THE EFFECT OF THE KETOGENIC DIET ON ANIMAL SEIZURE MODELS

by

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A thesis submitted in conformity with the requirements for the Degree of Master of Science in Pharmacology
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University of Toronto

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ABSTRACT

The Effect of the Ketogenic Diet on Animal Seizure Models


Graduate Department of Pharmacology, University of Toronto

The purpose of the present work was to develop an animal seizure model of the ketogenic diet that could be used in future experiments to study its mechanism of action. In the first experiment proconvulsant effects were seen in rats when the effects of the "medium chain triglycerides" ketogenic diet were tested in 4 standard seizure models (maximal electroshock, electroconvulsive shock threshold, metrazol threshold and maximal metrazol). It was hypothesized that these proconvulsant effects were due to the weight differences between the ketogenic diet and control subjects. In the second experiment, weight differences were eliminated, and anticonvulsant effects were seen in the electroconvulsive shock threshold test. In the third experiment, the "classic" ketogenic diet of Bough et al. was tested in the metrazol infusion model, and shown to have anticonvulsant effects. In the fourth experiment, the "classic" ketogenic diet was found to be anticonvulsant in two different seizure models - the electroconvulsive shock threshold model and the maximal metrazol model. Thus anticonvulsant effects of the ketogenic diet can reliably be seen in animals when the weights of the ketogenic diet and control subjects are equalized, and when sensitive tests are used. Experiments related to mechanism can now proceed.
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These two pages are not sufficient to express my gratitude to my supervisor as well as others who have been supportive in me completing my graduate work successfully, however, I will attempt.

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* Aum Sri Sai Ram *
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<td>AED</td>
<td>Antiepileptic Drug</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<td>ECS</td>
<td>Electroconvulsive Shock</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
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<tr>
<td>FLC</td>
<td>Forelimb Clonus</td>
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<tr>
<td>IV</td>
<td>Intravenous</td>
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<td>KD</td>
<td>Ketogenic Diet</td>
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<tr>
<td>MCT</td>
<td>Medium Chain Triglycerides</td>
</tr>
<tr>
<td>MCT KD</td>
<td>Medium Chain Triglycerides Ketogenic Diet</td>
</tr>
<tr>
<td>MES</td>
<td>Maximal Electroshock</td>
</tr>
<tr>
<td>MET</td>
<td>Metrazol (=Pentylenetetrazol)</td>
</tr>
<tr>
<td>MJ</td>
<td>Myoclonic Jerk</td>
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<td>MMT</td>
<td>Maximal Metrazol</td>
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CHAPTER 1

GENERAL INTRODUCTION

1.1 EPILEPSY: THE CLINICAL PROBLEM

Epilepsy is a central nervous system (CNS) disorder characterized by spontaneous, recurring seizures (Loscher, 1998). It is one of the most common CNS disorders, affecting approximately 1% of the world’s population (Guberman, Bruni, 1997).

While all epilepsies involve seizures, seizures themselves do not necessarily imply epilepsy (Engel, 1989). If subjected to the proper stimulus, every brain is capable of producing seizures (Engel, 1989). In epileptic patients, seizures are produced as the result of a chronic low seizure threshold, which results in spontaneous attacks (Burnham, 1998). It is the chronic low threshold which is the true hallmark of epilepsy.

In some cases, a chronic low threshold is caused by structural brain lesions, related to neoplasms, scars, strokes, vascular anomalies, birth traumas or brain injuries (Engel, 1989). These cases are called “symptomatic”. In other cases, the brains of epileptic patients show no lesion, suggesting a genetic cause (Burnham, 1998). These cases are called “idiopathic”. About 60% of all epilepsies fall into the idiopathic category (Guberman, Bruni, 1997).

1.2 SEIZURE TYPES

Seizures are generally classified into two major categories – “generalized” and “partial” - based on the areas of the brain involved in epileptic activity (Loscher, 1997; Anonymous, 1985). Generalized seizures involve the whole brain, while partial seizures,
at least initially, involve only a limited part of the brain (Wyllie, Luders, 1996). Generalized seizures have bilateral hemispheric involvement and consciousness is always absent (Wyllie, Luders, 1996). Partial seizures - depending on the subtype - have unilateral or bilateral hemispheric involvement, and consciousness is either present or “impaired” (Wyllie, Luders, 1996).

1.2.1 Subtypes of Generalized Seizures

There are a number of types of generalized seizures. The two most common types are “absence” and “tonic-clonic” (Wyllie, Luders, 1996; Anonymous, 1985). In absence seizures, the patient stares blankly, and the eyelids may flutter. The duration of the seizure is usually less than 30 seconds (Burnham, 1998). A three-per-second “spike and wave” pattern is seen in the electroencephalogram (EEG) (Burnham, 1998).

In tonic-clonic seizures, the whole body is involved in convulsions, which consist of a “tonic” phase followed by a “clonic” phase (Burnham, 1998). The duration of the seizure is usually less than 5 minutes (Burnham, 1998). A pattern of constant spiking is seen in the EEG (Burnham, 1998).

1.2.2 Subtypes of Partial Seizures

Partial seizures are subdivided into “simple partial” and “complex partial” seizures (Wyllie, Luders, 1996; Anonymous, 1985). In simple partial seizures, consciousness is preserved (Wyllie, Luders, 1996). There is usually unilateral hemispheric involvement (Wyllie, Luders, 1996). The attack typically involves sensory, motor, or perceptual/emotional signs (Burnham, 1998). The duration of the seizures
varies (Burnham, 1998). Localized spiking in a neocortical or limbic area is seen on the EEG (Burnham, 1998).

Complex partial seizures are associated with impairment of consciousness. Frequently, there is bilateral hemispheric involvement (Wyllie, Luders, 1996). The clinical attack involves "automatisms", and the patient has no subsequent memory of the episode (Burnham, 1998). These seizures often follow a simple partial attack of temporal-lobe origin (Burnham, 1998). The duration of the complex-partial seizures varies (Burnham, 1998). Spiking in both temporal lobes is often seen on the EEG (Burnham, 1998).

1. 2. 3 Secondary Generalization

Partial seizures may spread to the rest of the brain and thus become generalized (Laidlaw et al., 1988; Anonymous, 1985). These generalized seizures are then referred to as "secondarily generalized" seizures (Laidlaw et al., 1988). The partial seizure is often experienced as an "aura" just before the generalized seizure (Laidlaw et al., 1988). The generalized seizures may be symmetrical, asymmetrical, tonic or clonic, but are most often tonic-clonic in type (Laidlaw et al., 1988).
1.3 THERAPY FOR EPILEPSY

1.3.1 Antiepileptic Drugs (AEDs)

The major therapy for seizures is antiepileptic drug (AED) therapy (Burnham, 1998). Some commonly used AEDs include phenytoin, carbamazepine, valproate, primidone, phenobarbital and ethosuximide (Thomas et al., 1996).

Three major mechanisms of action are proposed for the AEDs:

Type 1: Type 1 AEDs bind to the voltage-dependent sodium channels (Burnham, 1998; Catterall, 1987). These channels play a role in the initiation of neuronal action potentials (Burnham, 1998; Catterall, 1987). The drugs that work on these channels exert their effect by holding the channel longer in its inactive state (Burnham, 1998; Catterall, 1987). Examples of drugs that work by this mechanism include phenytoin and carbamazepine (Burnham, 1998; Catterall, 1987).

Type 2: Type 2 AEDs enhance activity in the GABA\(_A\) system (Burnham, 1998; Catterall, 1987). The GABA system is the major inhibitory system in the brain (Burnham, 1998; Catterall, 1987). The drugs that work on this system enhance GABA\(_A\)-mediated Cl\(^-\) influx, which stabilizes the membrane near its resting potential (Burnham, 1998; Catterall, 1987). Examples of drugs that work by this mechanism include benzodiazepines and phenobarbital (Burnham, 1998; Catterall, 1987).

Type 3: Type 3 AEDs bind to T-type voltage-dependent calcium channels (Burnham, 1998; Catterall, 1987). These channels are particularly important in the thalamus, where they are thought to contribute to the genesis of absence attacks (Burnham, 1998; Catterall, 1987). Drugs that work on these channels decrease their activity (Burnham, 1998; Catterall, 1987). Examples of drugs that work by this mechanism include ethosuximide and trimethadione (Burnham, 1998; Catterall, 1987).
1.3.2 **Drug-Resistant Epilepsies**

Despite the large number of AEDs, approximately 20% of patients with epilepsy fail to achieve seizure control (Shorvon, 1996). This sort of epilepsy is said to be “drug-resistant”, “refractory”, or “intractable”. According to Leppick (in press), a patient is considered to have intractable epilepsy if “seizures continue to occur, with a therapeutic concentration of at least one appropriate AED, one year after diagnosis.”

Intractable epilepsy has severe physical, psychological, and psychosocial effects (Lannon, 1997). Patients with uncontrolled seizures face the loss of friends, jobs, housing, and their drivers' licenses (Devinsky, 1996; Lehman, 1996).

1.3.3 **Nondrug Therapies for Drug-resistant Epilepsy**

Nondrug therapies - such as surgery, vagal stimulation and the ketogenic diet (KD) - are used when the AEDs fail to be effective (Burnham, 1998).

1) **Seizure Surgery** - Surgical treatment of epilepsy involves removal of the part of the brain that is thought to originate the seizures (Duchowny, 1996). In order to qualify for surgery, a patient must have disabling seizures that are resistant to high therapeutic levels of first-line antiepileptic drugs, a well defined region of seizure onset, and an epileptogenic zone lying within functionally “silent” cortex (Duchowny, 1996). Even when these criteria are fulfilled, the selection of candidates and the timing of surgery remain challenging. A substantial body of evidence, however, indicates that individuals with medically resistant seizures can benefit from surgery, especially if it is performed during childhood (Jensen, 1976; Jensen, Vaernet, 1977).

2) **Vagal Stimulation** - As the name suggests, vagal nerve stimulation involves the intermittent stimulation of the patient’s vagus nerve (Uthman, Beydoun, 1996). The
efficacy of vagus nerve stimulation was first shown in animals (Woodbury, Woodbury, 1990; Lockard et al., 1990). More recently, its efficacy has also been shown in multicentre studies done on epileptic patients (Uthman et al., 1990; Uthman et al., 1993; Anonymous, 1995). Mechanisms that have been proposed to explain the antiepileptic effect of vagal stimulation include the desynchronization of neuronal discharges, an increase in seizure threshold, the stimulation of inhibitory pathways and the release of inhibitory transmitters (Uthman, Beydoun, 1996). Vagal stimulation may be used with either adults or children.

3) The KD - The KD is a high-fat, low-carbohydrate, low-protein diet, used to treat children with intractable epilepsy (Uthman, Beydoun, 1996). Since it is the main focus of the present study, it is discussed in detail in the following section.

1.4 THE KD

1.4.1 General Background

The KD is a high-fat, low-protein, low-carbohydrate diet used to control intractable seizures in children. It was developed by Dr. R. M. Wilder in 1921, who designed it to mimic the biochemical changes associated with fasting (Wilder R.M, 1921). Fasting had been known to prevent seizures since biblical times (Wilder R.M, 1921).

The KD was widely used in the 1920s and 1930s, before the discovery of the modern AEDs. Interest in the KD declined with the discovery of the modern AEDs, such as phenytoin (1930s), carbamazepine and valproate (1970s) (Wheless, 1995). Despite the discovery of many new AEDs, however, some types of seizures have remained drug
resistant. The KD has been shown to be effective against these drug-resistant seizures (Prasad et al., 1996). The KD has therefore re-gained popularity as a treatment for intractable epilepsy in infancy and childhood (Swink et al., 1997).

1.4.2 Physiological Effects of the KD

The KD was designed to mimic the physiological effects of starvation without actually starving the patient. Within 24 hours on the KD, ketone body production begins (Swink et al., 1997). Fatty acids are dehydrogenated in the liver and split into 2-carbon fragments of acetyl-CoA, which are exported from the liver in the forms of β-hydroxybutyrate and acetoacetate (ketone bodies) (Swink et al., 1997). Therefore, there is an increase in ketone bodies in the blood. As the levels in the blood increase, the brain can utilize the ketone bodies as metabolic fuel (Robinson, Williamson, 1980). Ketone bodies can provide 65% or more of the total energy requirement of the brain and also act as substrates for the synthesis of cholesterol, fatty acids, and complex lipids in the brain (Swink et al., 1997).

1.4.3 Anticonvulsant Effects Of The KD

The anticonvulsant efficacy of the KD has been established over the years in a variety of clinical studies (Huttenlocher et al., 1971; Schwartz et al., 1989; Kinsman et al., 1992; Freeman et al., 1998; Peterman, 1925), for review see (Prasad et al., 1996). A recent multicentre study by Vining et al., for instance, has reported that 40% of children, aged 1-8, who remained on the diet for 12 months had greater than 50% decrease in seizures, and 10% of the patients became totally seizure-free (Vining et al., 1998).
Studies reporting clinical experiences with the KD suggest that it can be used in the anticonvulsant treatment of all types of seizures (Freeman et al., 1994; Mak et al., 1999). Certain childhood seizure types seem to respond particularly well, including myoclonic, atypical absence and atonic (drop) seizures (Swink et al., 1997; Freeman et al., 1994).

The anticonvulsant efficacy of the KD seems to be higher in younger children than in older children, with the best effects being seen before puberty in children between the ages of 2 and 10 (Swink et al., 1997; Huttenlocher, 1976; Livingston, 1972).

1.4.4 The "classic" and "MCT" KD's

Two major types of KD exist – the "classic" KD and the "medium chain triglyceride" (MCT) KD (Huttenlocher et al., 1971). The "classic" form of the KD is rich in animal and dairy fats, and has a fat to carbohydrate and protein ratio of 4 to 1 (by weight) (Freeman et al., 1994).

The MCT form of the KD was devised by Huttenlocher in order to permit a more normal protein and carbohydrate intake (Huttenlocher et al., 1971). This form of the diet involves a lower fat to carbohydrate and protein ratio of 1.2 to 1 (by weight). Blood ketones are maintained at a high level (2 - 4 mM for β-hydroxybutyrate, (Huttenlocher, 1976)) by the addition of highly ketogenic MCT oil. The MCT KD has been shown to be as effective as the classic diet in controlling epileptic seizures (Schwartz et al., 1989).

A comparison of the principle constituents of the classic and the MCT KD is presented in Table 1:
Table 1: Comparison of the principle dietary constituents of the classic and MCT KD (Huttenlocher, 1976).

<table>
<thead>
<tr>
<th>Dietary Constituents</th>
<th>MCT KD (% of total calories)</th>
<th>Classic KD (% of total calories)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>Protein</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Dietary fat</td>
<td>12</td>
<td>87</td>
</tr>
<tr>
<td>MCT Oil</td>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>

1.4.5 **Side Effects of the KD**

The anticonvulsants used for the treatment of epileptic seizures have side effects which vary with the drug being used and with the individual. These side effects are both dose-related and non-dose-related in nature (Swink et al., 1997). The KD, however, has no non-dose-related side effects and only a few dose-related side effects (Swink et al., 1997). In general, these side effects are mild and easily avoided (Swink et al., 1997):

(1) **Elevated serum lipids.** The KD causes a significant elevation in serum cholesterol (Vining E.P.G et al., 1996). Possible alterations in other lipoproteins are also under investigation (Vining E.P.G et al., 1996). High-fat diets in adults are traditionally known to cause atherosclerosis (Swink et al., 1997). With the KD, however, there is no evidence of the diet being atherogenic (Swink et al., 1997).

