Potential Anticonvulsant Properties of the Three Major Ketone Bodies Elevated During the Ketogenic Diet

A Thesis
submitted to the Faculty of the
Department of Human Science
School of Nursing and Health Studies
of Georgetown University
in partial fulfillment of the requirements for the
degree of
Bachelor of Science
in Health Studies

By

Amy Lynn French

Washington, DC
May 1, 2006
Title: Potential anticonvulsant properties of the three major ketone bodies elevated during the ketogenic diet

Outline

I. INTRODUCTION

II. STUDIES OF THE EXOGENOUS ADMINISTRATION OF KETONE BODIES
   A. Acetone as an Anticonvulsant: Historical Studies
      i. Keith’s Pioneering Studies
      ii. Isopropyl Alcohol’s Potential Contributions to Seizure Control
   B. Recent in vivo Acetone Studies
      i. Description of Seizure Models
         1. Procedure for the Maximal Electroshock Test
         2. Procedure for the Pentylenetetrazol Seizure Test
         3. Procedure for Kindled Seizures
         4. Procedure for AY-9944 Seizure Test
      ii. Results of Likhodii and Colleagues’ 2003 Report
      iii. Additional Acetone Studies
   C. Acetoacetate
   D. β-Hydroxybutyrate

III. IN VITRO STUDIES

IV. KETOGENIC DIET STUDIES

V. CONCLUSION

VI. REFERENCES

VII. FIGURES AND CAPTIONS
I. INTRODUCTION

The ketogenic diet (KD) is a non-pharmacological therapy option typically employed by patients with resistant or refractory epilepsy. The diet, which mimics the biochemical changes of starvation, is a high-fat, low-carbohydrate regimen that has proven quite effective in decreasing seizure incidence, or in preventing seizures altogether, in modern clinical studies (Schwarzkroin PA, 1999), providing three-quarters of epileptic patients with substantial improvement (Vamecq J, et al., 2005). The KD was initially formulated and used with epilepsy patients in the early 1920s by R. M. Wilder, although similar dietary regimens have been described for the control of various seizure conditions since biblical times (Vamecq J, et al., 2005). Patients seeking to control their seizures via the KD are placed on a severely restricted dietary regimen composed of 80-90% fat intake and 10-20% combined protein and carbohydrate intake (Gasior M, et al., 2006). The high fat intake of the diet overwhelms the body’s ability to oxidize the products of fatty acid metabolism, leading to the build-up of ketone bodies (ketosis) in the blood (Gasior M, et al., 2006). In the face of the induced glucose deficit, these ketone bodies (so-called ketone bodies because they are not all true ketones), including β-hydroxybutyrate, acetoacetate, and acetone, become the brain’s main source of energy (Stafstrom CE and Bough KJ, 2002). The establishment and maintenance of ketosis appears paramount for
seizure control, and the slightest deviation from the diet can cause the loss of ketosis and subsequent breakthrough seizures (Stafstrom CE and Bough KJ, 2002). The KD places taxing requirements and restrictions on patients, and despite its effectiveness, it is often a last-ditch alternative for patients suffering from refractory epilepsy. The advent of effective antiepileptic drugs (AEDs) caused a gradual decline in the use of the KD for seizure control (Stafstrom CE, et al., 2003). Similarly, scientific interest in determining the still unknown mechanism behind the anticonvulsant efficacy of the ketogenic diet also waned.

The past fifteen years, however, witnessed renewed interest in the ketogenic diet as physicians began to once again utilize the diet as a treatment option for refractory epilepsy. Scientific interest in elucidating the anticonvulsant mechanism of the KD has yielded much new data (e.g. Rho JM, et al., 2002; Donevan SD, et al., 2003; Likhodii SS and Burnham WM, 2002; Likhodii SS, et al., 2003; Bough KJ, et al., 2002; Gasior M, et al., 2006) but has not provided conclusive evidence in favor of one particular theory. The studies cited here, and the remainder that will be examined in this review, have, in general, attempted to understand the mechanism of the KD through one of three main routes: exogenous administration of ketone bodies, implementation of the ketogenic diet in animal models, or in vitro studies of ketone bodies in brain slices. An underlying theme of these studies is to understand the potential anticonvulsant role of ketosis, the clinical hallmark of successful implementation of the KD in a patient. This review examines this one particular aspect of the KD - rising ketone body levels - and their potential role in the regimen’s anticonvulsant properties.
Scientists have long postulated that the brain’s utilization of ketones for energy is the source of the antiepileptic effects of the ketogenic diet (Schwartzkroin PA, 1999). Many studies have attempted to ascertain the role of ketones in the KD, but most of these reports provide conflicting results and conclusions. For example, establishment of ketonemia appears paramount for successful seizure control while on the ketogenic diet (Bough KJ, et al., 2000). Furthermore, acetone, one of the three major ketone bodies produced from fatty acid metabolism, has been recently shown in multiple studies to have anticonvulsant properties when administered exogenously (Likhodii SS, et al., Gasior M, et al., etc.) Other data, however, indicate that the degree of ketonemia does not correlate with the degree of seizure protection afforded by the diet (Bough KJ, et al., 2000). Thus, it appears that ketones play an important but indirect role in the seizure control provided by the KD, and that further research is needed to understand the direct cause of seizure protection afforded by the ketogenic diet. This review will examine these often divergent studies, both clinical and laboratory, in order to summarize and organize the results they present.

