection
Values with different superscripts are significantly different ($p<0.05$).

significantly greater than the control group (with the exception of the 3F group which was similar to the control group) ($p<0.05$). Plasma ketone levels were significantly higher in the MCT groups as compared to all other diet groups, but this did not translate into improved seizure protection for this group.

Despite significantly higher levels of free ALA in the plasma, liver, and total ALA in adipose of the flax group, this did not result in a significant increase in ketone levels (Figure 5.3F). The higher proportion of plasma free ALA and total brain ALA did not reflect a difference in improved seizure protection in the flax group as compared to the other ketogenic diet groups.

The proportion of free EPA in plasma, liver, and total EPA in brain was significantly higher in the flax and 3F group as compared to all other groups (Figure 5.4A,B,C). As seen with ALA, an increase in EPA did not relate to an increase in the level of ketosis, or increased protection from seizures in these diet groups.

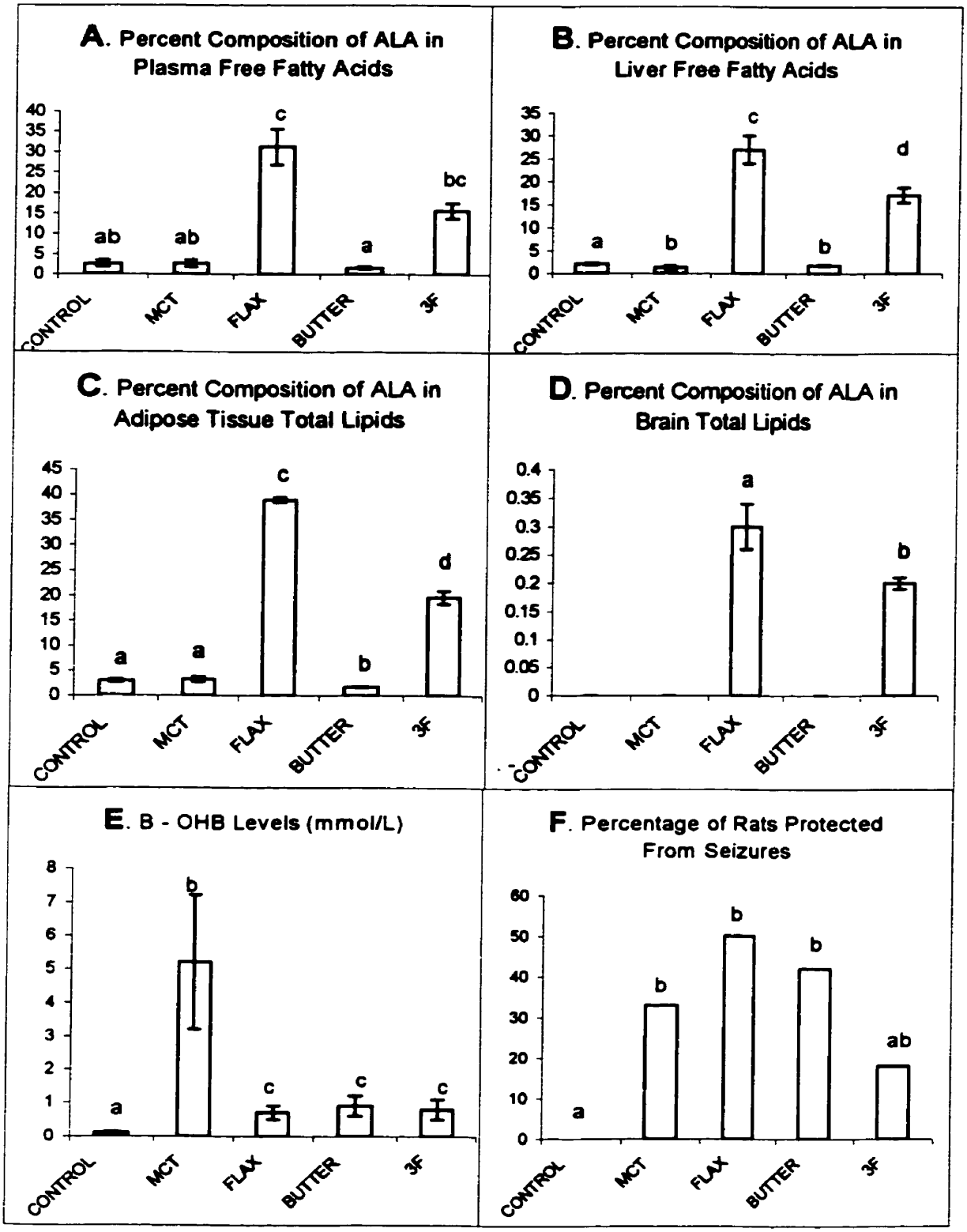

Figure 5.3 Overview of ALA % Composition in Tissues, Ketone Levels, and Seizure Protection
Values with different superscripts are significantly different ($p<0.05$).