(2) **Constipation.** Constipation is the most common problem with the KD. Fluid restriction - a common feature of the diet - may be one factor that leads to this problem.
(Swink et al., 1997). If constipation occurs, it can be treated with mineral oil, small amounts of MCT oil or non-sugar-containing laxatives (Wheless, 1995).

(3) **Mineral and vitamin deficiency.** The KD is deficient in water-soluble vitamins, trace minerals, magnesium, iron and calcium (Tallian et al., 1998). The water-soluble vitamins that are deficient include vitamins B and C. In clinical practice, however, vitamin and mineral deficiencies are circumvented by the use of multivitamin and mineral supplements (Swink et al., 1997). Calcium, magnesium, and iron supplements are also given to the patient if a deficiency occurs (Tallian et al., 1998).

(4) **Renal stones.** Renal stones occur in 5-8% of the children on the KD (Herzberg et al., 1990). These usually consist of calcium oxalate (Herzberg et al., 1990), and often appear as “sandy gravel” in the diaper (Herzberg et al., 1990). The passing of stones will only occasionally cause pain to the patient. Renal stones can be avoided by lowering calcium intake, increasing fluid intake, or, in some cases, adding citrates to alkalinize the urine (Swink et al., 1997).

(5) **Growth alteration.** In children on the KD, body weight only increases slightly if at all (Freeman et al., 1990). This is not of concern, since the caloric requirements of each patient are meticulously calculated, based on age, growth rate and perceived activity level (Swink et al., 1997). Once the child is taken off the diet (usually after 2 - 3 years), body weight increases normally (Freeman et al., 1990).

(6) **Acidosis and excess ketosis during illness.** Consumption of the KD causes compensated metabolic acidosis and stable ketosis, both of which are usually well tolerated (Swink et al., 1997). During illnesses such as influenza, severe colds, or pneumonia, the child may become dehydrated, and secondarily acidotic and/or too ketotic
(Swink et al., 1997). This is prevented in the early stages of illness by the consumption of fluids and oral rehydration formulae (carbohydrate-free) (Swink et al., 1997). If the condition becomes more severe, IV fluids (without glucose) can be used to overcome the problem (Swink et al., 1997).

1.4.6 Rigor of the KD

The rigor of the KD, along with the work required to initiate and maintain it, is the major disadvantage of the diet.

The diet is very hard for some children. Strict dietary restriction is required by children on the KD (Tallian et al., 1998). Glucose-containing substances must be avoided, including such commonly used items such as multi-vitamins, toothpaste and processed baby meats. Even the inactive ingredients in some medications (glucose or carbohydrates) can lead to reduced effectiveness of the KD. Furthermore, some children may simply reject the diet due to its un-palatability.

The diet is also difficult for parents. Parents are required to continually monitor the child and measure the child’s level of ketosis on a regular basis (Wheless, 1995). All meals, snacks and food consumed by the child have to be carefully calculated, weighed and prepared (Barron, Hunt, 1997). Parents must be constantly watchful to be sure that the child does not consume snack foods containing carbohydrates. Some children complain of hunger for weeks and seek food from other sources, leading to distress and frustration in the parents (Barron, Hunt, 1997). Therefore, parents may abandon the diet.

If the mechanism(s) of action of the KD were known, it is possible that a pharmacological substitute might be synthesized and administered to patients. If the
relevant brain changes could be accomplished with drugs, it would help to avoid the currently existing problems with the KD.

I. 4. 7 Proposed Mechanisms of Action for the KD

The mechanism by which the KD exerts its anticonvulsant effects is still not clearly understood, despite more than 70 years of clinical use and scientific investigations (Swink et al., 1997).

Over the years, five prominent hypotheses have been proposed regarding the KD’s mechanism of action (Swink et al., 1997). None of them is fully established:

1) Induction of acidosis. During the KD, the body’s metabolism shifts from glycogenolysis to ketosis, resulting in the production and accumulation of the ketoacids (β-hydroxybutyrate and acetoacetate) (Swink et al., 1997).

In 1928, Lennox (1928) proposed that the mild acidosis caused by the accumulation of ketoacids was responsible for the anticonvulsant effects of the KD. Others have also implicated acidosis as the critical factor (Bridge, Iob, 1931).

Clinical and experimental studies, however, suggest that acidosis may not be the factor that is critical for the diet’s anticonvulsant effect. In a clinical study, Huttenlocher (Huttenlocher, 1976) measured venous pH of children on the MCT KD, and showed that all of the children had a normal blood pH. In animal studies, Withrow demonstrated that, in rats maintained on the KD, there was initial acidosis in the blood, but that it was quickly compensated (within one week) by hyperventilation and a lowering of the partial pressure of carbon dioxide (Withrow, 1980). Assay studies, by Davidian et al. (Davidian et al., 1978), De Vivo et al. (DeVivo et al., 1978) and Al-Mudallal et al. (Al-Mudallal et
al., 1996) have also shown that there is no difference in the brain pH of animals fed the KD, as compared to a control diet.

The anticonvulsant effects of the KD are therefore, unlikely to be due to acidosis.

2) Dehydration and alteration of electrolyte balance. The KD induces a saline diuresis, which might alter electrolyte balance (Swink et al., 1997). Fluid restriction is often maintained on the KD and this might lead to dehydration (Swink et al., 1997).

In 1927, McQuarrie proposed tissue dehydration as the factor responsible for the anticonvulsant effects of the KD (McQuarrie, Keith, 1927). In 1930, Fay supported this hypothesis as well (Fay, 1930). Bridge and Iob later suggested that the loss of water and sodium were responsible for the effects of the KD (Bridge, Iob, 1931).

Early experimental studies by Millichap and Jones in mice and children did suggest that the anticonvulsant effects of the KD were associated with a negative balance of sodium and potassium ions in the blood (Millichap, Jones, 1964). Later studies by Appleton and De Vivo (1974) and DeVivo et al. (1978), however, showed that there was no consistent difference in whole-brain electrolytes or water contents in rats fed a KD, as compared to those fed laboratory chow (DeVivo et al., 1978; Appleton, DeVivo, 1974).

The data on dehydration and electrolyte balance, therefore, are contradictory. Further studies are needed to clearly determine whether dehydration and electrolyte balance play a role in the anticonvulsant effect of the KD.

3) Increased plasma lipids. The KD increases the lipid content of the blood (Dekaban, 1966). In patients on the classic KD there is a large increase in the concentrations of cholesterol, phospholipid, triglycerides and fatty acids (Dekaban,
1966). In animals on the classic KD, Appleton and De Vivo (1974) have also reported an increase in total serum lipids (Appleton, De Vivo, 1974).

Dekaban, therefore, has suggested that the increase in plasma lipids plays a major role in the anticonvulsant effects of the KD (Dekaban, 1966).

More recently, however, in clinical studies, Huttenlocher (1976) showed that the classic and MCT KDs have approximately equal anticonvulsant effects, but very different effects on plasma lipids (Huttenlocher, 1976). Cholesterol is elevated in children on the classic diet, but is normal in children on the MCT KD. Fatty acid levels show a smaller increase on the MCT KD than the classic KD (Huttenlocher, 1976). These observations led Huttenlocher to conclude that the KD’s mechanism of anticonvulsant action is not related to hyperlipidaemia (Huttenlocher, 1976).

Thus, studies examining lipidaemia as the potential mechanism of the anticonvulant effect of the KD have also produced ambiguous results. Further studies are therefore needed to clearly elucidate whether lipidemia plays a role in the anticonvulsant effects of the KD.

4) Ketone-body production. As mentioned previously, ketone bodies are elevated in patients on the KD ((Appleton, DeVivo, 1974; Uhlemann, Neims, 1972) see (Prasad et al., 1996).

Several theorists have suggested that this elevation leads to the diet’s anticonvulsant effect ((Appleton, DeVivo, 1974; Uhlemann, Neims, 1972) see (Prasad et al., 1996) for review).

Early experimental studies supported this idea. Uhlemann and Neims, who developed the first animal model of the KD, reported that mice with high ketone levels
were protected against maximal electroshock and hydration threshold electroshock, whereas animals with low levels were not (Uhlemann, Neims, 1972). Uhlemann and Neims also showed that when older mice that were fed the KD, little ketosis was achieved and there was little seizure protection (Uhlemann, Neims, 1972), whereas younger mice achieved both ketosis and seizure protection. Other studies, however, have reported a lack of correlation between blood ketone levels and seizure protection (Dekaban, 1966; Millichap, Jones, 1964; Bough, Eagles, 1999).

Although most theorists currently seem to feel that elevated ketone levels are a crucial factor in producing the anticonvulsant effects of the KD, the evidence is contradictory, and further work would be welcome.

5) Altered brain metabolism. Altered brain metabolism occurs as a consequence of the ketosis that results on the KD (Swink et al., 1997). Glucose utilization is decreased in rats fed the KD, for instance (DeVivo et al., 1978). ATP/ADP ratios and energy reserves are significantly increased (DeVivo et al., 1978). These changes presumably result from increased ketone-body use by the brain under low-glucose conditions.

Theorists such as DeVivo et al. (1978), Janaki et al. (1976) and Millichap et al. (1964), therefore, have suggested that the altered brain metabolism - rather than ketosis per se - is the cause of anticonvulsant effects of the KD (DeVivo et al., 1978). This is indicated because when glucose is administered to ketotic children on the KD, seizures quickly recur, even though ketone bodies are still present in the blood (Huttenlocher, 1976; Millichap, Jones, 1964; Janaki et al., 1976).
Summary

Most theorists now believe that altered cerebral metabolism - caused by ketosis - plays a major role in the anticonvulsant effect of the KD (Swink et al., 1997). Dehydration and hyperlipidemia, however have not been completely ruled out, and the precise relationship between ketosis, cerebral metabolism and the anticonvulsant effects of the KD still remain to be elucidated. Further experiments are needed to clarify the mechanism of the anticonvulsant effects of the KD. Since these studies will be invasive, they will require the use of animal seizure models.

1.5 ANIMAL SEIZURE MODELS

Animals, such as rats and mice, can be electrically or pharmacologically stimulated to produce seizures similar to those seen in humans. These "animal models" of the human condition can then be used to study the physiological, pharmacological and biochemical mechanisms underlying seizures and epilepsy (Fisher, 1989).

Animal models can be used to study the anticonvulsant effects of the KD since like humans, animals (rats) have the capacity use ketone bodies as a source of energy during starvation or when high fat diets are administered. The physiological changes that ensue as a consequence of KD feeding are very similar to those seen in humans. For example, the oxidative phosphorylation pathway that plays an important role in the fat utilization is very similar in rats and humans.

Animal models are needed in research since ethical considerations rule out the use of human subjects for invasive studies, such as seizure induction or the collection of brain tissue (Fisher, 1989). In animals, blood and brain samples can be freely obtained to
monitor and assess physiological, pharmacological and biochemical changes. Animal models also allow the experimenter to control the timing, duration, frequency and types of seizures. Furthermore, the age of the subjects can be varied to examine developmental effects. Animal models are therefore invaluable tools, and much of what is known about epilepsy today has been derived directly or indirectly from the study of animal seizure models (Fisher, 1989).

Animal seizure models - like human seizures - are divided into models of generalized seizures and models of partial seizures.

1.5.1 Animal models of generalized seizures

"Generalized seizure models" model generalized seizures in humans. Generalized seizure models can be subdivided into models of absence and tonic-clonic seizures (Fisher, 1989). Seizures which model absence attacks can be produced using low-level systemic chemical convulsants (pentylenetetrazol, penicillin and intravenous opiates) and low-intensity electrical stimulation (Fisher, 1989). Seizures which model tonic-clonic attacks can be produced in several different ways, including the application of high-level electricity or systemic chemical convulsants (pentylenetetrazol, picrotoxin, and bicuculline) and the production of metabolic derangements (hypoxia, hypoglycemia and uremia) (Fisher, 1989).

The two most common pharmacological models of generalized seizures are the maximal electroshock (MES) model and the threshold pentylenetetrazol (MET) model (Mody, Schwartzkroin, 1997). The MES model is the tonic-clonic model most widely used in anticonvulsant drug development (Mody, Schwartzkroin, 1997). In this model, a
high-intensity current (in adults rats: 150mA at 60Hz for 0.2s) is delivered through corneal electrodes, producing tonic hind-limb flexion and extension, followed by clonus (Fisher, 1989). In the MET model, a low dose of pentylenetetrazol (in rats: 70mg/kg) is administered subcutaneously, producing myoclonic jerks (MJ), followed by forelimb clonus (FLC) (Krall et al., 1978; Mody, Schwartzkroin, 1997).

1.5.2 Animal models of partial seizures

"Partial seizure models" model partial seizures in humans. Partial seizure models can be divided into models of simple partial seizures and models of complex partial seizures. Simple partial seizures can be modeled by topically applying convulsants such as penicillin, bicuculline, picrotoxin, strychnine, cholinergics, or metals (e.g. aluminum hydroxide, cobalt tungsten, zinc and iron) to the dorsal neocortex (Fisher, 1989). Complex partial seizures can also be modeled using systemic convulsants, kainic acid or tetanus toxin (Fisher, 1989). The technique most commonly used to model complex partial seizures, however, is kindling (Fisher, 1989). In this model, chronic depth electrodes are implanted and low currents are applied discretely to small areas of the brain (for rats: 1 sec, 60Hz, current adjusted to site, but usually < 1mA, peak to peak).

For a complete review of animal seizure models, see Fisher (Fisher, 1989).

1.6 THE KD AND ANIMAL SEIZURE MODELS – PAST STUDIES

A number of previous studies have used animal seizure models to test the anticonvulsant effects of the KD. These studies are summarized in Table 2. Taken as a whole, the results are variable and sometimes conflicting. While some studies show anticonvulsant effects (Appleton, DeVivo, 1974; Uhlemann, Neims, 1972; Millichap,
Jones, 1964; Hori et al., 1997; Nakazawa et al., 1983; Muller-Schwarze A.B. et al., 1998; Bough, Eagles, 1999), other studies show no effects in the standard tests (Otani et al., 1984) and some studies even show proconvulsant effects (Mahoney et al., 1983; Bough et al., 1998; Matthews et al., 1999).

There are several possible reasons for the conflicting results seen in this field: 1) different diets have been used in different experiments; 2) different species have been used in different experiments; 3) the age of the subjects has varied, with most studies involving adults although clinically the diet is most effective before puberty; 4) the time period on the diet has varied; and 5) the levels of ketosis achieved in most studies have been low compared to clinical levels.

1.7 OBJECTIVES

The initial goal of the present work was to develop an animal model of the KD that could be used in subsequent studies. The ultimate goal of these studies would be to identify the mechanism by which the KD exerted its anticonvulsant effects. Once the mechanism was clearly identified, drug therapies that mimicked the effects of the KD could be developed and administered much more easily than the KD.

Our first experiment (Experiment 1), however, showed proconvulsant effects rather than anticonvulsant effects. A secondary goal of these experiments then became to determine why the KD sometimes has proconvulsant effects and sometimes anticonvulsant effects.