II. STUDIES OF THE EXOGENOUS ADMINISTRATION OF KETONE BODIES

The first set of studies analyzed in this review are those in which the investigators have attempted to determine the anticonvulsant properties of a ketone body directly, testing the ketone in animals while utilizing a specific seizure test. Ketosis is generally measured in humans using $\beta$-hydroxybutyrate (BHB) levels. This ketone body is one of the three
major products of fatty acid metabolism in the human body during the ketogenic diet; the
other two are acetone and acetoacetate (Rho JM, et al., 2002) (see Figure 1).

Several recent studies, as well as others dating to the 1930s, have sought to
examine the potential anticonvulsant properties of these three major ketone bodies by
administering them exogenously in animal seizure models. Their results will be discussed
in this section of the review.

A. Acetone as an Anticonvulsant: Historical Studies

i. Keith’s Pioneering Work

Acetone has long been thought to possess anticonvulsant properties, due mainly to the
pioneering work of H. M. Keith in the early 1930s. In his initial investigation, published
in 1930 (Keith HM, 1930), Keith attempted to examine the protectiveness of three types
of ketone bodies – acetone, ethyl acetoacetate, and diacetone alcohol – against the
convulsing agent thujone. Ethyl acetoacetate and diacetone alcohol, though not
immediate products of fatty acid metabolism, are nevertheless similar in structure to
acetone and were tested in the following experiments for that reason. All experiments
were performed in rabbits. Thujone was suspended in 6 per cent gum acacia and
administered intravenously at a pre-determined convulsing dose of 0.35 cc per kilogram
of body weight. Acetone was delivered intravenously via the ear vein, undiluted, at a
dose of 1 cc per kilogram of body weight, followed five minutes later by the thujone
injection. Of six rabbits receiving the acetone pretreatment, only one experienced
convulsions. Another group of eight rabbits received the same acetone pretreatment but
were given twice the normal dose of thujone; all animals experienced convulsions. Three animals appeared to have a degree of protection from the acetone, however, experiencing mild seizures (Keith HM, 1930).

Ethyl acetoacetate (0.4 cc/kg), similar in structure to acetone, was administered to another group of seven animals five minutes before intravenous injection of thujone. All animals were protected from seizure but exhibited signs of motor depression. Again, the experiment was repeated with five animals receiving twice the normal convulsing dose of thujone; all responded with “a typical slight attack” (Ketih HM, 1930). A final group of animals were given undiluted diacetone alcohol (1 cc/kg), which was found to be a profound depressant, causing the animals to become comatose. Five of six animals were protected from convulsions with twice the normal convulsing dose of thujone; three of six were protected from a triple dose. These protective effects, however, appear to be related to diacetone alcohol’s depressant characteristics, which are unlikely to be useful in seizure treatment.

Keith’s report was the first to demonstrate the anticonvulsant properties of ketone bodies, and to illustrate their physiological effects as they might apply to seizure control. Acetone was shown to have the fewest detrimental side effects, while the toxicity and motor depression caused by ethyl acetoacetate and diacetone alcohol were noted. Keith continued to build on his initial report, releasing two additional reports in 1931 and 1932 that also studied “the control of experimentally produced convulsions” (Keith HM, 1931).

In his 1931 report, Keith continued experiments with diacetone alcohol, determining its toxicity in both rats and rabbits. Diacetone alcohol was found to be
effective and without serious side effects when administered orally, although a large dose (0.5 cc/kg) and an extended pretreatment time (5 hours) were required to prevent seizures. He furthermore established that diacetone alcohol’s anticonvulsant effect was due to the “diacetone portion of the molecule”, as similar experiments to those described above, performed with propyl alcohol, did not produce similar results (Keith HM, 1931).

**ii. Isopropyl Alcohol’s Potential Contributions to Seizure Control**

Isopropyl alcohol, a molecule that is rapidly converted to acetone in the body, was reported to have anticonvulsant properties by Robert L. Driver in 1947. In an attempt to determine the efficacy of the ketogenic diet he sought to examine its metabolic products to determine those that possessed potentially anticonvulsant properties (Driver RL, 1947). All experiments were performed using white rats (n=265), and clonic convulsions were induced by passing a ten second electrical current “through the brains” of the animals (Driver RL, 1947). The control threshold, ranging from 6 mA to 16 mA (average of 8 mA), was established for each rat at least 4 days prior to the experiment, and indicated the electrical current needed to produce a seizure in an untreated animal. Driver found isopropyl alcohol exerted “a tremendous anticonvulsant effect without ataxia or narcosis” (Driver RL, 1947) when utilizing a 1250 mg/kg dose, improving the threshold. The drug was given orally, and exhibited toxicity similar to ethyl alcohol. Driver postulated that isopropyl alcohol’s anticonvulsant properties were due, at least in part, to its partial conversion to acetone. Driver also determined other molecules sharing a similarity in structure with acetone, including diacetone alcohol and diacetone glycol, offered varying
but definite degrees of threshold improvement. Seizure threshold is defined as the amount of convulsing agent needed to induce seizures. Therefore, drugs that elevate seizure threshold increase the quantity of agent needed to induce seizures.