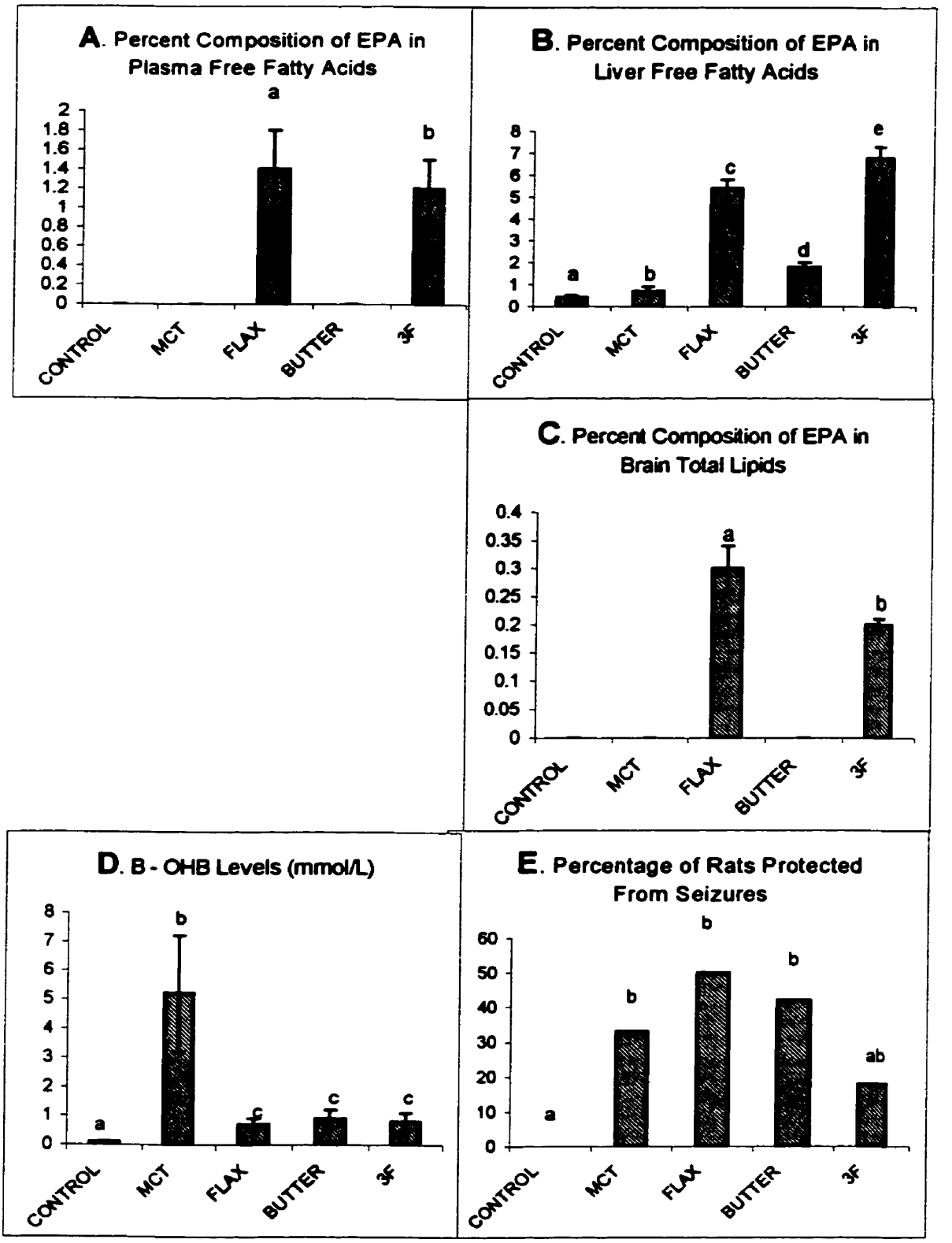

Figure 5.4 Overview of EPA % Composition in Tissues, Ketone Levels, and Seizure Protection
Values with superscripts are significantly different ($p<0.05$). No EPA detected in adipose tissue