During the course of our experiments, Bough et al. (1999), published two studies showing KD anticonvulsant effects with the MET infusion model (Bough, Eagles, 1999; Bough et al., 1999). A final goal became to replicate Bough et al. showing
anticonvulsant effects with the MET infusion model (Experiment 3) and other models (Experiment 4)

1.8 HYPOTHESIS

The KD is widely used today for the treatment of intractable epilepsy in infants and children. Its efficacy has been illustrated in several multicentre studies. I hypothesize that the KD will have anticonvulsant effects in animal seizure models as it has in people.
<table>
<thead>
<tr>
<th>STUDY</th>
<th>SUBJECTS</th>
<th>AGE INITIATED ON DIET</th>
<th>DIET</th>
<th>DAYS ON DIET</th>
<th>BOH LEVELS</th>
<th>SEIZURE TESTING</th>
<th>PROTECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Millichap et al. (1964)</td>
<td>Mice (females - Albino)</td>
<td>&quot;Young&quot;</td>
<td>100% beef fat</td>
<td>1</td>
<td>Not Measured</td>
<td>Hydration ECS</td>
<td>Maximal</td>
</tr>
<tr>
<td>Uhlmann And Neims (1972)</td>
<td>Mice (males and females - CD1)</td>
<td>16 days (infants)</td>
<td>70% lipid - based on raw pork sausage and casein</td>
<td>10</td>
<td>Total ketones KD 2.56 mM Control 0.30 mM</td>
<td>Electro-shock</td>
<td>Maximal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Threshold</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hydration</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MET (85mg/kg)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bicuculline</td>
<td>Clonic</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tonic hyper-extension</td>
<td>No</td>
</tr>
<tr>
<td>Appleton and DeVivo (1974)</td>
<td>Rats (males - Albino)</td>
<td>Adults</td>
<td>High fat diet resembling 4:1 KD. Made of corn oil, lard, casein, glucose, salt mixture, and vitamin mixture</td>
<td>10-20</td>
<td>KD 1.63 mM Control 0.13 mM</td>
<td>Threshold ECS</td>
<td>Maximal</td>
</tr>
<tr>
<td>Nakazawa et al. (1983)</td>
<td>Mice (females - ddY)</td>
<td>8-12 weeks (Adults)</td>
<td>MCT KD (milk powder)</td>
<td>14</td>
<td>Not measured</td>
<td>Threshold MES</td>
<td>Maximal</td>
</tr>
<tr>
<td>Mahoney et al. (1983)</td>
<td>Rats (females - Sprague Dawley)</td>
<td>5-6 weeks (Adults)</td>
<td>High fat diet resembling 4:1 KD, using corn oil and MCT oil</td>
<td>17</td>
<td>Not measured</td>
<td>Audiogenic (Magnesium Deficient Rats)</td>
<td>Maximal</td>
</tr>
<tr>
<td>Otani et al. (1984)</td>
<td>Mice (males - ddY)</td>
<td>4 weeks (Young)</td>
<td>MCT powdered milk</td>
<td>7 - 10</td>
<td>KD 3-5 mM Control 0.27 mM</td>
<td>MES</td>
<td>Maximal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MET (75 mg/kg)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Semicarbazide (100mg/kg)</td>
<td>No</td>
</tr>
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<td></td>
<td></td>
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<td>Hydration threshold ECS</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MET Kindling (45mg/kg)</td>
<td>Yes</td>
</tr>
<tr>
<td>Hori et al. (1997)</td>
<td>Rats (males - Sprague Dawley)</td>
<td>~75 days (Adults)</td>
<td>&quot;Classic&quot; KD</td>
<td>35 (weekly tests)</td>
<td>KD 0.75 mM Control 0.10 mM</td>
<td>Kindling</td>
<td>Maximal</td>
</tr>
<tr>
<td>Bough et al. (1998)</td>
<td>Rats (males - Sprague Dawley)</td>
<td>37 (Young)</td>
<td>Calorie restricted &quot;classic&quot; KD</td>
<td>20-21</td>
<td>KD 2.22 mM Control 0.75 mM</td>
<td>Kainic Acid (10mg/kg)</td>
<td>Maximal</td>
</tr>
<tr>
<td>Mull-Schwarze et al. (1998)</td>
<td>Rats (strain and sex not available)</td>
<td>56 days (Adults)</td>
<td>70% lipid 14% protein 0% carbohydrate</td>
<td>56</td>
<td>Not measured</td>
<td>Spontaneous recurrent seizures (SRS) following kainic acid administration</td>
<td>Maximal</td>
</tr>
</tbody>
</table>

BOH – β-hydroxybutyrate
<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Age Initiated on Diet</th>
<th>Diet</th>
<th>Days on Diet</th>
<th>B0H Levels</th>
<th>Seizure Tests</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lustig &amp; Niesen (1998)</td>
<td>Rats (males - Wistar)</td>
<td>3-4 weeks (Young)</td>
<td>KD (unspecified)</td>
<td>28</td>
<td>KD 1.8mM</td>
<td>MET (60 – 70mg/kg)</td>
<td>Yes - decreased seizure duration and deaths in KD group.</td>
</tr>
<tr>
<td>Bough and Eagles (1999)</td>
<td>Rats (males- Sprague Dawley)</td>
<td>63 days old (Adults)</td>
<td>Calorie restricted &quot;classic&quot; KD</td>
<td>35</td>
<td>KD =0.8 mM</td>
<td>MET infusion (10mg/min)</td>
<td>Yes – KD fed group had an elevated threshold for seizure induction</td>
</tr>
<tr>
<td>Bough et al. (1999)</td>
<td>Rats (males- Sprague Dawley)</td>
<td>Many different ages</td>
<td>Calorie restricted &quot;classic&quot; KD</td>
<td>&gt; 20</td>
<td>KD 1.0–7.5 mM</td>
<td>MET infusion (10mg/min)</td>
<td>Yes – most KD fed group had an elevated threshold for seizure induction</td>
</tr>
<tr>
<td>Matthews et al. (1999)</td>
<td>Rats (males- Sprague Dawley)</td>
<td>Unavailable</td>
<td>Calorie restricted &quot;classic&quot; KD</td>
<td>&gt; 20</td>
<td>Unavailable</td>
<td>MES</td>
<td>No – MES seizures were exacerbated, and more severe seizures were seen in the KD group</td>
</tr>
</tbody>
</table>

BOH – β-hydroxybutyrate
CHAPTER 2

GENERAL METHODS

2.1 SUBJECTS

Male Wistar (Experiments 1 and 2) or Sprague Dawley (Experiments 3 and 4) rat pups served as subjects. Subjects were 13 - 16 days old on arrival from the breeding farm (Charles River Canada, St. Constant, Quebec, Canada). Subjects were initially housed with their dams in wire cages in a temperature-controlled vivarium (22 ± 1°C) with a 12 hour day-night cycle (lights on at 7 a.m). At 20 days of age (Experiments 1 and 2), or 21 days of age (Experiments 3 and 4), subjects were weaned and randomly sorted into control and KD groups. They were then individually housed, and started on the diets. Subjects were weighed on the day of weaning, and every 2 days thereafter, plus on the day of seizure testing.

2.2 CONTROL DIET AND KD

Diets were obtained from commercial suppliers. The nutrient contents of the control diet (non-gavagable and gavagable), MCT KDs (non-gavagable and gavagable) and the classic KD are provided in Tables 3, 4 and 5. The MCT KD (lower fat + MCT oil) was used in Experiment 1 and 2, since pilot studies showed that it produced high (clinical) ketone levels in young rats. The “classic” KD (high fat, no MCT oil) was used in Experiments 3 and 4, since these were an attempt to reproduce the paradigm of Bough et al. (1999).

The MCT KD and the control diet used in Experiment 1 (Table 3) were in a non-gavagable form. The control diet (100218) and the basic diet (100219) used to make the KD were obtained from Dyets (Bethlehem, PA). MCT oil, a generous gift of Mead
Johnson (Division of Bristol-Myers Squibb Canada Inc., Ottawa, Ontario, Canada), was added to the diet in our own laboratory. Sodium cyclamate (Sucaryl®) was added in our laboratory to improve the flavor, as well as the consistency of the diet. Sodium cyclamate is a commonly used sweetening agent in foods given to children on the KD, and does not prevent ketosis.

The MCT KD and control diet used in Experiment 2 (Table 4) were in a gavagable form, so that, if necessary, the “high” KD group could be force-fed. The control diet (710079) and the basic diet (710093) used to make the KD were obtained from Dyets (Bethlehem, PA, USA). The constituents of this diet, however, are slightly modified, as compared to the diet used in Experiment 1, in order to make it gavagable. MCT oil was added in our laboratory. Sodium cyclamate was not added to this diet, since it would increase the consistency of the diet hence making it ungavagable.

For Experiments 3 and 4 the classic KD (F3666), was obtained from Bio-Serve (Frenchtown, NJ, U.S.A). The control diet used in both experiments was powdered rodent chow (Purina 5001) – obtained from Leis Pet Distributing Inc. (Wellesley, ON, Canada).
Table 3: Nutrient composition of the control diet and the MCT KD (non-gavagable form) used in Experiment 1.

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>Micronutrient</th>
<th>Control (g/kg)</th>
<th>MCT KD (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Casein</td>
<td>206.6</td>
<td>174.0</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>Corn Starch</td>
<td>404.5</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Dyetrose¹</td>
<td>134.3</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>101.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Non-Nutritive Fiber</td>
<td>Cellulose</td>
<td>51.0</td>
<td>200.0</td>
</tr>
<tr>
<td>Vitamins²</td>
<td>AIN-93G</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>Minerals²</td>
<td>AIN-93G</td>
<td>27.7</td>
<td>24.1</td>
</tr>
<tr>
<td>Fat</td>
<td>Soybean Oil³</td>
<td>71.3</td>
<td>60.1</td>
</tr>
<tr>
<td></td>
<td>MCT oil⁴</td>
<td>0.0</td>
<td>539.0</td>
</tr>
</tbody>
</table>

¹ Dyetrose – a selectively depolymerized corn starch which by weight is 1% monosaccharide, 4% disaccharides, 5% trisaccharides, and 90% tetrasaccharides, with an energy value of 16.0kJ/g
² Vitamin and mineral mixes contain no carbohydrates. All diets are supplemented with 2.5g of choline bitartrate.
³ Soybean oil is 15% saturated fatty acids, 54% linoleic acid, 8% α-linolenic acid, 23% monounsaturated fatty acids.
⁴ MCT oil is 6% caproic acid, 60-80% caprylic acid, 18-32% capric acid, 4% lauric acid.
⁵ Sucaryl (sodium cyclamate) was added at 70ml/kg to the KD.
Table 4: Nutrient composition of the control diet and the MCT KD (gavagable form) used in Experiment 2.

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>Micronutrient</th>
<th>Control (g/kg)</th>
<th>KD (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Casein</td>
<td>114.4</td>
<td>139.7</td>
</tr>
<tr>
<td></td>
<td>L-Cystine</td>
<td>1.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Maltose Dextrin</td>
<td>292.3</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>57.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Non-Nutritive Fiber</td>
<td>Cellulose</td>
<td>28.7</td>
<td>35.1</td>
</tr>
<tr>
<td>Vitamins&lt;sup&gt;1&lt;/sup&gt;</td>
<td>#310025</td>
<td>5.7</td>
<td>7.0</td>
</tr>
<tr>
<td>Minerals&lt;sup&gt;1&lt;/sup&gt;</td>
<td>#210032</td>
<td>20.0</td>
<td>24.5</td>
</tr>
<tr>
<td>Fat</td>
<td>Soybean Oil&lt;sup&gt;2&lt;/sup&gt;</td>
<td>40.2</td>
<td>49.0</td>
</tr>
<tr>
<td></td>
<td>MCT oil&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.0</td>
<td>205.6</td>
</tr>
<tr>
<td>Others</td>
<td>t-Butylhydroquinone (TBHQ)</td>
<td>0.009</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Choline Bitartrate</td>
<td>1.42</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>Xanthum Gum</td>
<td>6.5</td>
<td>7.9</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>431.8</td>
<td>527.3</td>
</tr>
</tbody>
</table>

<sup>1</sup>Vitamin and mineral mixes contain no carbohydrates.

<sup>2</sup>Soybean oil is 15% saturated fatty acids, 54% linoleic acid, 8% α-linolenic acid, 23% monounsaturated fatty acids.

<sup>3</sup>MCT oil is 6% caproic acid, 60-80% caprylic acid, 18-32% capric acid, 4% lauric acid.
Table 5: Nutrient content of the control and the classic KD used in Experiments 3 and 4.

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>Micronutrient</th>
<th>Control (g/kg)</th>
<th>KD (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>-</td>
<td>234.0</td>
<td>95.0</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Dextrose</td>
<td>490.0</td>
<td>7.6</td>
</tr>
<tr>
<td>Fiber</td>
<td>-</td>
<td>53.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>AIN-76</td>
<td>69.0</td>
<td>38.0</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>AIN-76</td>
<td>54.0</td>
<td>20.9</td>
</tr>
<tr>
<td>Fat</td>
<td>Lard / Saturated fat</td>
<td>15.0</td>
<td>475.0</td>
</tr>
<tr>
<td></td>
<td>Butter</td>
<td>0.0</td>
<td>199.5</td>
</tr>
<tr>
<td></td>
<td>Corn Oil / Unsaturated fat</td>
<td>85.0</td>
<td>114.0</td>
</tr>
</tbody>
</table>

2.3 PROCEDURES FOR SEIZURE TESTS

All seizure tests were recorded on video. This was done for the purpose of ensuring that a record of all seizure testing was present. All seizures were scored first at the time of occurrence, and then the scoring was confirmed by reference to the video record.

2.3.1 The MES Test (Experiments 1 and 4)

The MES test is the standard pharmacological model for tonic-clonic seizures (Krall et al., 1978). The MES test was administered according to a modification to the protocol of Krall et al. (Krall et al., 1978). The MES stimulus was generated by a purpose-built stimulator, and was administered via corneal electrodes using the following
parameters: pulse configuration - sine wave, pulse frequency - 60Hz, pulse intensity - 50 mA (peak-to-peak), train duration - 0.2sec. The intensity of 50 mA is lower than that used in adult rats (Swinyard, 1969). It was chosen after pilot studies with 30-day-old rats of the Wistar strain, which showed that 150 mA killed a number of subjects. Studies have shown that small rats are more sensitive to the MES stimulus (Davenport, Davenport, 1948). In our hands, 50mA produces hindlimb tonic extension in 90 - 100% of the subjects. In order to assure adequate electrical contact, the electrodes were dipped in 0.9% NaCl prior to application to the cornea. Maximal seizures were scored as "present" or "absent". Seizure absence was defined as a failure to extend the hindlimbs to an angle greater than 90°.

2.3.2 The Threshold ECS Test (Experiments 1, 2 and 4)

The threshold ECS test was administered using procedures identical to the MES test, except that subjects were tested 4 times, using different current levels. Current intensities were calculated by the half-split technique (Racine, 1972), starting with an initial current of 50mA. Current levels used include 50, 36.8, 25 and 11.8 mA. Tests were begun on post-natal day 26 (Experiment 1), day 31 (Experiment 2) or day 43 (Experiment 4) and continued at 2-day intervals until the last stimulation had been given. Subjects were stimulated only once on any given day. The threshold was calculated as the current level half way in between the lowest current that gives a minimal seizure (at least 5 seconds of FLC) and the highest that does not. Threshold was determined for both maximal seizures (defined as an extension of the hindlimbs to an angle greater than 90°) and minimal ECS seizures (defined as FLC).
2.3.3 The Threshold MET Test (Experiments 1 and 4)

The threshold MET test is the standard pharmacological model for absence seizures (Krall et al., 1978). The MET test was administered according to a modification of the procedure of Krall et al. (Krall et al., 1978). MET, as a 0.7% solution in 0.9% NaCl, was injected subcutaneously into a loose fold of skin on the back of the subjects' necks at a dose of 70mg/kg. This dose - which was near threshold - had been determined in pilot studies with 30-day-old rats of the Wistar strain. The volume of the injection was 1ml/100g of body weight. Following injection, the subjects were placed in a test chamber and observed for 30 minutes. Seizures were scored as "present" or "absent". Seizure absence was defined as the absence of FLC. The latency to the first MJ and to the first bout of FLC were also recorded.