Nai Chu, P. J. Hanzlik and Driver continued the study of isopropyl alcohol’s anticonvulsant properties in a thorough report published later in 1947. They examined isopropyl alcohol’s acute effects alone and together with other antiepileptic drugs, utilizing a similar seizure model as described above. Experiments were performed using rats, cats and rabbits. As demonstrated previously, isopropyl alcohol (dose of 1250 mg/kg) increased cortical threshold, which is the current at which a seizure is induced, between 50 and 65 per cent without ataxia. Further experiments examined isopropyl alcohol in combination with diphenylhydantoin, phenobarbital, and tridione; the pairings of diphenylhydantoin and phenobarbital with isopropyl alcohol each produced increases in cortical threshold equal to or above their individual increases in threshold.

Chu, et al., also confirmed 1250 mg/kg as the most effective dose of isopropyl alcohol that did not produce ataxia after trying seven doses in range of 250 to 4000 mg/kg. Higher doses produced increased cortical thresholds but also caused ataxia and motor depression. A time course of the anticonvulsant action of isopropyl alcohol was then determined. Animals (cats, rats, rabbits) were given three single doses and tested after 1, 4, 8, or 24 hours had passed. The greatest increase in cortical threshold was observed to occur after a one hour pretreatment, indicating that isopropyl alcohol is most likely a rapid-acting agent, quickly metabolized within the body (Chu N, et al., 1950).

Finally, Chu and colleagues (Chu N, et al., 1950) described the potential role of acetone in isopropyl alcohol’s apparent anticonvulsant efficacy. Isopropyl alcohol is
readily oxidized into acetone in the body, and the investigators postulated the anticonvulsant effects of isopropyl alcohol might be directly correlated with the degree of acetonemia occurring in the animal. They measured the blood acetone levels of cats (n=6) and rabbits (n=13), utilizing two different doses (500 mg/kg and 1250 mg/kg) of isopropyl alcohol, and found that, while maximal cortical depression occurred approximately one hour following isopropyl injection, acetone levels reached their peak approximately four hours following injection in both species and at both doses. The only exception was the 500 mg/kg dose in rabbits, which indicated peak acetonemia eight hours following injection. In either case, acetone levels were demonstrated to peak long after the effects of isopropyl alcohol faded, indicating a lack of correlation between peak acetone levels and seizure control. However, the authors did speculate that because acetone and isopropyl alcohol levels shared a general similarity in their metabolic paths, they could be related in a manner which could not be determined through their study.

The anticonvulsant properties of isopropyl alcohol were confirmed by the work of Ralph W. Schaffarzick in 1950. Twenty-two rats and six rabbits were given the “optimal single dose of isopropyl alcohol (1250 mg/kg)” gastrically (Schaffarzick RW, 1950). Electrical current was then passed through each animal’s brain for 2.0 seconds at 5 minute intervals until a tonic-clonic convulsive seizure was observed (Schaffarzick RW, 1950). Increases in cortical threshold closely paralleled blood levels of isopropyl alcohol, while acetone levels continued to rise long after the cortical threshold maximum was observed. These results, similar to those obtained by Chu, et al., indicate rising acetone levels are not correlated with increasing cortical threshold. Schaffarzick also performed a chronic gastric administration experiment with isopropyl alcohol, during which three
rabbits were given 1250 mg/kg isopropyl alcohol per day, split into three equal doses, for three days (nine total doses). Although acetone levels continued to rise, “the daily peaks in cortical threshold showed a diminishing magnitude and each day returned nearly to a zero value” (Schaffarzick RW, 1950).

Schaffarzick went on to determine the proportion of threshold increase attributed to acetone and isopropyl alcohol. Different groups of rats were injected intravenously with acetone (range of 30-100 ml/100ml) or isopropyl alcohol (range of 20-183 ml/100 ml) following cortical threshold determination; they were then immediately subjected to stimulation again in order to determine the new threshold. Rats injected with acetone showed variable responses; half had variable threshold increases, while half did not demonstrate any increase at all (Schaffarzick RW, 1950). Rats injected with isopropyl alcohol displayed more consistent results as higher doses of the compound were administered.

Isopropyl alcohol was then tested for its effectiveness against three chemical convulsing agents: metrazol (10%, s.c.), picrotoxin (0.3%, s.c.), and thujone (10% solution in 95% ethyl alcohol). Rats were injected one hour before testing with the convulsing agent. Results of the tests were compared against those for phenobarbital sodium and diphenylhydantoin, and both the incidence and severity of seizures were recorded. Isopropyl alcohol compared favorably with the other anticonvulsants in “preventing and ameliorating the convulsions of metrazol… [and] in antagonizing thujone” (Schaffarzick RW, 1950).