5.4.3 Correlations Between Plasma FFA Levels of LC-PUFA and Seizure Protection

Voskuyl et al. (1998) demonstrated anticonvulsant protection in rats after i.v. infusion of free DHA and EPA using the cortical stimulation model. They also observed increases in the plasma concentration of free DHA and EPA, but did not try to correlate this with the observed increase in anticonvulsant threshold. It was therefore one of my objectives to try to determine the strength of the association between plasma concentrations of free ALA, EPA, DHA and the seizure test results as determined by a series of correlations. There was a no association between plasma free DHA, and seizure score (Figure 5.5). Similar results were obtained when correlations between plasma free DHA, ALA and EPA were preformed against the other seizure parameters listed in Table 5.18. Correlations between the liver FFA levels of these n-3 PUFA were also performed against the seizure test parameters and were also non-significant. Table 5.19 lists the correlations performed and their respective correlation coefficients and p-values.

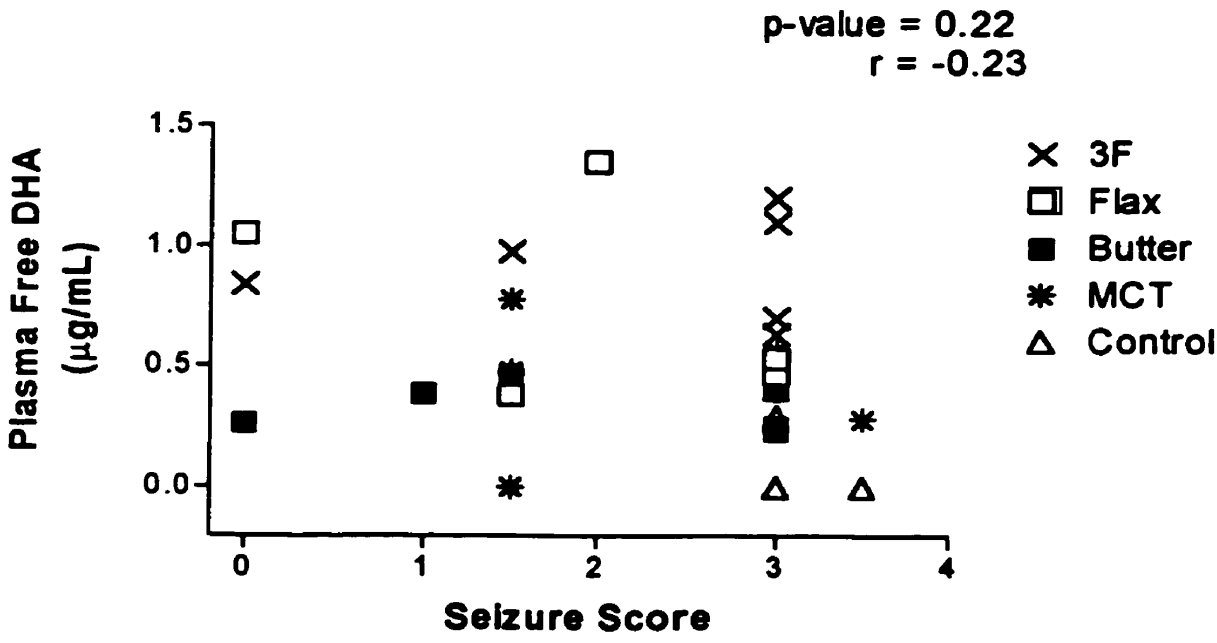

Figure 5.5 Relationship Between Plasma Free DHA and Seizure Score

Table 5.19

Relationships Between Liver and Plasma Free n-3 Polyunsaturated Fatty Acids and Seizure Test Results

		Percentage of Rats Protected from Seizures		Seizure Incidence		Seizure Score	
		r[1]	p-value	r	p-value	r	p-value
Plasma	ALA[2]	0.300	0.683	-0.300	0.683	0.218	0.263
	DHA[3]	-0.103	0.783	-0.154	0.783	-0.232	0.222
	EPA[4]	0.467	0.503	-0.467	0.503	-0.467	0.503
Liver	ALA	0.200	0.783	-0.200	0.783	0.101	0.605
	DHA	-0.564	0.350	0.564	0.350	-0.292	0.130
	EPA	0.467	0.503	-0.467	0.503	-0.467	0.503