2.3.4 The MMT Test (Experiments 1 and 4)

A modified MMT test was administered using procedures identical to the threshold MET test, except that a dose of 85mg/kg MET was used. Our procedure was not identical to that typically used for the MMT test (Desmedt et al., 1976), since this dose of MET was not expected to produce maximal seizures in all animals. The dose was chosen in pilot studies so that pro- as well as anticonvulsant effects could be seen (It was clear by the start of this test that proconvulsant effects might be seen). Seizures were scored as "present" or "absent". Seizure absence was defined as a failure to extend the hindlimbs to an angle greater than 90°. The latency to the first MJ and to the first bout of FLC were also recorded.

2.4 MEASUREMENT OF β-HYDROXYBUTYRATE LEVELS

In Experiments 1, 2 and 4, immediately after the seizure tests, subjects were anesthetized with pentobarbital (40mg/kg) and blood samples were drawn from the heart.
Blood samples were taken after seizure testing to avoid any possible effects on seizure threshold. Pilot studies had indicated that seizure activity had no significant effect on β-hydroxybutyrate levels (data not shown). In Experiment 3, one day prior to seizure testing, a needle was inserted into the tail vein and blood was drawn (~ 0.1 ml). The analysis of the blood was consistent for all studies and the procedure for this analysis is provided below.

Blood was analyzed for β-hydroxybutyrate levels, within 1 - 2 hours of being obtained from the subjects, using Keto Site test cards and a Stat-Site meter (GDS Diagnostics) (Div. of GDS Technology Inc., Elkhart, IN, USA). Whole blood from the control subjects (25μl) was analyzed without dilution. In the case of the experimental subjects, however - where, β-hydroxybutyrate levels were higher - following the manufacturer's instructions, 20μl of whole blood were diluted in 200μl of Ketosite Serum Diluent (GDS Diagnostics) and then 22μl of the diluted sample were analyzed using the Stat-Site meter. The result was multiplied by 11 in order to obtain the undiluted concentration (dilution of 1 part blood in 10 parts of diluent). This dilution was required because the Stat-Site meter measures β-hydroxybutyrate levels in blood only between concentrations of 0 and 2 mM. Since our KD subjects had β-hydroxybutyrate levels higher than 2mM, dilution was required. The blood from the control subjects was not diluted since their β-hydroxybutyrate levels were low (below 0.5mM) and any dilution would reduce the levels to an extent that they could not be reliably measured by Stat-Site meter. For the diluted samples, in order to eliminate any interference from β-hydroxybutyrate concentration contained in the diluent, 22μl of the serum diluent was assayed on the Stat-Site meter. This value was multiplied by 10 and subtracted from the previous value obtained.
2.5 STATISTICAL ANALYSIS

Different statistical tests were used in the various chapters. For interval-ratio data, either the unpaired t-test (for normally distributed data), the Mann-Whitney Rank Sum test (for data not normally distributed) or the ANOVA test was used. For the quantal data, the Chi-Square test was used as the non-parametric test. The different tests used are described in the Specific Methods section of each chapter. All data are presented as mean ± SD.
CHAPTER 3

EXPERIMENT 1

THE EFFECT OF THE MCT KD ON FOUR STANDARD ANIMAL SEIZURE MODELS

3.1 RATIONALE

As indicated in the General Introduction, past animal studies on the KD have reported variable and conflicting results. Some of the reasons for these conflicting results may include: 1) different diets have been used; 2) different species have been used; 3) the age of subjects has varied; and 4) the time on the diet has varied.

Experiment 1 was designed to examine the effect of the MCT KD on four standard seizure tests – the maximal electric shock (MES), the threshold electroconvulsive shock (threshold ECS), the threshold MET and the maximal MET (MMT) – using a standard diet, species, age and time on the KD. Young animals (20-30 days of age) were used, since clinical studies show that the KD is more effective before the onset of puberty (Huttenlocher, 1976; Livingston, 1972). The MCT form of the KD was used because it induced higher (clinical) levels of ketosis in pilot studies. (Pilot studies with the classic diet had produced β-hydroxybutyrate levels below 2 mM). A 10-day period on the diet was chosen in order to ensure that the subjects were pre-pubertal during the period of seizure testing. Puberty occurs in male Wistar rats between the age of 35–60 days (Ojeda, Urbanski, 1994). Past studies have reported anticonvulsant effects after 10 days on the KD (Uhlemann, Neims, 1972).
3.2 SPECIFIC METHODS

3.2.1 Subjects

Male Wistar rat pups served as subjects. They were 14 days old on arrival from the breeding farm (Charles River, St. Constant, Quebec, Canada). Subjects were weaned at 20 days of age and begun on the control or KD on the same day. Subjects were housed as described in the General Methods. They were weighed on the day of weaning, and every 2 days thereafter, plus the day of seizure testing.

3.2.2 Control Diet and MCT KD

Beginning on postnatal day 20, the control and MCT KD (Table 3) were administered as described in the General Methods. Diets were continued for 10 days.

3.2.3 Measurement of β-hydroxybutyrate Levels

Immediately after the seizure tests, subjects were anesthetized with pentobarbital (40 mg/kg) and blood samples were drawn from the heart. β-hydroxybutyrate levels were assayed following procedures described in the General Methods.

3.2.4 Seizure Testing / Statistical Analysis

On post-natal day 31 – after 10 days on the diets – the four seizure tests were administered as described in the General Methods. Subjects were used only once, different subjects being used for the different tests. Numbers of subjects and statistical tests were as follows:
The MES Test - Twenty male pups served as subjects (10 control and 10 KD). Seizures were scored as "present" or "absent", and seizure severity was categorized as: "sub maximal" (= no tonic hindlimb extension), "maximal" (= tonic hindlimb extension) or "two phase maximal" (= 2 tonic hindlimb extension). Two-phase maximal, which is not commonly seen, seems to be an unusually strong form of maximal seizure. This two-phase pattern consists of a complete extension of hindlimbs, followed by a brief relaxation, then a second extension. Differences between the groups were evaluated using the Chi-Square test.

The Threshold ECS Test - Nineteen male pups served as subjects (9 control and 10 KD). Current levels needed to produce "threshold" (= FLC) and "maximal" (= tonic hindlimb extension) seizures were calculated. Differences between the groups were evaluated using the Mann-Whitney Rank Sum test, since the data were not normally distributed.

The Threshold MET Test - Twenty-four male pups served as subjects (12 control and 12 KD). Threshold seizures (= FLC) were scored as "present" or "absent", and the latencies to the first MJ and to the first bout of FLC were measured. The number of animals surviving 30 minutes after injection was also recorded. (Deaths may occur in tests involving convulsant drugs.) Differences between the groups were evaluated using the Chi-Square test (seizure/death occurrence) or unpaired t-tests (latency data).

The MMT Test - Forty-eight male pups served as subjects (24 control and 24 KD). Seizures were scored as "present" or "absent" and ranked as "submaximal" (= no tonic hindlimb extension) or "maximal" (= tonic hindlimb extension). (Two-phase maximal seizures were not seen in this test.) The latency to the first MJ and to FLC were
measured. The number of subjects surviving 30 minutes after injection was also recorded. Differences between the groups were evaluated using the Chi Square test (seizure/death occurrence), the Mann-Whitney Rank Sum test (MJ latency, which was not normally distributed) or unpaired t-tests (FLC latency data).

3. 2. 5  **General Statistical Analysis**

Weight differences between the control and KD groups were analyzed using the unpaired t-test. The differences in β-hydroxybutyrate were analyzed using the Mann-Whitney Rank Sum test, due to the absence of normality in the data.

3. 3  **RESULTS**

3. 3. 1  **Weights**

Table 6 presents body weights for the control and KD subjects. The control weights were higher than the KD weights. The KD subjects usually gained less than 10 g during the study period, and weighed 45-65 g on the day of testing. The control subjects usually gained over 50 g, and weighed 100-175 g on the day of testing. Statistical analysis showed that the KD subjects were significantly lighter on the day of testing in every pair of test groups (p < 0.001, unpaired t-test).

3. 3. 2  **β-hydroxybutyrate Levels**

Table 6 (last column) also presents β-hydroxybutyrate levels for the control and KD groups. As indicated, β-hydroxybutyrate levels were 10 - 30 times higher in the KD groups. Levels varied between experiments, from a high of 7.14 mM to a low of 3.36
mM, but they were always within or above the clinical range (2 - 4 mM). Control values were much lower, varying between 0.17 and 0.42 mM. KD/control differences were statistically significant in every pair of test groups (p < 0.001, Mann-Whitney Rank Sum test).

Table 6: Body weights (mean ± SD) on the day of weaning, on the day of seizure testing, and β-hydroxybutyrate levels on the day of seizure testing.

<table>
<thead>
<tr>
<th>Seizure Test</th>
<th>Diet Groups</th>
<th>N</th>
<th>Wean-day body weights ± SD (g)</th>
<th>Seizure-test day body weights ± SD (g)</th>
<th>β-hydroxybutyrate levels ± SD (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES</td>
<td>Control</td>
<td>10</td>
<td>44.8 ± 5.4</td>
<td>103.5 ± 11.9</td>
<td>0.42 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>10</td>
<td>44.2 ± 3.8</td>
<td>46.8 ± 9.2*</td>
<td>7.14 ± 1.51*</td>
</tr>
<tr>
<td>Threshold</td>
<td>Control</td>
<td>9</td>
<td>49.0 ± 3.7</td>
<td>175.6 ± 10.3</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>ECS</td>
<td>KD</td>
<td>10</td>
<td>49.9 ± 3.8</td>
<td>63.6 ± 13.1*</td>
<td>6.09 ± 1.95*</td>
</tr>
<tr>
<td>Threshold</td>
<td>Control</td>
<td>12</td>
<td>48.3 ± 3.1</td>
<td>105.5 ± 7.7</td>
<td>0.20 ± 0.08</td>
</tr>
<tr>
<td>MET</td>
<td>KD</td>
<td>12</td>
<td>47.5 ± 4.4</td>
<td>64.2 ± 5.1*</td>
<td>3.36 ± 0.93*</td>
</tr>
<tr>
<td>MMT</td>
<td>Control</td>
<td>24</td>
<td>43.5 ± 6.1</td>
<td>103.8 ± 12.4</td>
<td>0.37 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>24</td>
<td>48.0 ± 4.6*</td>
<td>54.8 ± 9.2*</td>
<td>3.49 ± 1.34*</td>
</tr>
</tbody>
</table>

* Significantly different from control group (p < 0.05, unpaired t-test or Mann-Whitney Rank Sum test)

3.3.3 The MES Test

Figure 1 presents the results of the MES seizure test. As indicated, the majority of subjects in both groups experienced maximal seizures. The percentage of maximal seizures was actually higher in the KD group (100%) than in the control group (70%),
Percentage of subjects showing maximal and two-phase seizures in the MES test. 

**Figure 1:** Percentage of control and KD subjects showing maximal and two-phase seizures.

Subjects in the KD group showed this two-phase pattern, while none of the control subjects showed this pattern. The difference between KD and control subjects was not statistically significant (p > 0.05, Chi Square test).
3.3.4 The Threshold ECS Test

Figure 2 presents the results of the threshold ECS test. As indicated, thresholds for minimal seizures (FLC) in the control and the KD group were very similar (17.1 ± 1.6 mA and 16.2 ± 1.6 mA, respectively). There was no significant difference between the groups on this measure (p = 0.234, Mann-Whitney Rank Sum test). Thresholds for maximal (MES) seizures, however, were significantly lower in the KD group (control group: 49.2 ± 8.7 mA; KD group: 23.5 ± 8.7 mA) (p = 0.004; Mann-Whitney Rank Sum test).

* Significantly different from control group (p = 0.004, Mann-Whitney Rank Sum test)

Figure 2: Mean thresholds (mA) for minimal and maximal seizures in the control and KD groups (mean ± SD).
3.3.5 *The Threshold MET Test*

Table 7 presents the results of the threshold MET seizure test. Seizure occurrence is indicated in column 3. All subjects in the control group displayed FLC, as did all but one of the subjects in the KD group. There was no significant difference in seizure occurrence between the groups (p > 0.05, Chi-Square test). Seizure latencies are indicated in columns 4 and 5. There was also little difference in the seizure latencies. In the control subjects, the average latency to the first MJ was 307 ± 153 seconds, while in the KD subjects it was 301 ± 291 seconds. In the control subjects, the average latency to the first bout of FLC was 592 ± 229 seconds, while in the KD subjects it was 490 ± 286 seconds. There were no significant differences between the two groups on either parameter (p = 0.952 and 0.350 respectively, unpaired t-tests). Survival data are presented in column 6. At the end of the 30-minutes observation period, only 75% of the control subjects were alive, while in the KD group 100.0% of the subjects were alive. This difference was, however, not statistically significant (P > 0.05, Chi-Square test).

Table 7: Minimal seizure occurrence, latencies and survival for the control and MCT KD groups in the threshold MET test.

<table>
<thead>
<tr>
<th>Diet Groups</th>
<th>N</th>
<th>% showing FLC</th>
<th>Avg. latency to first MJ (sec. ± SD)</th>
<th>Avg. latency to FLC (sec. ± SD)</th>
<th>Number surviving 30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>100.0</td>
<td>307 ± 153</td>
<td>592 ± 229</td>
<td>9</td>
</tr>
<tr>
<td>KD</td>
<td>12</td>
<td>91.7</td>
<td>301 ± 291</td>
<td>490 ± 286</td>
<td>12</td>
</tr>
</tbody>
</table>
3.3.6 The MMT Test

Table 8 presents the results of the modified MMT seizure test. Seizure occurrence is indicated in column 3. In the control group, all of the subjects had seizures, and 1/24 had maximal seizures. In the KD group, all of the subjects had seizures, and 14/24 had maximal seizures. Thus, significantly more maximal seizures were seen in the KD group (p < 0.05, Chi Square Test). Seizure latencies are indicated in columns 4 and 5. In the control subjects, the average latency to the first MJ was $211 \pm 120$ seconds, while in the KD subjects it was significantly less at $158 \pm 76$ seconds ($p = 0.041$, Mann-Whitney Rank Sum test). In the control subjects, the average latency to the first bout of FLC was $214 \pm 99$ seconds, while in the KD subjects it was considerably less at $165 \pm 76$ seconds, although this difference just failed to reach significance ($p = 0.063$, unpaired t-test). Survival data are indicated in column 6. There was also a large difference in mortality. At the end of the 30-minute observation period, only 20.8% of the control subjects were alive, while in the KD group 91.7% of the subjects were alive. This difference was statistically significant ($p < 0.05$, Chi-Square test).

Table 8: Maximal seizure occurrence, latencies and survival for the control and MCT KD groups in the MMT test.