Although the reports issued from Driver, Chu and colleagues, and Schaffarzick indicate isopropyl alcohol is a potential anticonvulsant, it is not a major product of fatty
acid oxidation in the ketogenic diet. Their experimental results also show acetonemia is
not correlated with increased cortical threshold, conflicting with the results of Keith.
Several factors may have contributed to the differing results. Keith, Driver, Chu, et al.,
and Schaffarzick each utilized different convulsing agents, different dosages and different
dilutions and each had a different method of quantifying his results. Further, Keith found
treatments with isopropyl alcohol, given intravenously (0.5 cc/kg) and followed by the
“normal convulsing dose of thujone” (0.4 cc/kg), were toxic and lacked any
anticonvulsant effects (Keith HM, 1931). These results led Keith to conclude that the
anticonvulsant properties of diacetone alcohol were due to the acetone portion of the
molecule. Thus, reports of isopropyl alcohol’s potential anticonvulsant properties have
not only contradicted one another but have also provided conflicting evidence regarding
acetone’s role in seizure protection. No definite conclusions may be drawn from the
results offered by the reports described in this section. While these studies provide an
intriguing sketch of the potential relationship between acetone and isopropyl alcohol,
additional research is needed to determine if isopropyl alcohol and acetone could indeed
work in concert to provide seizure control.

B. Recent in vivo Acetone Studies

Renewed interest in the ketogenic diet during the past fifteen years has resulted in
numerous studies which have attempted to elucidate the mechanism of the diet by
examining its immediate metabolic products (e.g. Rho JM, et al., 2002; Donevan S, et al,
2003; Likhodii SS and Burnham WM, 2002; Likhodii SS, et al., 2003; Gasior M, et al.,
Acetone, as described earlier in this report, has long been thought to have a role in the anticonvulsant mechanism of the ketogenic diet. Keith demonstrated that it is an anticonvulsant in the 1930s in a thujone-induced seizure model using exogenous administration in rabbits. Recent literature appears to confirm Keith’s original research, showing that exogenous administration of acetone is an anticonvulsant in a variety of seizure models.

In 2002 Sergei S. Likhodii and W. McIntyre Burnham reported the anticonvulsant properties of two doses of acetone (1 and 10 mmol/kg) in thirty male Albino Wistar rats. They also conducted a chronic experiment in thirty other rats (fifteen control animals and fifteen experimental animals), exposing the experimental animals to acetone in their drinking water (1% v/v) for 10 days. On the day of testing, these rats were given a 1 mmol/kg injection of acetone 15 minutes before testing. Doses used in these experiments were considered to match acetone levels found in patients on the KD (Likhodii SS and Burnham WM, 2002). Both groups of rats (acute and chronic acetone exposure) were administered the pentylentetrazol (PTZ) seizure test, a subcutaneous injection of a 50 mg/kg dose of PTZ into the skin on the back of the neck, fifteen minutes after the acetone injection. The animals were then placed in a box for observation. Animals were considered protected if no whole body clonus occurred within 30 minutes of the PTZ injection (Likhodii SS and Burnham WM, 2002).

Likhodii and Burnham found a statistically-significant increase in seizure control among animals administered the 10 mmol/kg dose of acetone (six of ten animals were seizure-free), as well as those given acetone chronically, via their water supply (seven of fifteen animals protected). Animals given these pretreatments also demonstrated a
statistically-significant increase in latency to seizure. Animals receiving the 1 mmol/kg acute acetone pretreatment also showed a slight, but statistically insignificant, increase in latency and protection from seizures. These results suggest that acetone, one of the ketogenic diet’s main metabolic products, does indeed possess anticonvulsant properties in a single seizure model. However, in order to validate acetone’s role in the KD itself, acetone needed to demonstrate anticonvulsant properties in a variety of seizure models.

Likhodii, et al., in a major report published in 2003, validated acetone’s anticonvulsant abilities in a variety of seizure models. They detailed the testing of acetone in four different seizure tests: the maximal electroshock (MES) seizure test, the pentylenetetrazol (PTZ) seizure test, kindling, and the AY-9944 seizure test. A large range of acetone doses (2 – 32 mmol/kg) were utilized, and a dose-response curve was established which indicated the percentage of animals protected at each dose. Male rats were used in all experiments, and were matched for age and weight where appropriate. The age of the animal is an important factor in developing ketosis, and consequently in benefiting from KD treatment, as younger animals typically develop greater ketosis than adult animals (Bough KJ, Chen RS, and Eagles DA, 1999). Animals were matched for age in the MES seizure test, and animals of similar weight were used in the kindling and AY-9944 experiments.

\[\text{i. Description of Seizure Models}\]

This section describes the \textit{in vivo} seizure models used by Likhodii, et al. to determine the broad spectrum of acetone’s anticonvulsant properties, the results of which were
published in their 2003 report. The models are common seizure tests, frequently
described in other literature, and will be referred to at other points in this review.

1. Procedure for the Maximal Electroshock (MES) Test

Pretreatment (acetone injection) is administered approximately 30 to 32 minutes before
MES. The MES stimulus consists of a 60 Hz sine-wave current of 150 mA with a train
duration of 0.2 seconds, and is applied via corneal electrodes. Animals are exposed to the
test once, and seizures are determined to be “present” or “absent.” Seizure absence,
according to Likhodii, et al. (2003), is defined as failure to extend the hind limbs to an
angle greater than 90 degrees during the tonic period of the convulsion. To ensure the
validity of the results, rats are prescreened six days prior to testing for hindlimb extension
using the same stimulus; only rats with the desired extension are included. The detailed
description of this test is given by Likhodii, et al. in their 2003 report.