[1] correlation co-efficient
[2] α- linolenic acid
[3] docosahexaenoic acid
[4] eicosapentaenoic acid

CHAPTER 6

GENERAL DISCUSSION

CHAPTER 6. GENERAL DISCUSSION

6.1 OVERVIEW

The ketogenic diet involves the consumption of dietary fat that is greater than 60% by weight of the total macronutrients in the diet. An extensive review of the literature has indicated that the effects of a ketogenic diet on tissue fatty acid profiles have not been reported. As a result, one of the important metabolic effects of the ketogenic diet still remains largely unknown. The Cunnane group appears to be the first to have compared models of the ketogenic diets used clinically to one that contains flaxseed oil. Initial observations are submitted (Likhoddii et al., 1999) and the current data suggest that the use of flaxseed oil as the primary fat source of the ketogenic diet provides the same degree of seizure protection as the classic and MCT ketogenic diets used clinically. This study was unable to prove that a flaxseed oil-based ketogenic diet would result in higher levels of ketosis, and greater seizure protection than the classic and MCT ketogenic diets.

Although the pathways of lipid metabolism (digestion, transport, absorption, and oxidation) during the consumption of diets containing greater than 40% fat are well understood, the exact partitioning of fatty acids (particularly the PUFA) between oxidation and esterification on a ketogenic diet are not. ALA and LA have several fates within the body including accumulation in storage and structural lipids, elongation to longer chain derivatives, and β-oxidation which yields respiratory CO_2 or acetate (Cunnane et al, 1999). The effects of a ketogenic diet on PUFA profiles in tissues may reveal important relationships between tissue lipid constituents and seizure protection.

The biological importance of n-3 and n-6 PUFA have been implicated in neuronal tissue growth and repair, the development of learning patterns and memory

(Agostoni et al. 1995, Farquharson et al 1995), and, most recently, anticonvulsant protection (Yehuda et al., 1994, Voskuyl et al 1998). The work of Yehuda et al (1994) and Voskuyl et al (1998) demonstrating the anticonvulsant effects of PUFA (i.e. ALA, LA, DHA, and EPA) suggested that the free form of these fatty acids in the plasma, or their accumulation and incorporation in the brain contributes to their protective effects. The focal point of my project was to examine the tissue fatty acid profiles induced by the ketogenic diet, and to determine whether any significant relationships existed between plasma free n-3 PUFA and the seizure results obtained by Musa (1999). Musa (1999) conducted a parallel project using the same design but studied breath acetone production.

6.2 NUTRITIONAL STATUS, MEMBRANE COMPOSITION AND THE KETOGENIC DIET

The ketogenic diet in the clinical situation is nutritionally complete only when parents provide their child with the use of a sugar-free multivitamin (Freeman et al 1996). No studies exist to date which have examined the effects of nutrient absorption, or adverse effects from the absence of micronutrients on such a high fat diet. Similarly no studies have reported the compliance rate on the use of vitamin supplements for children on a ketogenic diet. Thus, the nutritional status of children on a ketogenic diet requires further exploration.

In the clinical situation in children, lower weight gain on a ketogenic diet is experienced as a result of intentional energy restriction, which is used in order for the children to sustain ketosis (Freeman, 1996). In this study, with the exception of the 3F group that was similar to the control group, the ketotic rats weighed between 10-40% less than the control rats ($p<0.05$). The MCT group did not adjust to the diet as readily

as the other diet groups and this resulted in significantly lower body weight gain ($p<0.05$). Lower body weights in the ketogenic diet groups (with the exception of the 3F group) may have been a result of lower food intakes (no data obtained) because of the significantly high level of ketones in their blood (Table 5.18). High ketone levels are known to have an appetite suppressing effect (Cahill 1982, Scharrer 1999). MCTs, however, even at similar overall energy intakes have been shown to decrease fat deposition, increase metabolic rate, and enhance thermogenesis (Seaton, 1986).

Lower intakes in the ketogenic diet groups may have put them at a potential risk for nutrient deficiency, particularly because of the unbalanced nature of the ketogenic diets. The data in Table 4.2 provide the diet ingredient compositions in g/kg and mg/kcal. Although on a weight basis the protein and nutrient compositions of the ketogenic diets appear similar to the control diet, the differences become more apparent when they are expressed in terms of energy. The lower amounts of protein and minerals in the ketogenic diets as opposed to the control diet indicate that the MCT group in particular ate less protein and other essential nutrients. It is not possible to say whether the ketogenic diet groups could have been malnourished but if so, this would only likely have occurred in the MCT group. In this study however, diets were prepared in an attempt to model the clinical situation in children as closely as possible.