<table>
<thead>
<tr>
<th>Diet Groups</th>
<th>N</th>
<th>% showing maximal seizures</th>
<th>Avg. latency to first MJ (sec. ± SD)</th>
<th>Avg. latency to FLC (sec. ± SD)</th>
<th>Number surviving 30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>4.2</td>
<td>$211 \pm 120$</td>
<td>$214 \pm 99$</td>
<td>4</td>
</tr>
<tr>
<td>KD</td>
<td>24</td>
<td>58.3*</td>
<td>$158 \pm 76$*</td>
<td>$165 \pm 76$</td>
<td>22</td>
</tr>
</tbody>
</table>

* Significantly different from control group ($p < 0.05$, Chi-Square test or Mann-Whitney Rank Sum test).
3.4 DISCUSSION

Experiment 1 was designed to examine the effect of the MCT KD on four standard seizure tests, using a standard species, age, and time on the diet.

Despite high β-hydroxybutyrate levels, no anticonvulsant effects were seen on any test. These results are at variance with several reports in the literature (Table 2). Millichap et al. (1964), Uhlemann and Neims (1972), and Nakazawa et al. (1983) for instance, have all reported that the KD suppresses maximal (MES) seizures, while Appleton and DeVivo (1974) and Bough et al. (1999) have reported an elevation of threshold for minimal (FLC) seizures (see Table 2). It seemed possible that anticonvulsant effects in the present experiment were masked by the occurrence of proconvulsant effects.

Proconvulsant effects were seen in several tests. MES threshold was significantly lower in the KD subjects in the threshold ECS experiment and significantly more subjects displayed maximal seizures in the MMT test. The time to the first MJ was significantly shorter in the MMT test and more subjects showed the maximal two phase pattern in the MES test (although this was not statistically significant). These findings - though unexpected - are in agreement with three previous reports (Table 2). Mahoney et al. (1983) found that the KD increased the incidence and severity - and decreased the latency - of audiogenic seizures in magnesium-deficient rats. Bough et al. (1998) have recently reported that the KD increases the severity of kainic-acid induced seizures, and Matthews et al. (1999) have found an increase in MES severity in KD subjects.

The factors that caused the proconvulsant effects in Experiment 1 - as opposed to anticonvulsant effects - were not clear at this point. Comparing our experiments to past
experiments, however, some factors were noted that could play a role, including: (1) the difference in weights between the control and KD subjects; (2) the sensitivity of the seizure tests used; (3) the form of KD used (i.e. MCT instead of classic); (4) the strain of rats used; and (5) the duration of time on the diet. It is also important to note that the KD subjects ate only about half as much as the controls, and that they may not have been getting a full complement of vitamins and minerals. Therefore vitamin and mineral deficiencies in the KD subjects could have also played a role in the proconvulsant effects.

The role of these factors was examined in the 3 subsequent experiments. Experiment 2 addressed the role of weight. Experiment 3 – a strict replication of Bough et al. (1999) - used a more sensitive seizure test, the classic KD, a different strain of rats and a longer duration on the diet. Experiment 4 replicated the seizure tests from Experiment 1 with the classic KD, a different strain of rats and a longer duration on the diet.
CHAPTER 4

EXPERIMENT 2

EFFECTS OF THE KD IN FOOD-RESTRICTED AND FOOD-AUGMENTED SUBJECTS

4.1 RATIONALE

In Experiment 1, four common seizure models showed either no effect, or proconvulsant effects, with the MCT KD. One possible reason for this result is the substantial difference in weight between the control and KD diet groups, due to voluntary food restriction by the KD subjects.

A review of the past published studies reporting anticonvulsant effects (Table 2) indicated that control and KD subjects often had similar weights, which were normal for their age (Appleton, DeVivo, 1974; Uhlemann, Neims, 1972; Millichap, Jones, 1964; Hori et al., 1997). In most studies reporting proconvulsant effects, the KD subjects were considerably lighter than the control subjects (Otani et al., 1984; Mahoney et al., 1983; Bough et al., 1998).

A classic study by Davenport and Davenport (1948) provides a possible explanation of how low weight could affect seizure thresholds. Davenport and Davenport reported that partial starvation (caloric restriction) enhances seizure susceptibility in rats (Davenport, Davenport, 1948). Seizure thresholds drop as weight is lost and increase with increasing body weight (Davenport, Davenport, 1948). The supplemental administration of carbohydrate, fat or protein to rats having low thresholds as a results of caloric restriction, raises the threshold back towards normal (Davenport, Davenport,
In brief, this report shows that normal food consumption maintains seizure thresholds, while food restriction decreases them.

In Experiment 1, our control subjects ate normally, and their body weight increased. The MCT KD group, however, self-restricted its food intake and gained little weight (after initially losing weight). This voluntary food restriction in the MCT KD subjects, along with a lack of weight gain, may have lowered seizure thresholds. This might explain the proconvulsant effects that were found, and the absence of KD anticonvulsant effects.

Experiment 2 was designed to examine the effect of weight on seizure outcomes with the KD. The weights of the subjects were regulated using special diet paradigms. Subjects were divided into four groups – Control Low-Weight, MCT KD Low-Weight, Control High-Weight and MCT KD High-Weight. The Low-Weight groups (Control and MCT KD) were given a calorie-restricted diet (100 - 110% of their regular caloric requirement). This kept controls close in weight to the MCT KD group (Low-Weight MCT KD), and both group relatively light. This group was regarded as the low weight group since their weight gain was much less than the control subjects fed ad libitum in Experiment 1 (compare weights to that of ECS threshold group).

The High-Weight groups (Control and MCT KD) were given a calorie augmented diet (180 – 200% of their regular caloric requirement). This kept the High-Weight groups heavier than the Low-Weight groups. This group was regarded as the high weight group since their weight gain was the same as the control subjects fed ad libitum in Experiment 1 (compare weights to that of ECS threshold group).
All subjects – and in particular the MCT KD High-Weight - were induced to eat by putting them on a “limited access” feeding paradigm (Linseman et al., 1994). Subjects were only fed once a day, and were allowed to feed for only a restricted amount of time ($\geq 3$ hours). When food is presented for a limited time, subjects tend to eat all that they are given (Linseman et al., 1994). Bough et al. (1999) had previously used the restricted access paradigm in their KD studies.

In addition, the MCT KD was presented in a liquid form. A liquid form was used in order to ensure that the diet was gavagable, if necessary. The original plan was to gavage subjects – especially the MCT KD High-Weight subjects – if they did not eat their entire daily meal. In practice, the limited access paradigm made this unnecessary.

The seizure test used in this experiment was the threshold ECS test. This test was chosen because it provides more information than the other tests, including the presence or absence of maximal seizures (first stimulus = 50mA), the threshold for maximal seizures and the threshold for minimal seizures.

It was hypothesized that anticonvulsant effects of the MCT KD would be seen if the weight differences between the control and KD diets were eliminated.

4.2 SPECIFIC METHODS

4.2.1 Subjects

As in Experiment 1, male Wistar rat pups ($N = 48$) served as subjects. They were 15 days old on arrival from the breeding farm (Charles River, St. Constant, Quebec, Canada). Subjects were housed as described in the General Methods. They were weaned at 20 days, and randomly separated into 4 groups of twelve (Control Low-Weight,
Control High-Weight, MCT KD Low-Weight and MCT KD High-Weight). Subjects were weighed on the day of diet initiation and every two days thereafter, plus the day of seizure testing.

4. 2. 2  **Control Diet and MCT KD**

As in Experiment 1, on post-natal day 20, subjects were started on the control diet (710093) or the gavagable MCT KD (710079). The diets were obtained from a commercial supplier (Dyets, Bethlehem, PA). MCT oil, a generous gift of Mead Johnson, was added to the MCT KD in our own laboratory.

The subjects’ weight gains were regulated by controlling the amount of food available to them. The calorie requirement for each group was calculated by multiplying the average weight of the group by 0.3Kcal/g/day (the caloric requirement for a 20-40 day old laboratory rat) (Rogers, 1979). The subjects in the Control Low-Weight and MCT KD Low-Weight groups were fed exactly their daily caloric requirement (allowing for a modest weight gain), while the subjects in the Control High-Weight group and MCT KD High-Weight Group were fed 1.8 - 2 times their caloric requirement per day (allowing for a large weight gain).

In accordance with the limited access paradigm, subjects were fed for only 3 hours per day, between 15:00 and 17:00h. On the limited access paradigm, even the MCT KD subjects tended to eat all their food.

4. 2. 3  **Seizure Testing**

Starting on post-natal day 31 – after 10 days on the diets – the ECS Threshold seizure test was administered. The test was administered as described in the General
Methods, except that one extra current level was added to give better threshold determination. The current levels used were 50, 36.8, 25, 17.8, and 11.8 mA. The "threshold" current levels needed to produce minimal seizures (= FLC) and maximal seizures (= tonic hindlimb extension) were calculated for every subject. Seizures were induced between 11:00 and 13:00 h.

4.2.4 Measurement of β-hydroxybutyrate Levels

Following seizure testing, subjects were anesthetized with pentobarbital (40 mg/kg) and blood samples were drawn from the heart. β-hydroxybutyrate levels were assayed following the procedures outlined in General Methods.

4.2.5 Statistical Analysis

Differences in weights between the control and KD groups were analyzed using the one-way ANOVA on ranks (due to the absence of normality). When differences were present Dunn's post hoc t-test was used to identify the differences. Differences in β-hydroxybutyrate levels were compared using the unpaired t-test or Mann-Whitney Rank Sum test (when data was not normally distributed).

Differences in minimal and maximal seizure thresholds between the groups were evaluated using the Mann-Whitney Rank Sum test (due to a lack of normality in the data).
4.3 RESULTS

4.3.1 Weights

Table 9 presents body weights for the control and MCT KD subjects. The subjects in the Low-Weight groups – both control and MCT KD - gained 43 – 45g during the experimental period, while the subjects in the High-Weight groups – both control and MCT KD - gained 120 -130g. On the day of seizure testing, the High-Weight groups were significantly heavier than the Low-Weight groups (p < 0.001, One-Way ANOVA on Ranks). There was no significant difference between the Low-Weight Control and the Low-Weight MCT KD groups or the High-Weight Control and the High-Weight MCT KD groups (p > 0.05, Dunn’s post hoc test).

Table 9: Body weights (mean ± SD) on the day of diet initiation, on the day of seizure testing, and β-hydroxybutyrate levels on the day of seizure testing.

<table>
<thead>
<tr>
<th>Weight Group</th>
<th>Diet Groups</th>
<th>N</th>
<th>Diet Initiation day body weights ± SD (g)</th>
<th>Last seizure test day body weights ± SD (g)</th>
<th>β-hydroxybutyrate levels ± SD (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Weight</td>
<td>Control</td>
<td>11</td>
<td>48.6 ± 4.1</td>
<td>91.8 ± 6.2a</td>
<td>0.35 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>12</td>
<td>49.5 ± 3.3</td>
<td>93.8 ± 3.6a</td>
<td>1.54 ± 0.68*</td>
</tr>
<tr>
<td>High Weight</td>
<td>Control</td>
<td>12</td>
<td>48.8 ± 4.0</td>
<td>176.0 ± 5.7b</td>
<td>0.50 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>12</td>
<td>50.5 ± 3.3</td>
<td>172.2 ± 6.3b</td>
<td>0.80 ± 0.28*</td>
</tr>
</tbody>
</table>

ab weights with the same letter designation are not statistically different (p > 0.05, one-way ANOVA on ranks), and weights with different letter designation are statistically different (p < 0.05, Dunn’s post hoc test).

* Significantly different from control group (p < 0.05, Mann-Whitney Rank Sum test or unpaired t-test).
4.3.2 β-hydroxybutyrate Levels

Table 9 (last column) also presents β-hydroxybutyrate levels for the control and MCT KD groups. As indicated, β-hydroxybutyrate levels were 1.6 to 4.4 times higher in the MCT KD groups. They varied between 0.80 mM in the High-Weight MCT KD group and 1.54 mM in the Low-Weight MCT KD group. Control values were lower, varying between 0.35 mM in the Low-Weight Control group and 0.50 mM in the High-Weight Control group. KD/control differences were statistically significant in both the groups (p < 0.01, Mann-Whitney Rank Sum test).

In this particular experiment, for reasons that are not clear, β-hydroxybutyrate levels were below the desired clinical range (2 - 4 mM).

4.3.2 Seizure Test

Figures 3 - 6 present the results of the ECS threshold test. The MES occurrence data for the MCT KD Low-Weight and Control Low-Weight groups are presented in Figure 3. All subjects had maximal seizures, with 50% of the control subjects showing the maximal two-phase pattern, while 33% of the MCT KD subjects showed this pattern. The difference in the occurrence of the two-phase pattern is not statistically significant (p > 0.05, Chi-Square test).

Figure 4 presents the seizure threshold results for the MCT KD Low-Weight and Control Low-Weight groups. As indicated in Figure 4, the threshold for minimal seizures (FLC) in the MCT KD group was significantly higher than that in the control group (19.5 ± 2.90 and 15.8 ± 1.87 mA respectively). This difference was statistically significant (p = 0.016, Mann-Whitney Rank Sum test). The threshold for maximal seizures was also
slightly higher in the MCT KD group (22.2 ± 2.7 versus 21.4 ± 3.7 mA in the controls), however this difference not statistically significant (p = 0.533, Mann-Whitney Rank Sum test).

The MES seizure occurrence data for the MCT KD High-Weight and Control High-Weight groups are presented in Figure 5. All subjects had maximal seizures, with 41.6% of the control subjects showing the maximal two-phase pattern, while 25% of the MCT KD subjects showed this pattern. This difference in the occurrence of the two phase pattern was not statistically significant (p > 0.05, Chi-Square test).

Figure 6 presents the results of the seizure threshold test for the MCT KD High-Weight group and the control High-Weight group. As indicated in Figure 6, the threshold for minimal seizures in the MCT KD High-Weight group was slightly greater than that in the Control High-Weight group, (20.2 ± 4.1 and 19.0 ± 2.5 respectively). This difference, however, was not statistically significant (p = 0.62, Mann-Whitney Rank Sum test). The threshold for maximal seizures was the same for the KD and control groups (34.5 ± 8.5 and 34.5 ± 8.5, p = 0.97, Mann-Whitney Rank Sum test).
Maximal seizures occurrence in the Control Low-Weight and the Ketogenic Diet Groups.

Percentage of Subjects Showing Maximal Seizures (%)

N = 12

Maximal Two Phases
Maximal
* Significantly different from control (p = 0.016, Mann-Whitney Rank Sum test)

**Figure 4:** Maximal and minimal seizure thresholds (mA) for Control Low-Weight and MCT KD Low-Weight groups (mean ± SD).
Figure 5: Maximal seizure occurrence in the Control High-Weight and the MCT KD High-Weight groups.
Figure 6: Maximal and minimal seizure thresholds (mA) for the Control High-Weight and the MCT KD High-Weight groups (mean ± SD).
4.4 DISCUSSION

Experiment 2 was designed to examine the role of weight gain in the anticonvulsant efficacy of the MCT KD in the ECS threshold model. It was hypothesized that anticonvulsant effects of the MCT KD would be seen if weight differences between the control and the MCT KD subjects were eliminated. Using caloric restriction, the weights of the subjects were regulated. In Experiment 2, the diet paradigms also ensured that the control and the KD subjects had similar amounts of vitamins and minerals per kilogram. The groups were: Control Low-Weight, KD Low-Weight, Control High-Weight and KD High-Weight.

Presenting measured amounts of food in a limited access paradigm was a successful maneuver. Weights in the Low-Weight Control and MCT KD groups were very similar. Unfortunately, clinical levels of β-hydroxybutyrate were not achieved in this MCT KD groups. For unknown reasons, the gavagable KD was not as successful as the non-gavagable form used in Experiment 1. Ketone levels in the MCT KD High-Weight group were particularly low. This should be kept in mind when interpreting the seizure data.