2. Procedure for the Pentylenetetrazol Seizure Test (PTZ)

Pentylenetetrazol (PTZ) solution is injected subcutaneously at the back of the neck of the
rat in a dose of 50 mg/kg. This dose, as determined in previous experiments, will produce
seizures in 90 to 99% of rats. The injection volume is equal to 7 ml/kg of bodyweight,
and is administered 15 to 17 minutes after acetone pretreatment. Seizures are scored as
“present” or “absent.” Absent indicates a lack of whole body clonus within 30 minutes of
PTZ injection. The full description of this test is given by Likhodii, et al. in their 2003 report.

3. Procedure for Kindled Seizures

Likhodii and colleagues utilized the protocol of Albright and Burnham, which is published in their 1980 report. For the kindling procedure, bipolar stimulating/recording electrodes are surgically implanted into the right basolateral amygdala of rats. Rats are allowed to recover for two weeks following surgery, at which time kindling is initiated. Stimulation is usually carried out on a daily basis. Each stimulus consists of a 1-second train of 60 Hz biphasic 1-millisecond square-wave pulses with a 400 μA peak-to-peak intensity. Electrographic activity is recorded during each trial and the convulsions are ranked according to the 5-point scale of Racine (1972). Stimulations are continued until the rats achieve a grade five convolution in ten consecutive tests.

The desired experiment is performed once rats have been appropriately kindled. Likhodii, et al. (2003), utilized kindled rats in a dose-response study in which each rat was given one of six possible doses of acetone (injected intraperitoneally). Rats were then kindled to determine if seizure threshold had been altered by the injected acetone. Tests were administered at least 48 hours apart, and each rat eventually received all six doses of acetone. The detailed description of this test is given by Likhodii, et al. in their 2003 report.

4. Procedure for the AY-9944 Seizure Test
AY-9944 is a trans-1,4-bis(2-chlorobenzylaminomethyl)-cyclohexane dihydrochloride that inhibits the biosynthesis of cholesterol. When it is given to rat pups (dose of 7.5 mg/kg, subcutaneously) from postnatal day (P) 2 until P20, the injections result in atypical absence seizures, which recur throughout the rat’s life. Two frontal and two parietal monopolar epidural electrodes are surgically implanted at P50 as described by Cortez, et al (2001). The rats are allowed to recover, and on P55, experimental studies may begin.

Likhodii, et al. (2003) used AY-9944 rats to begin dose-response studies using electrocorticography as the means of measuring changes in the rat brains. A one-hour baseline recording of each rat was conducted prior to injection of acetone or saline. A twenty-minute recording was then conducted which demonstrated the post-acetone effects ten minutes after the injection. The full description of this test is given by Likhodii, et al. in their 2003 report.

**Table 1: Seizure Models**

<table>
<thead>
<tr>
<th>Model</th>
<th>Method/Dose</th>
<th>Seizure Scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal Electroshock (MES)</td>
<td>60 Hz sine-wave current of 150 mA with a train duration of 0.2 seconds</td>
<td>Presence/absence of tonic hindlimb extension greater than 90 degrees</td>
</tr>
<tr>
<td>Pentylenetetrazol (PTZ)</td>
<td>Subcutaneous injection of 50 mg/kg; total volume is 7 ml/kg of body weight</td>
<td>Presence/absence of whole body clonus within 30 minutes of PTZ injection</td>
</tr>
<tr>
<td>Kindling</td>
<td>Repeated electrical stimulation via surgically implanted amygdala electrodes. One stimulus consists of a 1-second train of 60 Hz biphasic 1-millisecond square-wave pulses with a 400 μA peak-to-peak intensity.</td>
<td>Convulsions are analyzed according to the 5-point scale of Racine (1972): (1) immobility, eye closure, twitching of vibrissae, facial clonus (2) head nodding associated with</td>
</tr>
</tbody>
</table>
Stimulations are continued until rat achieved 10 grade 5 convulsions. more severe facial clonus (3) clonus of forelimb (4) rearing, bilateral forelimb clonus (5) rearing with loss of balance

<table>
<thead>
<tr>
<th>AY-9944</th>
<th>AY-9944 is given to rat pups from P2 – P20, subcutaneously, in a dose of 7.5 mg/kg.</th>
<th>Yields atypical absence seizures throughout the lifetime of the animal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-aminopyridine (4-AP) (to be discussed in Section II.B.iii)</td>
<td>Subcutaneous injection of 13 mmol/kg of 4-AP at the back of the neck.</td>
<td>Presence/absence of tonus within 45 minutes after injection.</td>
</tr>
</tbody>
</table>