The differences in the total liver and plasma TG between the flax, MCT groups as compared to the 3F and butter groups also mitigates the concern that the ketogenic diets may have resulted in malnourishment due to protein deficiency. Studies examining the effects of protein deficiency have reported lower amounts of very low density lipoprotein (VLDL) apo B and total apo C (which originate in the liver) in rats fed protein deficient diets (Meghelli-Bouchenak et al 1987). As a result of protein

malnutrition the rats they examined had higher concentrations of liver TG, as a result of the inability to export fatty acids out of the liver (Meghelli-Bouchenak et al 1987). It should be noted that the diets used in the study by Meghelli-Bouchenak et al (1987) were 2 and 5% protein by weight, which is much less than what was used in the present study.

The data presented in Table 5.5 illustrate that the total concentrations of liver TG were not similar between the ketogenic diet groups. Only the butter and 3F groups had an elevation in liver TG, while the MCT and flax diet groups were similar to control. Given that the total amount of fat and protein was the same between the ketogenic diet groups, the changes observed in liver TG are a function of the type of fat, and not the result of a protein deficiency. If protein deficiency was present in the ketogenic diet groups it would have been expected to observe increases in liver TG across all groups.

Although the flax and MCT diet groups had similar plasma and liver total concentrations of TG as controls, the amount of fatty acids in liver PL was significantly greater in all the ketogenic diet groups compared to controls. Differences in liver weights were observed, suggesting that differences in PL fatty acid content may be a result of differences in the number of cells, and therefore membranes in the liver (Table 5.1). Differences in liver weights among diet groups were no longer present when the liver weights were expressed as a percentage of body weight (Table 5.1). This suggests that the ketogenic diet groups did not have an increase in liver membrane synthesis, but an increase in fatty acid accumulation. Whether or not increased incorporation of fatty acids in liver PL is related to seizure protection is not known.

6.3 ADIPOSE TISSUE COMPOSITION ON A KETOGENIC DIET

Adipose tissue, the repository of dietary fat and supplier of free fatty acids is considered to reflect the composition of dietary fat intake, but is not an exact mirror (Berry, 1997). In the present study, the proportions of fatty acids in the diets were generally similar to their proportions in adipose tissue (Table 4.2 and Table 5.8). The exceptions to this arose in the MCT and 3F groups where the dietary short and medium chain fatty acids are directly transported to the liver and not deposited in adipose to an appreciable extent. An interesting discovery from the analysis of the adipose tissue of the 3F and MCT groups was that they had much higher proportions of LA and ALA than were given in the diet. In fact, the proportion of LA in the adipose tissue of the MCT group was similar to the controls and significantly higher than the flax, butter, and 3F groups ($p<0.05$). The amount of AA in the MCT group was also significantly higher than all other groups.

Thus, it appears in these two diet groups that the rat is conserving or sparing the release of ALA and LA. A possible explanation for this result may be that the higher proportion of MCT in these diets drives fat oxidation because of their direct transport to the liver and therefore allows PUFA to be spared. Although the proportion of ALA in the MCT group was higher in adipose tissue than in the diet, the proportion in adipose tissue was still significantly lower than in the flax and 3F groups reflecting the higher dietary n-6 to n-3 PUFA ratio of the MCT diet.

The amount and type of fatty acid coming to the liver on TG or indirectly from FFA release by adipose tissue will vary with diet and has been shown to influence the hepatic secretion of TG. Kohout et al (1971) found that the total output of TG by the liver was a function of the chemical structure of the fatty acid in the diet. Perfusion of

fatty acids containing 12 carbons or more resulted in more TG secretion and was greatest in the presence of 16:0. However, long chain fatty acids of 18 carbons or more decreased the output of TG as the number of double bonds in the fatty acid increased (Kohout et al., 1971).

The higher proportions of long chain saturated fatty acids compared to PUFA in the butter and 3F group may explain the increased plasma and liver TG. Subsequently, the total liver TG concentration was significantly higher in the butter and 3F groups compared to the control, MCT, and flax group (Table 5.5). The more rapid oxidation to CO_2 of short and medium chain saturated fatty acids, and of long chain unsaturated fatty acids may explain why MCT and PUFA tend to lower the level of TG in plasma in the flax and MCT groups.