In the Low-Weight groups, the minimal seizure threshold was significantly higher in the MCT KD group. This was true even though the ketone levels were slightly below the clinical range. In the High-Weight groups, the minimal seizure threshold was non-significantly higher in the MCT KD group.

These data generally confirm our hypothesis. Proconvulsant – not anticonvulsant – effects were seen in Experiment 1, where the KD groups were much lighter than the
control groups. In Experiment 2, however, where control and KD weights were matched, a protective effects of the MCT KD was evident.

In the Low-Weight groups, the maximal seizure threshold was non-significantly higher in the MCT KD group. In the High-Weight groups, however, there was no difference. One reason for the lack of effect in the High-Weight MCT KD group could be the low β-hydroxybutyrate levels (0.80 mM).

In maximal seizure occurrence, there were a fewer number of subjects both in the Low-Weight and High-Weight MCT KD groups that showed the maximal two-phase seizure pattern (although not statistically significant, p > 0.05, Chi-Square test).

As suggested by Davenport and Davenport (1948), the present data also suggest a decrease in seizure threshold with caloric restriction. Comparing Figures 4 and 6, there is a clear reduction in the maximal seizure threshold for both the control and MCT KD subjects in the Low-Weight groups (where caloric restriction was present) as compared to the High-Weight groups.

It would be useful to repeat Experiment 2, perhaps using the non-gavagable MCT KD, and see the results when higher ketone levels are present.
5.1 RATIONALE

Experiment 3 - which was run at the same time as Experiment 2 - was an attempt to replicate the KD anticonvulsant effects recently reported by another group.

Anticonvulsant effects have recently been reported by Bough et al. (1999) in the MET infusion model. In this study, a diet regulation paradigm was also used, where the subjects were limited to 90% of their regular caloric requirement in order to ensure there was no alteration in body weight during the period of the experiment. Bough et al. (1999) also used the classic KD, a different strain of rats (Sprague-Dawley), and a longer time on the diet (\( \geq 20 \) days).

The MET infusion model is one in which MET is administered at a controlled rate into the tail vein of the subjects. The time required to produce the first FLC is recorded. Then, based on the time elapsed and the infusion rate, the dose (mg/kg) required to cause forelimb clonus is calculated. This model is somewhat similar to the classic MET test (Experiment 1), but it avoids the variability in absorption that may arise when MET is administered sub-cutaneously, and provides a true measure of threshold. The infusion model is, therefore, highly sensitive to subtle threshold changes. It might be expected to detect small changes in threshold, which would be missed by the threshold MET test.
5.2 SPECIFIC METHODS

All methods used duplicated those used by Bough et al. (1999).

5.2.1 Subjects

Male Sprague-Dawley rat pups served as subjects. They were 14 days old on arrival from the breeding farm (Charles River, St. Constant, Quebec, Canada). Subjects were housed as described in the General Methods. They were weaned at 21 days of age onto standard rodent chow for one day (Purina 5001). They were weighed on the day of diet initiation, every two days thereafter, plus on the day of seizure testing.

5.2.2 Control Diet and classic KD

Beginning on postnatal day 22 - after one day on the rodent chow - subjects were fasted for 6 hours and then started on the control diet (Purina 5001) or the classic KD (F3666). The classic KD was obtained from Bioserv (Frenchtown, NJ, USA) (for details of the control and KD diets, see table 5 in the General Methods). Subjects were fed individually once each day, between 15:00 and 17:00 h, and allowed to feed for 2.5 - 3 hours. They were calorie-restricted, and were limited to approximately 90% of their calculated daily requirement. This was done in order to maintain stable body weights for the duration of the experiment. Water was provided at libitum.

5.2.3 Measurement of β-hydroxybutyrate Levels

One day prior to seizure testing (post-natal day 42), blood samples were drawn from the tail vein of the subjects. β-hydroxybutyrate levels were assayed following procedures in the General Methods.
5.2.4 Seizure Testing

On post-natal day 43 – after 21 days on the diet – the MET infusion test was administered. MET (Sigma Chemicals) was dissolved in physiological saline to a concentration of 10mg/ml. This solution was then infused into the tail vein using an infusion pump (Syringe Pump model 351, Sage Instruments) via a 27-gauge butterfly needle. The infusion rate was 1.0ml/min. The infusion was stopped, and the needle removed, after the first bout of bilateral FLC. The time to the onset of forelimb clonus was recorded, and motor seizures were ranked as “maximal” (= tonic hindlimb extension to 90° of more) or “submaximal” (= absence of tonic hindlimb extension to 90° or more). (Note: although infusion was stopped after the first bout of bilateral clonus, most subjects proceeded to maximal seizures within 10 seconds). Testing was done between 11:00 and 16:00 h.

5.2.5 Data Analysis

The dose of drug needed to produce the first bout of bilateral FLC was calculated, using the infusion rate, the time needed to produce the bilateral FLC, the concentration of drug used and the weight of the subject. The dose was expressed as a “threshold” dose in mg/kg.

5.2.6 Statistical Analysis

Differences in weights between the control and KD groups were analyzed using the unpaired t-test. Differences in β-hydroxybutyrate levels were analyzed using the Mann-Whitney Rank Sum test (due to absence of normality in data).
Differences in the weights of the control and KD groups, and the threshold MET doses, were analyzed using unpaired t-tests. Differences in β-hydroxybutyrate levels were compared using the Mann-Whitney Rank Sum test, since the data were not normally distributed.

5.3 RESULTS

5.3.1 Weights

Table 10 presents mean body weights for control and KD subjects on the day of diet initiation and on the day of seizure testing. Neither group gained weight during the experiment, both groups staying at about the weight seen on the day of diet initiation. There was no significant difference in weights between the control and KD group on the day of seizure testing (p > 0.05, unpaired t-test).

Table 10: Body weights (mean ± SD) on the day of diet initiation, on the day of seizure testing, and β-hydroxybutyrate levels one day prior to seizure testing.

<table>
<thead>
<tr>
<th>Diet Groups</th>
<th>N</th>
<th>Body weights ± SD (g) on the day of diet initiation</th>
<th>Body weights ± SD (g) on the day of seizure testing</th>
<th>β-hydroxybutyrate levels ± SD (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>59.2 ± 7.1</td>
<td>57.3 ± 4.5</td>
<td>0.33 ± 0.10</td>
</tr>
<tr>
<td>KD</td>
<td>12</td>
<td>55.8 ± 6.7</td>
<td>53.8 ± 4.3</td>
<td>2.72 ± 1.21*</td>
</tr>
</tbody>
</table>

* Significantly different from control group (p < 0.001, Mann-Whitney Rank Sum test)
5.3.2 β-hydroxybutyrate Levels

Table 10 (last column) also presents β-hydroxybutyrate levels for the control and KD groups. As indicated, β-hydroxybutyrate levels were 8 times higher in the KD group than in the control group, and were within the clinical range (2 – 4 mM). The difference between the two groups was statistically significant (p < 0.001, Mann-Whitney Rank Sum test).

5.3.4 Seizure Test

Figure 7 presents the results of the MET infusion test. The dose required to induce threshold (FLC) seizures in the KD group was significantly higher than that in the control group (p < 0.001, unpaired t-test).

Although thresholds were higher in the KD group, the seizures (severity) themselves were not attenuated. Eighty-three percent of the control subjects showed maximal seizures while 92% of the KD subjects showed maximal seizures. This difference was not statistically significant (p > 0.05, Chi-Square test).
Figure 7: Comparison of the dose of MEI (mean ± SD) required to produce forelimb clonus in the control and KD groups. Subjects significantly different from control group (p < 0.01, unpaired t-test).

Ketogenic Diet | Control

Dose required to produce forelimb clonus (mg/kg)

N = 12

N = 12
Experiment 3 was designed to replicate the study of Bough et al. (1999), and to determine whether the reported anticonvulsant effects of the classic KD could be shown. The diet, the time on the diet, the strain of subjects and the seizure model used were altered in comparison to Experiment 1.

KD and control subjects did not gain weight during the experiment. This is similar to Bough’s findings, and to the clinical observation that children on the KD only gain minimal weight, if at all (Kinsman et al., 1992; Freeman et al., 1994).

The KD used in this Experiment (Table 4) had lower vitamins and minerals content as compared to the control diet on a per kilogram basis. The KD used was exactly the same as that used by Bough et al. (1999) and was obtained from the same supplier. It should however, be noted that the presence of lower vitamins and minerals in the KD works against us, since this deficiency could have lead to proconvulsant effects.

β-hydroxybutyrate levels in the KD group were moderately high, as desired (2.72 ± 1.21 mM). The levels were, however, not high as that reported by Bough et al. (1999) (~ 7.5mM) for the same age subjects - despite an exact replication of diet administration. The reason for this difference is not clear at present. The β-hydroxybutyrate levels in Experiment 3 were, however, within the clinical range (2 –4 mM).

We successfully replicated the anticonvulsant effects reported by Bough et al. in the MET infusion model. Even though the effect of the KD was small, it was still significant. (Bough’s reported effects were similarly small.)

Anticonvulsant effects were seen in Experiment 2 with the MCT KD and Wistar rats, and in Experiment 3, with the classic KD and Sprague-Dawley rats. From the
results of these two experiments, it appears that the anticonvulsant effects of the KD do not depend on type of KD diet or the strain of rats used. The effect of duration on the diet is not clear, since, in Experiment 2, the ECS threshold subjects were also on the diet for a total period of 19 days, even though seizure testing was begun on the 11th day. (The test itself took 8 days.)
CHAPTER 6

EXPERIMENT 4

EFFECT OF THE CLASSIC KD ON FOUR ANIMAL SEIZURE MODELS

6.1 RATIONALE

Experiment 4 was designed to test Bough's "classic" diet paradigm on the four standard models used in Experiment 1. The goal was to see whether Bough's paradigm would have anticonvulsant effects in the standard animal seizure tests.

6.2 SPECIFIC METHODS

6.2.1 Subjects

Male Sprague Dawley rat pups served as subjects. They were 14 days old on arrival from the breeding farm (Charles River, St. Constant, Quebec, Canada). Subjects were housed as described in the General Methods. They were weaned at 21 days of age onto standard rodent chow for one day (Purina 5001). They were weighed on the day of diet initiation and every two days thereafter, plus the day of seizure testing.

6.2.2 Control Diet and classic KD

Beginning on postnatal day 22 — after 1 day on the standard rodent chow — subjects were fasted for 6 hours and then started on the control diet (Purina 5001) or the classic KD (F3666). The classic KD was obtained from Bioserv (Frenchtown, NJ, USA). Subjects were fed individually once each day, beginning between 15:00 and 17:00 h, and allowed to feed for 2.5 - 3 hours. They were calorie-restricted, and were limited to
approximately 90% of their calculated daily requirement. This was done in order to maintain stable body weights for the duration of the experiment. Water was provided at libitum.

6.2.3 Seizure Testing / Statistical Analysis

On post-natal day 43 – after 21 days on the diets – 4 standard seizure tests (below) were administered as described in the General Methods. Subjects were tested only once on any given day, with different subjects being used for the different tests. The four standard tests, the number of subjects and the statistical tests used for each were as follows:

The MES Test – Twenty-four male pups served as subjects (12 control and 12 KD). As in Experiment 1, seizures were scored as “present” or “absent”. Seizure severity was categorized as “sub maximal” (= no tonic hindlimb extension), “maximal” (= tonic hindlimb extension) or “maximal two-phase” (= 2 tonic hindlimb extension). Differences between the groups were evaluated using the Chi Square test.

The Threshold ECS Test – Twenty-four male pups serves as subjects (12 control and 12 KD). Current levels needed to produce “threshold” (= FLC) and “maximal” (= tonic hindlimb extension) seizures were calculated. Current levels used included 50, 25, 18, 15.4, 14, 11.8, 10.8 and 8.8 mA. More current levels were used, as compared to Experiments 1 and 2, in order to attain better threshold determination. Differences between the groups were evaluated using the Mann-Whitney Rank Sum test.

The Threshold MET Test – Twenty-four male pups served as subjects (12 control and 12 KD). As in Experiment 1, threshold seizures (= FLC) were scored as “present” or
absent, and the latencies to the first MJ and to FLC were measured. The number of animals surviving 30 minutes after injection was also recorded. Differences between the groups were evaluated using the Chi Square test (seizure/death occurrence), unpaired t-tests (latency data) or Mann-Whitney Rank Sum test (latency data not normally distributed).

**The MMT Test** – Twenty-four male pups served as subjects (12 control and 12 KD). Seizures were scored as “present” or “absent” and ranked as “submaximal” (= no tonic hindlimb extension) or “maximal” (= tonic hindlimb extension). The latency to the first MJ and to FLC were measured. The number of subjects surviving 30 minutes after injection was also recorded. Differences between the groups were evaluated using the Chi Square test (seizure/death occurrence) or the Mann-Whitney Rank Sum test (latency data).

### 6.2.4 Measurement of β-hydroxybutyrate Levels

Immediately following seizure testing (post-natal day 43), blood samples were drawn from heart and β-hydroxybutyrate levels were assayed following procedures in the General Methods.

### 6.2.5 General Statistics

Weight differences between the groups on the day of seizure testing were analyzed using the one-way ANOVA, while differences in β-hydroxybutyrate levels were compared using the Mann-Whitney Rank Sum test (lack of normality in data).
RESULTS

6.3.1 Weights

Table 11 presents body weights for the control and KD subjects. The subjects did not gain weight during the experiment. There were no statistically significant differences in body weights between the control and KD subjects on any seizure test on the day of seizure testing (p = 0.568, one-way ANOVA).

6.3.2 β-hydroxybutyrate Levels

Table 11 (last column) also presents β-hydroxybutyrate levels for the control and KD groups. As indicated, β-hydroxybutyrate levels were 4 - 7 times higher in the KD groups. They varied from a high of 2.75 mM to a low of 2.12 mM, but were always within the clinical range (2-4 mM). Control values were much lower, varying between 0.43 and 0.50 mM. KD/control differences were statistically significant in every test (p < 0.001, Mann-Whitney Rank Sum test).
Table 11:  Body weights (mean ± SD) on the day of weaning, on the day of seizure testing, and β-hydroxybutyrate levels on the day of seizure testing.

<table>
<thead>
<tr>
<th>Seizure Test</th>
<th>Diet Groups</th>
<th>N</th>
<th>Wean day body weights ± SD (g)</th>
<th>Seizure test day body weights ± SD (g)</th>
<th>β-hydroxybutyrate levels ± SD (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES</td>
<td>Control</td>
<td>12</td>
<td>44.7 ± 3.2</td>
<td>47.0 ± 4.5</td>
<td>0.43 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>12</td>
<td>44.8 ± 3.0</td>
<td>46.9 ± 3.6</td>
<td>2.75 ± 1.37*</td>
</tr>
<tr>
<td>Threshold ECS</td>
<td>Control</td>
<td>12</td>
<td>42.5 ± 4.8</td>
<td>44.7 ± 2.9</td>
<td>0.42 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>12</td>
<td>44.0 ± 4.4</td>
<td>45.8 ± 4.0</td>
<td>2.07 ± 0.67*</td>
</tr>
<tr>
<td>Threshold MET</td>
<td>Control</td>
<td>12</td>
<td>42.3 ± 3.6</td>
<td>45.3 ± 2.8</td>
<td>0.50 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>12</td>
<td>43.3 ± 3.7</td>
<td>45.6 ± 3.5</td>
<td>2.12 ± 0.89*</td>
</tr>
<tr>
<td>MMT</td>
<td>Control</td>
<td>12</td>
<td>44.7 ± 4.3</td>
<td>46.6 ± 3.7</td>
<td>0.48 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>12</td>
<td>43.8 ± 2.6</td>
<td>45.6 ± 3.3</td>
<td>2.62 ± 1.17*</td>
</tr>
</tbody>
</table>

* Significantly different from control group (p < 0.05, Mann-Whitney Rank Sum test)

6.3.3 The MES Test

Figure 8 presents the results of the MES seizure test. As indicated, all of the subjects in both groups produced maximal seizures (hindlimb extension). Approximately 25% of the subjects in the control and KD group showed the two-phase maximal (p > 0.05, Chi-Square test).
Figure 8: Percentage of control and KD subjects showing maximal and maximal two-phase seizures in the MES test.