**ii. Likhodii and Colleagues’ 2003 Acetone Anticonvulsive Findings**

Acetone demonstrated appreciable anticonvulsant effects in each of the four seizure models described above. It was most potent in the AY-9944 model, with an ED$_{50}$ of 4.0 mmol/kg. The ED$_{50}$ for the three other models ranged from 6.6 to 13.1 mmol/kg. Thus, Likhodii, et al. (2003), successfully demonstrated acetone’s ability to suppress a variety of seizure types including tonic-clonic seizures, typical and atypical absence seizures, and complex partial seizures. This discovery reveals an important correlation between the ketogenic diet and acetone: both are capable of suppressing a wide range of seizure types, including those that are likely to be refractory to antiepileptic drugs (AEDs). This may indicate that acetone does indeed play an active anticonvulsant role in the ketogenic diet. Furthermore, as acetone does not demonstrate any evidence of serious toxicity or behavioral alterations (Likhodii, et al., 2003) and is naturally present within the body (Kalapos MP, 1999), it may prove an exceptional agent for seizure protection.
**iii. Additional Recent Acetone Studies**

J. M Rho and colleagues released their findings of exogenous administration of ketone bodies in the Frings audiogenic seizure-susceptible mouse model in 2002, just prior to the publication of the initial report of Likhodii and Burnham. The Frings audiogenic seizure-susceptible mouse is a model of sensory-evoked reflex seizures, which are characterized by a sequence of wild running, loss of righting reflex, tonic flexion, and ultimately tonic extension in response to a high-intensity sound stimulus (Rho, et al., 2002). This variety of mouse was selected for this experiment because it has been successfully used in the testing of antiepileptic drugs (AEDs). One-month-old mice were injected intraperitoneally with 1-30 mmol/kg of acetone fifteen minutes prior to initiation of the seizure test. The seizure test consists of exposure to 110-dB, 11-KHz sound for a total of 20 seconds, or until a full tonic extension is achieved (Rho, et al., 2002). Rho and colleagues found that a 10 mmol/kg dose of acetone was one-hundred percent effective at blocking seizures in Frings audiogenic mice. The $ED_{50}$ of acetone 3.1 mmol/kg, with a 95 percent confidence interval of 1.8 to 4.7 mmol/kg. The experimental results obtained using the Frings audiogenic model were comparable to those of Likhodii and colleagues, who demonstrated acetone’s anticonvulsant properties in four other seizure models. The work of Rho, et al., therefore adds support to the findings of Likhodii, et al (2003). The remainder of Rho’s work will be discussed at a later point in this review.

Gasior and colleagues sought to further extend the work of Likhodii, Rho and colleagues by examining the metabolites of acetone in two seizure models. Up to two-thirds of circulating acetone is metabolized (Gasior, et al., 2006) (see Figure 1), and four
intermediate metabolites were tested in the PTZ seizure test (described previously) and 4-AP seizure test, in which male mice were given a 13 mg/kg dose of 4-aminopyridine (4-AP), injected subcutaneously at the back of the neck, following a specified pretreatment with acetone, a metabolite, or saline. 4-Aminopyridine is a powerful convulsant that induces seizures by stimulating the release of neurotransmitters (Pena F and Tapia R, 1999).

Gasior and colleagues first confirmed acetone’s anticonvulsant properties in the PTZ seizure model, simultaneously establishing a time course of acetone’s anticonvulsant activity. A pretreatment time of 30 minutes was selected because the three benchmarks of PTZ-induced seizures in mice – tail twitch, clonic seizure, and tonic seizure – could be readily observed. All agents were tested in doses ranging from 1-32 mmol/kg. The four metabolites of acetone - acetol, 1,2-propanediol, methylglyoxal, and pyruvic acid – were then tested in the PTZ model. In nearly all cases, equimolar doses of the metabolites did not share anticonvulsant effects similar to those of acetone. Higher doses of the metabolites did increase seizure threshold; however, the increase was accompanied by significant motor impairment. Only one equimolar dose of a metabolite, the 10 mmol/kg dose of methylglyoxal, produced similar increases in seizure threshold as acetone. This dose, however, was accompanied by severe respiratory distress in all mice tested, and was lethal in 20% of them. The 4-AP seizure test yielded similar results: acetone produced significant protection against tonic seizures, and the ED_{50} was determined to be 26.3 mmol/kg. None of the four metabolites tested had any effect against 4-AP induced seizures.
Gasior and colleagues’ work indicates that the metabolites of acetone do not appear to be related to its potential anticonvulsant role in the ketogenic diet. This investigation is therefore crucial in establishing that acetone is likely alone in responsibility for the anticonvulsant effect observed in the studies described above.

C. Acetoacetate

Acetoacetate is the second of the three major metabolites of the ketogenic diet that will be discussed in this review. Acetoacetate has not also been studied in many experiments involving exogenous administration. Nevertheless, the data that has been collected is summarized below in order to determine acetoacetate’s potential as an anticonvulsant.

Haddow M. Keith, the pioneer of acetone studies, studied acetoacetic acid, which is another term for acetoacetate, and sodium acetoacetate, its sodium salt, in rabbits using the thujone seizure model described previously. A dose of 1 g/kg acetoacetic acid was given intravenously to each rabbit, followed seven minutes later by a convulsing dose of thujone. Five of six animals were protected from convulsions. Furthermore, when the same experiment was repeated with sodium acetoacetate, all six animals tested were protected. Keith concludes that acetoacetic acid and sodium acetoacetate are both more effective, requiring one-fifth the amount of agent to product an equivalent effect, than either diacetone alcohol or ethyl acetoacetate, two substances whose results in the thujone-induced seizure model were described previously in this review.