6.4 BRAIN FATTY ACID PROFILES

Tables 5.9 and 5.10 contain data on the fatty acid profiles of the brain lipids. The amount of LA, ALA, and EPA was significantly higher, and the amount of AA was lower in the flax group as compared to all other diet groups ($p<0.05$). These data confirm other studies which have shown that it is possible to significantly alter the fatty acid composition of the brain through dietary manipulation in rats post-weaning (Greenwood et al 1989, Bourre et al., 1988). What is also interesting is that, despite the dramatic contrasts in the dietary amounts of ALA or the n-6 to n-3 PUFA ratios between the groups, there was no difference in the amount of DHA found in the brain. It had been predicted given that the flax group had the highest levels of ALA in diet, plasma, and brain, that this would have lead to a greater increase in DHA in the brain. Although free brain DHA was not measured, it is possible that it could have been higher in the groups

given flaxseed oil. However, the brain fatty acid data suggest that in the ketogenic diet groups not given flaxseed oil the ALA and LA is partitioned more towards elongation into LC-PUFA than towards oxidation. They also suggest, given the similarity in DHA levels between the groups, that there is a limit to the amount of ALA that is elongated into brain DHA, or to the amount of exogenous DHA, or other LC-PUFA precursors of DHA that can enter the brain.

The level of AA was significantly higher in the brains of animals in the MCT group ($p<0.05$). Although AA is an important component in membrane PL, free arachidonic acid is known to produce a variety of detrimental neurotoxic effects on membrane structure and activities of membrane-bound enzymes (Faroqui et al., 1997). The seizure data from this study do not suggest any deleterious effects of an MCT diet, though pro-convulsant effects of an MCT based ketogenic diet have been reported (Mahoney et al., 1983, Otani et al., 1984, Thavendiranathan et al 1999).

6.5 BRAIN AND PLASMA CHOLESTEROL

Exogenous cholesterol contributes little to the pool of cholesterol in the brain, and it is known that the brain synthesizes de novo all of the cholesterol it requires (Edmond et al., 1991). It has been demonstrated that when dietary cholesterol is high (>200% normal) blood concentrations of cholesterol increase 2-3 fold, yet normal concentrations of cholesterol in the brain are maintained (Edmond et al., 1991). In my study the MCT, butter, and 3F groups had significantly higher brain cholesterol than that of the control and flax group ($p<0.05$, Table 5.11). These results illustrate that although manipulation of dietary cholesterol itself does not affect brain cholesterol,

manipulation of dietary fatty acids and, possibly, plasma ketones can alter the level of cholesterol in the brain.

The lower brain cholesterol levels of the flax group versus the butter, MCT, and 3F groups might be explained by the very high ALA content of the ketogenic diet prepared with flaxseed oil. ALA is known to have a high oxidative capacity, resulting in increased ketone body synthesis. Thus the resulting ketones in this group may have been oxidized via the citric acid cycle, or preferentially contributed to the de novo synthesis of other fatty acids over cholesterol synthesis. No ketone body data were obtained from the brains of these animals so differences in ketone levels can only be speculated upon.

PUFA such as ALA and LA are known to lower serum cholesterol, and the mechanism by which this occurs is a highly controversial topic in lipid nutrition. For this study, it would have been predicted that high saturated fat feeding (particularly of fatty acids 14:0, 16:0, and 18:0) would have resulted in higher cholesterol levels than those rats on an MCT or high PUFA diet. Although this trend is apparent between the groups, there were no statistically significant differences in plasma cholesterol. One reason accounting for the similarity in plasma cholesterol values may be due to the weight differences between the diet groups. Lower weight gain in the ketogenic diet groups may have resulted in less carbon going into cholesterol synthesis and more carbon routed towards energy metabolism. Beynen and Katan (1985) hypothesized that the mechanism for lowering serum cholesterol through PUFA and MCT dietary manipulation was through their preferential oxidation into ketone bodies.

The brain and plasma cholesterol data in this study contradict other studies which have not only shown increases in tissue levels of cholesterol on a ketogenic diet,

but have also suggested that their relative increases provide anticonvulsant protection (Vining et al 1996, Dekaban et al 1966). In this study seizure protection was observed in all of the ketogenic diet groups regardless of the similarity in piasma cholesterol levels, or in the case of the flaxseed diet group, the lack of change in brain cholesterol. The present data do not support the concept that increases in tissue lipid constituents are important in providing seizure protection.