6.3.4 The Threshold ECS Test

Figure 9, presents the results of the ECS threshold test. As indicated, thresholds for minimal seizures (FLC) in the KD group were higher than in the control group (11.51 ± 1.14 and 9.44 ± 1.38 mA, respectively). This difference was statistically significant (p = 0.002, Mann-Whitney Rank Sum test). The threshold for maximal seizures was also
higher in the KD group (16.1 ± 3.0 versus 13.7 ± 1.5 mA in the controls). This difference was also statistically significant (p = 0.03, Mann-Whitney Rank Sum test).

![Graph showing thresholds for minimal and maximal seizures in control and KD groups.](image)

*Statistically different from control (p < 0.05 Mann-Whitney Rank Sum test)

**Figure 9:** Thresholds (mA) for minimal and maximal seizures in the control and KD groups (mean ± SD).
6.3.5  \textit{The Threshold MET Test}

Table 12 presents the results of the threshold MET seizure test. Seizure occurrence is indicated in column 3. In the control group, 11/12 subjects showed FLC while in the KD group, 10/12 subjects showed FLC. This difference was not statistically significant (\( p > 0.05 \), Chi-Square test). Seizure latencies are indicated in columns 4 and 5. In the control subjects, the average latency to the first MJ was 226 ± 126 seconds, while in the KD subjects it was longer at 365 ± 244 seconds. In the control subjects, the average latency to the first bout of FLC was 374 ± 222 seconds, while in the KD subjects it was longer at 566 ± 477 seconds. Although latencies were always greater in the KD group, there was no significant difference between the two groups on either parameter (\( p = 0.10 \), unpaired t-test and 0.70 Student-Newman Keuls test, respectively), perhaps because of variability of the data. Survival data are presented in column 6. At the end of the 30-minute survival period, 91.6\% of the control subjects were alive, while 100\% of the KD subjects were alive. This difference was not statistically significant (\( p > 0.05 \), Chi-Square test)

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Diet Groups} & \textbf{N} & \% showing FLC & \textbf{Avg. latency to first MJ (sec. \pm SD)} & \textbf{Avg. latency to FLC (sec. \pm SD)} & \textbf{Number surviving 30 minutes} \\
\hline
Control & 12 & 91.6 & 226 \pm 126 & 374 \pm 222 & 11 \\
KD & 12 & 83.3 & 365 \pm 244 & 566 \pm 477 & 12 \\
\hline
\end{tabular}
\end{table}

Table 12: Minimal seizure occurrence, latencies and survival for the control and classic KD groups in the threshold MET test.
6.3.6  **The MMT Test**

Table 13 presents the results of the modified MMT seizure test. Seizure occurrence is indicated in column 3. In the control group, all of the subjects had seizures of some sort and 11/12 subjects had maximal seizures. In the KD group, all subjects had seizures of some sort and 10/12 had maximal seizures. The difference in maximal seizure occurrence was not statistically significant (p > 0.05 Chi-Square test). Seizure latencies are indicated in columns 4, 5 and 6. In the control subjects, the average latency to the first MJ was 153 ± 43 seconds, while in the KD subjects it was greater at 215 ± 103 seconds. This difference was not statistically significant (p = 0.149, Mann-Whitney Rank Sum test). In the control subjects, the average latency to the first bout of FLC was 182 ± 41 seconds, while in the KD subjects it was greater at 497 ± 283 seconds. This difference was statistically significant (p = 0.013, Mann-Whitney Rank Sum test). In the control subjects, the average latency to the first maximal seizure was 266 ± 145, while in the KD subjects it was greater at 497 ± 293 seconds. This difference was also statistically significant (p = 0.038, Mann-Whitney Rank Sum test). Survival data are indicated in column 6. At the end of the 30-minute observation period, 25.0% of the control subjects were alive, while in the KD group 83.3% of the subjects were alive. This difference was statistically significant (p < 0.05, Chi-Square test).
Table 13: Maximal seizure occurrence, latencies and survival in the control and classic KD groups during the MMT test.

<table>
<thead>
<tr>
<th>Diet Groups</th>
<th>N</th>
<th>% showing maximal seizures</th>
<th>Avg. latency to first MJ (sec. ± SD)</th>
<th>Avg. latency to FLC (sec. ± SD)</th>
<th>Avg. latency to maximal seizure (sec. ± SD)</th>
<th>Number surviving 30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>91.7</td>
<td>153 ± 43</td>
<td>182 ± 41</td>
<td>266 ± 145</td>
<td>3</td>
</tr>
<tr>
<td>KD</td>
<td>12</td>
<td>83.3</td>
<td>215 ± 103</td>
<td>323 ± 156*</td>
<td>497 ± 283*</td>
<td>10*</td>
</tr>
</tbody>
</table>

* Significantly different from control group (p < 0.05, Mann-Whitney Rank Sum test or Chi-Square test).

6.4 DISCUSSION

Experiment 4 was designed to repeat the four standard seizure tests from Experiment 1 using the "classic" KD, and the paradigm of Bough et al. (1999).

β-hydroxybutyrate levels in the KD group were high as desired, and in the clinical range (2 – 4 mM).

Anticonvulsant effects were seen in several seizure tests. KD seizure latencies were longer (though not significantly) in the threshold MET test, and were significantly longer in the MMT test. In the threshold ECS test, there was a significant increase in both the minimal seizure threshold and the maximal seizure threshold in the KD group.

These data show that the classic diet works not only in MET infusion test, but in other tests as well, including those involving electric stimulation as well as chemical convulsants. Effects were seen in both minimal and maximal seizure tests. The differences were relatively small, however, and were not seen in the standard tests when seizure occurrence was measured.
In Experiment 1, proconvulsant effects were seen with a non-gavagable MCT KD. In Experiment 2, however, anticonvulsant effects were seen with a gavagable MCT KD. In Experiment 3, clear anticonvulsant effects were seen with the classic KD. Similarly in Experiment 4, anticonvulsant effects were shown in several models with the classic KD. It is not clear, however, that the classic diet is more effective than the MCT KD, due to the lack of weight similarity between the control and KD subjects in Experiment 1. In order to make any definite conclusion regarding the efficacy of the two different forms of the KD, Experiment 1 has to be repeated with the restricted feeding paradigm.

SUMMARY OF EXPERIMENTAL RESULTS (EXPERIMENTS 1 – 4)

A summary of all experimental results is provided in Table 14 for comparison.
Table 14: Summary of experimental results from Experiments 1 - 4

<table>
<thead>
<tr>
<th>Seizure Test</th>
<th>Diet Group</th>
<th>Parameter Measured</th>
<th>Experimental</th>
<th>Experimental 2</th>
<th>Experimental 3</th>
<th>Experimental 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES</td>
<td>Control</td>
<td>Maximal (%)</td>
<td>70</td>
<td>N/A</td>
<td>N/A</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Maximal (%)</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maximal Two-Phase (%)</td>
<td>100</td>
<td>N/A</td>
<td>N/A</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maximal Two-Phase (%)</td>
<td>40</td>
<td>N/A</td>
<td>N/A</td>
<td>25</td>
</tr>
<tr>
<td>ECS Threshold</td>
<td>Control</td>
<td>Minimal Seizure threshold (mA)</td>
<td>17.1 ± 1.6</td>
<td>Low wt. 15.8 ± 1.9</td>
<td>N/A</td>
<td>9.4 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Maximal Seizure Threshold (mA)</td>
<td>49.2 ± 8.7</td>
<td>Low wt. 21.4 ± 3.7</td>
<td>N/A</td>
<td>13.7 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mininal Seizure threshold (mA)</td>
<td>16.2 ± 1.6</td>
<td>Low wt. 19.5 ± 2.9</td>
<td>N/A</td>
<td>11.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maximal Seizure Threshold (mA)</td>
<td>23.5 ± 8.7</td>
<td>Low wt. 22.2 ± 2.7</td>
<td>N/A</td>
<td>16.1 ± 3.0</td>
</tr>
<tr>
<td>MET Threshold</td>
<td>Control</td>
<td>% showing FLC</td>
<td>100</td>
<td>N/A</td>
<td>N/A</td>
<td>91.6</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Time to MJ (seconds)</td>
<td>307 ± 153</td>
<td>N/A</td>
<td>N/A</td>
<td>226 ± 126</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Time to MJ (seconds)</td>
<td>301 ± 291</td>
<td>N/A</td>
<td>N/A</td>
<td>365 ± 244</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Time to FLC (seconds)</td>
<td>490 ± 286</td>
<td>N/A</td>
<td>N/A</td>
<td>566 ± 477</td>
</tr>
<tr>
<td>MMT</td>
<td>Control</td>
<td>% showing maximal seizures</td>
<td>4.2</td>
<td>N/A</td>
<td>N/A</td>
<td>91.7</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Time to MJ (seconds)</td>
<td>211 ± 120</td>
<td>N/A</td>
<td>N/A</td>
<td>153 ± 43</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Time to FLC (seconds)</td>
<td>214 ± 99</td>
<td>N/A</td>
<td>N/A</td>
<td>182 ± 41</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Time to maximal seizures</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>266 ± 145</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>% surviving</td>
<td>16.7</td>
<td>N/A</td>
<td>N/A</td>
<td>25</td>
</tr>
<tr>
<td>MET infusion</td>
<td>Control</td>
<td>% showing maximal seizures</td>
<td>58.3</td>
<td>N/A</td>
<td>N/A</td>
<td>83.3</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Time to MJ (seconds)</td>
<td>158 ± 76</td>
<td>N/A</td>
<td>N/A</td>
<td>215 ± 103</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Time to FLC (seconds)</td>
<td>165 ± 76</td>
<td>N/A</td>
<td>N/A</td>
<td>323 ± 156</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Time to maximal seizures</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>497 ± 283</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>% surviving</td>
<td>91.7</td>
<td>N/A</td>
<td>N/A</td>
<td>83.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dose to produce FLC (mg/kg)</td>
<td>N/A</td>
<td>N/A</td>
<td>34.5 ± 2.9</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>KD</td>
<td>Dose to produce FLC (mg/kg)</td>
<td>N/A</td>
<td>N/A</td>
<td>83.3</td>
<td>N/A</td>
</tr>
</tbody>
</table>
CHAPTER 7

GENERAL DISCUSSION

7.1 OVERVIEW / GOALS

The overall goal of the present work was to develop an animal model of the KD, which could be used in subsequent studies to identify the mechanism by which the KD exerts its anticonvulsant effects (Experiment 1). Once the mechanism was clearly identified, drug therapies that could mimic the effects of the KD could be developed and administered more easily than the KD.

In Experiment 1 we were unable to show anticonvulsant effects with the MCT KD. Proconvulsant effects were seen instead. Following this experiment, a secondary goal of the present work was to understand why the KD sometimes shows proconvulsant effects and sometimes anticonvulsant effects in animal seizure models (Experiment 2).

Finally, with the publication of the work of Bough et al. (1999) – showing anticonvulsant effects in the MET infusion test – a third goal was to replicate and extend Bough’s work with the classic KD (Experiments 3 and 4).

Each experiment will be considered in detail below.

7.2 EXPERIMENT 1

Experiment 1 was designed to examine the effect of the MCT KD on four standard seizure tests, using a single species, age and time on the diet. Contrary to expectation, anticonvulsant effects were not found in any test. Instead, in several of the tests, the MCT KD appeared to have proconvulsant effects.
The lack of anticonvulsant effects in Experiment 1 was at variance with several reports in the literature (Table 2). Millichap at al. (1964), Uhlemann and Neims (1972) and Nakazawa et al. (1983), for instance, had all reported that the KD suppresses maximal (MES) seizures, and Appleton and DeVivo (1974) and Bough et al. (1999) had reported an elevation in the threshold for minimal seizures(Appleton, DeVivo, 1974; Bough, Eagles, 1999; Bough et al., 1999).

The reasons for the failure to find anticonvulsant effects in Experiment 1 were not immediately clear. Previous successful studies had involved the same species (Appleton, DeVivo, 1974; Hori et al., 1997), similar ages and the MCT form of the KD (Nakazawa et al., 1983). None of these factors was unique to our study.

The time spent on the diet in Experiment 1 was shorter than the duration of exposure in some previous studies, but Uhlemann and Neims had reported anticonvulsant effects in several different seizure tests following only 10 days on the diet. Millichap et al. (Millichap, Jones, 1964), in fact, had reported anticonvulsant effects after only 1 day on the diet, and Nakazawa et al. (Nakazawa et al., 1983) reported anticonvulsant effects 24 hours after an injection of MCT oil (preceded by a 24 hr fast, = 2 days of treatment). Clinically, anticonvulsant effects have been reported in children after 5 to 10 days of treatment (Huttenlocher et al., 1971; Huttenlocher, 1976). It seemed unlikely, therefore, that our failure to find anticonvulsant effects related to duration of ingestion of the KD.

Experiment 1 was unusual in that blood levels of β-hydroxybutyrate were higher than those in most previous studies - and, in some cases, higher than clinical levels. Otani et al. (Otani et al., 1984), who reported an absence of anticonvulsant effects in most standard seizure tests, also had high clinical β-hydroxybutyrate levels. This raised the
question of whether our ketone levels were too high. In two of our seizure tests (threshold MET and MMT), however, β-hydroxybutyrate levels were exactly in the clinical range (2-4 mM). Furthermore, Bough et al. (1999) had just shown anticonvulsant effects in young rats (28 days old at initiation of diet) with β-hydroxybutyrate levels in the 7 mM range (Bough et al., 1999). Therefore, it seemed unlikely that high β-hydroxybutyrate levels were causing the proconvulsant effects - and the lack of anticonvulsant effects - seen in Experiment 1.

One possible explanation for our findings - explored in Experiment 2 - was that anticonvulsant effects were present, but that they were masked by the proconvulsant effects which occurred.

Proconvulsant effects were seen in several different tests in Experiment 1. MES threshold was significantly lower in MCT KD subjects in the threshold ECS experiment, and significantly more KD subjects displayed maximal seizures in the MMT test. The time to the first MJ was also significantly shorter in the MMT test.

The finding of proconvulsant effects in Experiment 1 is in agreement with three previous reports. Mahoney et al. (Mahoney et al., 1983) in the 1980s found that the MCT KD increased the incidence and severity - and decreased the latency - of audiogenic seizures in magnesium-deficient rats. More recently, Bough et al. (Bough et al., 1998) have reported that the classic KD increases the severity of kainic-acid-induced seizures, and Matthews et al. (Matthews et al., 1999) have found an increase in MES severity in classic KD subjects.