Rho and colleagues, whose work with Frings audiogenic seizure-susceptible mice ascribed anticonvulsant properties to acetone, also studied acetoacetate in the same
model, the results of which are included in that same report (Rho, et al., 2002). As with acetone, a dose of 10 mmol/kg of acetoacetate was completely protective in eight mice. The ED$_{50}$ of acetoacetate was determined to be 7.3 mmol/kg, a notably higher concentration than the ED$_{50}$ determined for acetone. Nevertheless, the report does not indicate any negative physiological effects of acetoacetate.

The reports of Keith and Rho and colleagues describe the results of the small number of in vivo studies that have been performed using acetoacetate. Acetoacetate, unlike acetone, has not been demonstrated to possess anticonvulsant properties in a broad range of seizure models. Furthermore, in vitro studies that will be discussed at a later point in this paper do not support the findings of the in vivo studies discussed here.

**D. β-Hydroxybutyrate**

β-Hydroxybutyrate (BHB) is the third metabolite of the ketogenic diet. It is also the source of the most conflicting data, both in vitro and in vivo. BHB is typically the metabolite measured when determining ketosis in patients on the ketogenic diet, and many speculations have been made regarding BHB levels and their relationship to seizure control, a controversy addressed below when other studies of the ketogenic diet are examined. This section discusses the exogenous administration of BHB and its correlation with seizure control in vivo.

Again, one must return to the pioneering studies of Haddow M. Keith to begin the study of beta-hydroxybutyrate. In his report released in 1931, Keith details his experiments with beta-hydroxybutyric acid. Unlike acetone or acetoacetic acid, both of
which were tested in the same manner in a thujone-induced seizure model, Keith reported that BHB had no appreciable affect on thujone-induced seizures, with five of six rabbits having convulsions. BHB was given in the same dose as diacetone alcohol (0.5 cc/kg), and all animals were reported to show signs of irritation. Thus, Keith’s findings seem to indicate BHB does not possess any anticonvulsant properties of its own.

In 1982, Mahoney and colleagues studied beta-hydroxybutyrate in a new seizure model: magnesium-deficient rats. These rats, which are fed a magnesium-deficient diet, are prone to audiogenic seizures. The majority of this study focused on the effects of the ketogenic diet itself: groups of animals were fed different variations of the diet or were fasted in order to determine their respective efficacies against seizure induction. However, in one experiment, rats fed a specific ketogenic diet were given 1.5 ml of 1 M beta-hydroxybutyrate solution gastrically. Mahoney, et al., reported that the high-fat diets employed in their experiments actually increased seizure susceptibility in the magnesium-deficient rats, and made no mention of any changes caused by the administration of BHB. Thus, one may conclude beta-hydroxybutyrate had no effect upon improving seizure protection.

Rho and colleagues, in a 2002 study previously described, also examined the effects of exogenous BHB administration in seizure protection. Beta-hydroxybutyrate was tested in each of its forms: L-(+)-β-hydroxybutyrate, D(-)-β-hydroxybutyrate, and DL-β-hydroxybutyrate (the racemate). Doses in the range of 1 to 30 mmol/kg were tested. Each dose of each stereoisomer was injected intraperitoneally into Frings audiogenic seizure-susceptible mice. Thirty mmol/kg L-(+)-BHB was protective in seven of eight mice tested, yet the same the dose of D(-)-BHB, with an identical pretreatment
time, was not protective in any mice. Furthermore, the racemate was not protective, and all forms of BHB exhibited signs of toxicity in the animals at the 30 mmol/kg dose.

The researchers were struck by the racemate’s lack of protection, and used gas chromatography and mass spectrometry to confirm their suspicions: the L form of BHB was contaminated with dibenzylyamine (DBA), a chemical that has been “listed in the Environmental Protection Agency inventory under the Toxic Substances Control Act” (Rho, et al., 2002). DBA is a bioactive contaminant that was identified in several commercial lots of L- (+)-BHB (Donevan, et al., 2003). When tested independently of L- (+)-BHB by Rho and colleagues, it was determined that DBA possesses anticonvulsant properties, providing complete seizure protection in a range of doses from 50-200 μmol/kg. Thus, the authors demonstrate that BHB is not able to block seizures in Frings audiogenic seizure-susceptible mice.

As is the case with acetoacetate, little data exist that have examined the exogenous administration of β-hydroxybutyrate. Those that have been published (Rho JM, et al., 2002), however, seem to indicate that BHB does not possess anticonvulsant properties of its own. This result is important because, as will be discussed later in this paper, the establishment of ketosis in a patient on the KD is often verified by BHB levels found in the blood. Thus, while it is possible that BHB levels may truly indicate the successful establishment of the ketosis, and consequently seizure control, a more accurate measuring tool is likely to exist.