Several studies have demonstrated that the oxidation rates of ALA are comparable to those of the highly ketogenic MCT (Kohout et al. 1971, Beynen and Katan, 1985, Jones et al., 1985, Leyton et al., 1987). Therefore we hypothesized that a ketogenic diet with a highly oxidized dietary fat source would have higher levels of ketosis. Although this was not a primary focus of this thesis, it is interesting to note that the levels of ketosis were not similar between the diet groups (Table 5.18). The MCT group had significantly higher levels of β-OHB than all other groups. The level of ketosis of the flax group (although higher than controls) did not differ from the butter or 3F group. Subsequently the ketone data from this study do not support the evidence in the literature on the high oxidation rates of PUFA.

6.6 SEIZURE PROTECTION

One of the objectives of this work was to determine whether or not plasma FFA levels of ALA, EPA, and DHA hypothesized to be anticonvulsant (Yehuda1994, Voskuyl 1998), were associated with seizure protection. Figures 5.2, 5.3, and 5.4 illustrate the levels of the various n-3 PUFA on plasma and liver FFA, adipose total lipid, β-OHB levels, and the percentage of rats protected from seizures in all groups.

The relative amounts of free ALA and EPA were significantly higher in the flax group than all other groups ($p<0.05$). Plasma free DHA was higher in the 3F group as compared to the control, MCT and butter group, but did not differ from the flax group. The similarity of the amount of plasma free DHA in the flax group compared to the other groups was surprising but, given the higher amounts of free ALA and EPA, this group still would have been predicted to protect more rats from seizures. The percentage of rats protected from seizures ranged from 18-50% (Table 18). Although 50% of rats on the flax ketogenic diet were protected, this was not significantly different from any of the other ketogenic diet groups. Thus, despite the higher free n-3 PUFA in plasma, there was no relationship to seizure protection. Similarly, seizure protection was not associated with the level of DHA in the brain total lipid as all of the diet groups were similar. To test the strength of association between the plasma free n-3 PUFA and seizure protection, a series of correlations were preformed (Table 5.19, Figures 7 and 8). No statistically significant relationships between any of the plasma free n-3 PUFA and the seizure parameters were found.

6.7 SUMMARY

In summary, this thesis has presented data on the dietary effects of a ketogenic diet on the fatty acid profiles of liver, adipose, brain, and plasma, as well as total brain and plasma cholesterol. Despite higher levels of free n-3 PUFA in the liver, brain and plasma of rats fed an n-3 PUFA enriched diet, no relationship was found between this and the seizure test results obtained by Musa (1999) in animals receiving the same diets. Although we achieved one of our objectives in comparing the lipid profiles of the ketogenic diets presented in this study, we were not able to establish a relationship

between seizure protection and increased plasma free n-3 PUFA as demonstrated by Voskuyl et al (1998). Our data suggest that the seizure protection observed on a ketogenic diet is not due to the increased plasma concentrations of n-3 PUFA.

The hypothesis that rats consuming greater numbers of fatty acids partitioned towards β-oxidation and, subsequently, ketogenesis would have a higher states of ketosis than rats consuming greater amounts of long chain saturated fatty acids was partially supported. Rats consuming a ketogenic diet containing primarily MCT oil were ketotic, but the MCT diet resulted in higher levels of ketosis than a ketogenic diet prepared with flaxseed oil. Given that ALA has been shown to be highly oxidized (Kohout et al. 1971, Beynen and Katan, 1985, Jones et al., 1985, Leyton et al., 1987), my data contradict the results reported in the literature. My data also contradict studies that suggest that the seizure protection of a ketogenic diet is a direct function of plasma ketone levels (Uhelmann and Neims 1972, Bough and Eagles 1998).

6.8 IMPLICATIONS FOR FUTURE STUDIES

A major implication of this work appears to be that a ketogenic diet comprised of flaxseed oil is capable of providing the same degree of seizure protection as the clinically used classic and MCT diets. As well, it was demonstrated that a flaxseed oil-based diet comprised of 60% fat by weight resulted in similar plasma and liver TG and plasma cholesterol as a control diet that was 7% fat by weight. Increasing the amount of n-3 PUFA may be an important consideration for the dietician initiating a ketogenic diet as a way of affording seizure protection without increasing the lipidemia of the patient unnecessarily.