Two of the studies reporting proconvulsant effects have involved the MCT KD (the present study and Mahoney et al. (Mahoney et al., 1983)), but one of them did not
(Bough et al., 1998). One of the studies reporting anticonvulsant effects also involved
the MCT KD, so MCT oil does not seem to be the crucial factor in determining the
appearance of proconvulsant effects of the KD.

A review of the literature, however, suggested that differences in food intake, and
therefore weight, might be important. It has been known for some time that food
restriction (less than normal food intake) - in contrast to total fasting (no food intake) -
makes rats more sensitive to maximal (Davenport, Davenport, 1948). In most of the
studies reporting anticonvulsant effects, control and KD subjects had similar weights,
which were normal for their age. In most of the studies reporting proconvulsant effects,
however, KD subjects were considerably lighter than the control subjects, suggesting
voluntary or involuntary food restriction. In Experiment 1, for instance, the MCT KD
subjects were only about half the weight of the controls, due to voluntary food restriction.
This voluntary food restriction could have been due to the high ketosis in the KD
subjects. It is known that high ketosis suppresses appetite and thirst in rats (Swink et al.,
1997). It may be that, when food restriction is present, there are proconvulsant effects
which outweigh the possible anticonvulsant effects of the KD. Experiment 2 was
designed to test this hypothesis.

An unexpected observation in Experiment 1 was that the MCT KD diet protected
subjects against death in the MMT test. This was true even though the MCT KD subjects
had stronger seizures, and significantly more maximal seizures. The actual cause of
death in the MMT test is not known, and the mechanism by which the KD could prevent
such deaths is unclear. The protective effects of the KD may be specific to the MMT test,
since no protective effect was seen in the recent kainic-acid experiments of Bough et al. (Bough et al., 1998).

7.3 EXPERIMENT 2

Experiment 2 was designed to examine the potential role of weight gain (food restriction) on seizure outcomes with the MCT KD. The weights of the subjects in Experiment 2 were regulated using special diet paradigms. When weights were equalized, anticonvulsant effects were seen in the ECS threshold seizure test. There was an increase in the minimal seizure threshold in the MCT KD Low-Weight group, as compared to the control Low-Weight group. This occurred even though β-hydroxybutyrate levels were slightly below the clinical range.

These data support our hypothesis that the anticonvulsant effects of the MCT KD would be seen if weight differences between the control and KD subjects were eliminated. Our data on KD and threshold rise are now in agreement with those of Appleton and DeVivo (1974), who found an increase in minimal seizure threshold (with electrical stimulation) in rats fed a classic KD, and Hori et al. (1997), who found an increase in kindled after-discharge threshold in rats fed a classic KD. Our results are also in general agreement with Bough et al. (1999), who reported an increase in the threshold for minimal MET infusion seizures in rats fed a classic KD. Our results are, however, contradictory to those of Uhlemann and Neims (1964), who reported no change in minimal seizure threshold (with electrical stimulation) in mice fed a high fat diet, and also to those of Millichap et al. (1964) and Otani et al. (1984) who failed to find an
increase hydration ECS threshold. Further work will be required to resolve these differences (see Proposed Experiments, below).

Although minimal seizure thresholds were significantly elevated in the MCT KD Low-Weight group, there was no significant elevation in maximal seizure thresholds. This may relate to technical considerations. The threshold determination was cruder in the “maximal” range (interval = 10 - 15 mA) than in the “threshold” range (interval = 5 - 8 mA), and subtle changes in maximal threshold may have been missed.

While anticonvulsant effects were seen in the MCT KD Low-Weight group, they were not seen in the MCT KD High-Weight group. The reason for the lack of anticonvulsant effects in the High-Weight group may relate to the low β-hydroxybutyrate levels in this group, which were only 0.8mM. Bough et al. (1999), however, did show anticonvulsant effects in some of his animals with β-hydroxybutyrate levels in the same range. This is also a question that requires further research.

Experiment 2 should be repeated with non-gavagable (Experiment 1) MCT KD (and presumably higher ketone levels) and with better discrimination in the threshold tests. This is proposed below (see Proposed Experiments, below).

7.4 EXPERIMENT 3

Experiment 3 was an attempt to replicate the KD anticonvulsant effects recently reported by Bough et al. (1999) in the MET infusion model. The classic KD was used, coupled with a diet-restriction paradigm where subjects were limited to 90% of their regular caloric requirement. In agreement with the results of Bough et al., anticonvulsant effects were seen in Experiment 3. There was an elevation in the dose of MET needed to
produce minimal seizures (FLC) in the classic KD subjects. The effects were small, but significant.

These results are similar to those in Experiment 2, where an elevation in minimal seizure threshold (with electrical stimulation) was seen. Comparing Experiments 2 and 3, it is clear that the KD can cause an elevation in the threshold for minimal seizures, and that this elevation can be achieved with different forms of the diet (MCT KD versus classic KD), strains of rat (Wistar versus Sprague-Dawley) and types of epileptogenic stimulation (electrical versus chemical).

The results of Experiment 3 are also in general agreement with the findings of Appleton and DeVivo (1974) and Hori et al. (1997), both of whom reported threshold elevations with the KD.

7.5 EXPERIMENT 4

Experiment 4 was designed to test Bough's classic KD paradigm on the four standard models used in Experiment 1. The goal of Experiment 4 was to repeat Experiment 1 with the KD paradigm shown to work in Experiment 3.

In the MES test, no significant difference was seen in the occurrence of maximal or maximal two-phase seizures. In the ECS threshold test, however, there was a significant elevation in the minimal seizure threshold and the maximal seizure threshold, and, in the MMT test, there was a significant increase in latency to the first bout of FLC, as well as to the first maximal seizure. In the MET threshold test – although there was no difference in seizure occurrence – there was also a trend (non-significant) toward an increase in latency to the first MJ and FLC. Thus, Bough's classic KD paradigm shows
anticonvulsant effects in some of the standard seizure tests, as well as in the MET infusion model.

7.6 SUMMARY: FINDINGS WITH THE CLASSIC KD

To summarize our findings in Experiments 3 and 4, the classic KD – administered according to the procedure of Bough et al. – did not significantly alter seizure occurrence in either the MES test, the threshold MET test or the MMT test. These standard models – where the stimulus is well above seizure threshold – are apparently not sensitive enough to detect the anticonvulsant effects of the KD.

In the MET infusion test and the ECS threshold test, however, there was a small, but clear, elevation in minimal seizure threshold. Increased latencies, although not significant, were also seen in the threshold MET test. (Latency is essentially a threshold measure.) Thus, the KD causes a subtle but significant increase in minimal seizure thresholds.

As mentioned above, the findings of increased minimal seizure thresholds is in agreement with several previous studies. Appleton and DeVivo (1974) reported an increase in minimal seizure threshold in the ECS threshold test and Hori et al. (1997) reported an increase in AD threshold in the kindling model. Bough et al. (1999) have recently reported an increase in the threshold for minimal seizures in the MET infusion model. Appleton and DeVivo (1974) and Hori et al. (1997) used adult rats, but Bough et al. (1999) used subjects of varying ages including some at the same age as those used in Experiment 4.

The minimal seizure test results, however, are contradictory to those of Uhlemann and Neims (1964), who reported no change in minimal seizure threshold (with electrical
stimulation) in mice fed a high fat diet. They also fail to agree with Millichap et al. (1964) and Otani et al. (1984), who reported no increase in hydration ECS threshold. Further studies will be needed to resolve these conflicts.

There was also an increase in the maximal seizure threshold in the threshold ECS test, and an increased latency to the maximal seizure in the MMS test. Thus, the KD causes a subtle, but significant, increase in maximal seizure thresholds as well.

The finding of an increase in maximal seizure thresholds is in general agreement with two past studies. Uhlemann and Neims (1972) found protection in the maximal hydration ECS and in the standard MES seizure test in infant rats fed a high-fat diet. Nakazawa et al. (1984) also found a lower percent of maximal seizures in KD fed rats.

The finding of elevated maximal seizure thresholds is, however, contradictory to studies that have reported an absence of effects, or proconvulsant effects, in the maximal seizure models. Otani et al. (1984), for instance, reported an absence of effects in the MES model in mice fed an MCT KD and Matthews et al. (1999) found proconvulsant effects in rats fed the classic KD. In both of these studies, the KD subjects were light in weight. It seems possible that the proconvulsant effects of diet restriction (low weight) may have masked the KD’s anticonvulsant effects.

7.7. SUMMARY: RESULTS WITH THE CLASSIC KD COMPARED TO RESULTS WITH THE MCT KD.

Comparing Experiments 3 and 4 to Experiments 1 and 2, it might appear that the classic KD is more effective than the MCT KD. The classic KD produced anticonvulsant effects not only in the MET infusion test, but also in several of the standard models. It must be remembered, however, that in Experiment 1 (proconvulsant effects) the MCT
KD group was much lighter than the control group. Food-restriction effects may have masked anticonvulsant effects of the MCT KD. In Experiment 2 (anticonvulsant effects only on minimal – not maximal – thresholds), β-hydroxybutyrate levels were sub-clinical, particularly in the High-Weight subjects, and threshold measures were crudely done, so, once again, anticonvulsant effects may have been missed. Experiments 1 and 2 need to be repeated with improved techniques (see Proposed Experiments).

7.7 GENERAL ISSUES

7.7.1 Has our overall goal been reached?

The ultimate goal of the present work was to develop an animal model of the KD that could be used in subsequent studies to identify the mechanism by which the KD exerts its anticonvulsant effects. Our experiments have shown that clinical β-hydroxybutyrate levels can be obtained, and also that anticonvulsant effects can be demonstrated using either the MCT KD (Experiment 2) or the classic KD (Experiments 3 and 4). Experiments related to mechanism can now proceed.

7.7.2 Are anticonvulsant effects of the KD found in minimal seizure models, maximal seizure models, or in both?

A review of the literature (Table 2) shows that anticonvulsant effects of the KD have been reported in both minimal and maximal seizure models. Anticonvulsant effects on maximal generalized seizure models have been reported by Uhlemann and Neims (1964) and Nakazawa et al. (1983), while anticonvulsant effects on minimal generalized seizure models have been reported by Uhlemann and Neims (1964), Appleton and DeVivo (1974) and Bough et al. (1999). Hori et al. (1997) have reported an elevation of
focal limbic thresholds. Thus, the findings in Experiments 2 – 4 are in general agreement with the literature. Anticonvulsant effects have been shown in a maximal generalized model (the MMT model) as well as in two minimal generalized models (ECS threshold and MET infusion).

Traditionally, minimal generalized seizures have been believed to model absence attacks in humans, while maximal generalized seizures have been believed to model tonic-clonic attacks (Lustig, Niesen, 1998). Focal limbic seizures model complex partial attacks (Albright, Burnham, 1980). Thus, the KD has a wide spectrum of effects in animal seizure models. This corresponds to its wide spectrum of effects in the clinical setting (Swink et al., 1997).

7. 7. 3 Does weight regulation play a role in the anticonvulsant effects of the KD?

In Experiment 1, proconvulsant effects were seen when weights of the control and KD subjects were significantly different. We hypothesized that anticonvulsant effects could have been present in this experiment, but that they were masked by the proconvulsant effects that resulted from the weight differences. Once weight differences were eliminated (Experiments 2 – 4), anticonvulsant effects were seen. Our data, therefore – in agreement with Davenport and Davenport (1948) – suggest that food restriction leads to proconvulsant effects.

Bough et al. (1999), however, have argued that food restriction elevates seizure thresholds. The reason for this difference in results in not clear, but it might relate to the amount of food restriction. Total fasting does raise thresholds. It may be, therefore, that severe restriction is anticonvulsant and partial restriction is proconvulsant.
It is interesting to note that in the clinical situation, children on the KD do not gain weight either, and are also food restricted. Since it is known that food restriction in rodents in proconvulsant, it might be worth checking if a similar effect is seen in children as well. The KD might work better without food restriction.

What is clear is that to obtain valid data in animal models of the KD, one must ensure that differences in weight between the control and KD subjects are absent. The limited access paradigm provides a powerful tool for accomplishing this.

7.7.4 What factors play a role in demonstrating the anticonvulsant effects of the KD?

From Experiments 2 - 4, it is clear that the strain of rats, the form of KD and the type of epileptogenic stimulus do not play an essential role in demonstrating the anticonvulsant effects of the KD. Anticonvulsant effects can be seen with both the MCT KD and the classic KD, with the Wistar strain as well as the Sprague-Dawley strain and with the ECS threshold model (electrical stimulation), the MET infusion model (chemical stimulation) and the MMT model (chemical stimulation). What is crucial, however, is that the test must pick up small changes in threshold. Cruder tests, such as the MES and the MET (standard MET) models, are unable to pick up the anticonvulsant effects of the KD, since the effects are small. It should be noted that AEDs in comparison to the KD produce a large increase in seizure threshold. Therefore the anticonvulsant effects seen with the KD are far less those seen with the conventional AEDs.

The importance of the time on the diet still needs investigation. Since only proconvulsant effects were seen in Experiment 1, anticonvulsant effect data for 10 days on the diet are not available. (Experiment 1 does demonstrate that proconvulsant effects

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can develop in 10 days.) Experiment 2 showed anticonvulsant effects, but the ECS threshold test takes several days. By its completion, the subjects had been on the MCT KD for about 20 days. Bough et al. (1999) argued that more than 20 days are required to produce an anticonvulsant effect. Clinical effects are seen in 5 – 10 days, however. This question needs further research.

7.7.5 What is (are) the best seizure model (s) to use?

Anticonvulsant effects have been shown in three different models in Experiments 2 – 4 – the ECS threshold, MET infusion and MMT models. In general, a good model to study the anticonvulsant effects of the KD should be able to identify small rises in threshold, since the KD appears to only produce a small rise in threshold. Of the models used in Experiments 2 – 4, the best model is probably the MET infusion model. This model, identifies small rises in threshold and provides a true measure of threshold. A second model, which might be used, is the ECS threshold model. The ECS threshold model is technically easier, and it provides a threshold for maximal as well as minimal seizures. In the ECS threshold model, however, it is important to use many different current levels in order to identify small changes in threshold.

The MET infusion test is probably the best, but it is technically difficult. The ECS threshold test can be used by researchers who desire an easier preparation to work with.
7.8 PROPOSED EXPERIMENTS

1. Experiment 1 – the MCT KD in 4 standard tests - should be repeated with the KD and control subjects matched for weight. Subjects should be restricted to 90 - 100% of their daily caloric requirements (as in Experiments 2 - 4) and all four seizure tests should be re-administered. This experiment will indicate whether the MCT KD is as effective as the classic KD.

2. Experiment 2 – ECS threshold in matched low or high-weight groups - should be repeated with the non-gavagable MCT KD used in Experiment 1. Higher levels of β-hydroxybutyrate will hopefully be achieved and clear anticonvulsant effects should be seen in the High-Weight KD group.

3. Experiment 3 – MET infusion - should be repeated (with the classic or MCT KD) and the duration of exposure to the diet should be varied. Time periods such as 5, 10, 20, 30, 40 days should be examined. This time-course experiment will determine how long it takes to achieve anticonvulsant effects, and whether they increase over time.

4. The effects of the MCT and classic KD’s should also be examined in other models such as those involving spontaneous seizures. One such model is the cholesterol inhibition model being studied at the Hospital for Sick Children.
5. The effect of the MCT and classic KDs in partial models - such as the kindling model - should be examined. All of the models used in Experiments 1 - 4 are generalized seizure models.

6. Finally, having evolved a viable animal model of the KD, the next step should be to attempt to identify the mechanisms by which the KDs exert their anticonvulsant effects.
REFERENCES


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