Although there is currently no literature available that has performed a comprehensive plasma lipid analysis in children on the ketogenic diet composed of PUFA, caution should be exercised in its recommendation. Oxidation of low density lippoproteins (LDL) begins with the abstraction of hydrogen from a PUFA and oxidized LDL are one the determinants in the pathogenesis of artherosclerosis (Reaven and Witztum, 1996). Thus LDL fatty acid composition contributes to the process of LDL oxidation, and dietary fat influences the fatty acid composition of LDL, and may effect the susceptibility of LDL to oxidative damage. One of the factors affecting the overall extent of LDL oxidation is the number of LDL present in the artery wall. PUFA are known to lower the amount of LDL that enter the arterial wall and theoretically would reduce the amount of LDL available for oxidation (Reaven and Witztum, 1996). No markers of oxidative damage, such as plasma thiobarbituric acid-reactive substances (TBARS) were measured in the present study, and no data are currently published on the extent of LDL oxidative damage while on a ketogenic diet. If this were to be a concern, perhaps the use of supplementation with an anti-oxidant such as vitamin E could be considered to prevent the possibility of enhanced lipid peroxidation while on a ketogenic diet.

Little is known about the metabolic effects of the ketogenic diet, or the mechanism of its anticonvulsant effects. A future experiment that could be considered might be the use of marine fish oils with preformed sources of EPA and DHA in the context of a ketogenic diet. Almost all fish oils obtained commercially contain vitamin E to reduce the rate of oxidation during storage (Reaven and Witztum, 1996). Thus, fish oil may be a beneficial dietary fat source to try in the context if a ketogenic diet, and may also reduce the extent of lipid peroxidation that could arise from the high n-3

content of this fat source. The use of fish oil as the main dietary fat on a ketogenic diet may result in a more dramatic increase in the levels of n-3 PUFA than what was observed in this study, and subsequently may have a more dramatic improvement on seizure protection. Another area of this study that could be explored further is the relationship between seizure protection and the amount of free n-3 PUFA in the brain. The analysis in this study of brain total lipids did not indicate differences in the amount of DHA between the different diet groups, but perhaps if the lipids had been separated into esterified and free fractions differences would have been detected.

The role dietary fatty acids play in anticonvuslant effects of the ketogenic diet is still unclear. Tracer/isotope or whole body fatty acid balance studies in a similar context as the experiments performed by Voskuyl et al (1998) and Cunnane and Anderson (1997) may provide useful answers through the localization of administered PUFA upon reaching seizure threshold through cortical stimulation, or some other seizure testing modality. Tracer/ isotope or whole body fatty acid balance methodology could also be used simply to understand more about the partitioning of fatty acids between oxidation, de novo synthesis and membrane incorporation while on such a high fat diet.

CHAPTER 7

REFERENCES

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APPENDIX

Appendix 1. GAS LIQUID CHROMATOGRAPHY (GLC) – PARAMETERS

Type: Chromatograph (Hewlett Packard Model 5090A); Integrator (Hewlett Packard Model 3393A); Autosampler (Hewlett Packard Model 7673A)

Column: Fatty Acids: J&W Durabond 23, 30m X 0.25mm i.d.
Cholesterol: Alltech Econo Cap SE-30, 30m X 0.25mm i.d.

Stationary Phase: Fatty Acids: 0.25µm cyanopropyl phenyl film
Cholesterol: 0.25µm polydimethylsiloxane film

Carrier Phase: compressed helium

Detector Type: flame ionization detector

Column Head Pressure: 140 kPa

Column Flow: 1.6 ml/min

Purge Vent: 4.1 ml/min (on 2min after sample injection)

Split Ratio: 100:1

H_2 (19 psi): 33 ml/min

Compressed Air (40 psi): 400 ml/min

Auxiliary (50 psi): 22 ml/min

Fatty Acid Run Time: 45min ⟶ Initial Temperature = 50 °C held for 2 min
Rate ① = 10 °C/min (for 13 min)
Final Temperature = 180 °C held for 5 min
Rate ② = 5 °C/min (for 10 min)
Final Temperature = 230 °C held for 5 min
Rate ③ (cool- down) = 25 °C/min (for 7.2min)
Final Temperature = 50 °C held for 2.8 min

Cholesterol Run Time: 19min ⟶ Initial Temperature = 240 °C held for 1 min
Rate ① = 5 °C/min (for 12 min)
Final Temperature = 300 °C held for 6 min