III. IN VITRO STUDIES
Few \textit{in vitro} studies involving acetone, β-hydroxybutyrate, or acetoacetate have been conducted, and the results they have yielded provide conflicting data as to whether or not these ketone bodies possess anticonvulsant properties. Furthermore, following an exhaustive search of the available literature, it appears that no \textit{in vitro} studies of acetone in an epilepsy model have been conducted. Thus, this particular section of the review will be rather limited due to the small number of studies which have been performed.

Sean D. Donevan and colleagues, all members of the original research team that discovered the DBA contaminant in L-(+)-BHB in 2002 (Rho JM, et al., 2002), examined acetoacetate and β-hydroxybutyrate’s effects on several neuronal membrane receptors, including NMDA, AMPA, and GABA\textsubscript{A}, in order to determine if these ketones were directly able to affect synaptic activity (Donevan S, et al., 2003). These receptors are of particular interest because they are often the target of antiepileptic drug (AED) action. The results of this probe were released in report published in 2003. This study found that only the L-isomer of BHB demonstrated any anticonvulsant properties: 30 mM of L-BHB, a non-physiologically relevant dose, was able to block 23% of currents evoked by 100 \textmu M kainate, and a range of L-BHB (300 \textmu M to 10 mM) was found to evoke a “rapid and reversible concentration-dependent block of currents activated by 10 \textmu M NMDA” (Donevan, et al., 2003). Acetoacetate did not demonstrate any anticonvulsant activity. However, the researchers once again determined that the anticonvulsant properties displayed by the L form were due to the dibenzyamine (DBA) contaminant. Identical experiments conducted with DBA revealed nearly identical concentration- and voltage-dependent blocks of NMDA receptors, enabling the investigators to conclude that BHB does not itself possess anticonvulsant properties in the \textit{in vitro} models utilized.
Liu Lin Thio and colleagues had performed a prior study, published in 2000, of the effects of acetoacetate and β-hydroxybutyrate on excitatory and inhibitory neurotransmission in the mammalian CNS. Using cultured rat hippocampal neurons as the medium for testing, the researchers studied the ketone bodies’ ability to affect changes in neurotransmission due to kainate, NMDA, AMPA, GABA, and glycine whole cell currents. No currents were significantly altered by BHB or acetoacetate, which were infused in physiologically relevant concentrations. Furthermore, studies using rat hippocampal slices to examine synaptic transmission also found no effect due to the ketone bodies. A further study of spontaneous seizure activity in the hippocampal-entorhinal cortex slice model also witnessed no changes in seizure activity due to either acetoacetate or BHB. Therefore, the work of Thio, et al., seems to correlate with that of Donevan and colleagues, indicating that the ketone bodies beta-hydroxybutyrate and acetoacetate do not appear able to alter neurotransmission \textit{in vitro} and therefore do not appear to possess intrinsic anticonvulsant properties.

In contrast, an abstract published in 1998 by Niesen and Ge indicates that both β-hydroxybutyrate and acetoacetate do possess anticonvulsant effects, based on their ability to alter recordings of two brain slice seizure models (Niesen CE and Ge S, 1998). The results of the study indicate that 1-3 mM of BHB and acetoacetate were both able to significantly reduce the size and number of spikes due to high K+ and 4-AP. High K+ (8 mM) and 4-AP (100 μM) were among the four brain slice seizure models examined in the study, all of which were performed on CA1 neurons from hippocampal slices. Spontaneous bursting was eliminated, and post-synaptic seizure-like discharges
diminished, in the two models mentioned as well as in the bicuculline (50 µM) and rapid kindling models.

Both Thio, et al., and Donevan, et al., recognize the work of Niesen and Ge, and postulate their different results may be attributed to slightly different brain slice models, recording sites, and other technical differences. A scientific explanation for the observed differences, however, had not been determined at the time of each of their respective publications. Thio, et al., and Donevan, et al., do confirm that their own work complements the others.

These three reports encompass the in vitro studies that have been published in an attempt to ascertain ketone bodies’ potential anticonvulsant properties as a part of the ketogenic diet. The results of these reports are few and conflicting, and have not provided conclusive evidence of either BHB or acetoacetate’s anticonvulsant properties. Furthermore, acetone, another of the three main metabolites of the ketogenic diet, has yet to be studied in vitro, thereby preventing the establishment of its anticonvulsant efficacy. Donevan and colleagues do note, however, that acetone’s volatility makes it difficult to perform comparative in vitro studies (Donevan, et al., 2003).

IV. KETOGENIC DIET STUDIES

Many animal and clinical studies in the past decade have analyzed the biochemical manifestations of the ketogenic diet in an attempt to learn and understand the anticonvulsant mechanism which underlies the ketogenic diet’s ability to protect patients from convulsions. The ultimate goal of many of these scientists and clinicians is to create
a pharmacological alternative to the strict regimen of the ketogenic diet, thereby allowing patients refractory to AEDs to reap the benefits of the diet without dealing with the strict compliance required by the diet. Furthermore, a pharmacological solution would likely allow many more patients to benefit, as a large percentage of patients on the KD still do not achieve seizure control.

That fact brings us to the final discussion of this review. Though many studies of the ketogenic diet in both animals (Bough KJ, Chen RS, Eagles DA, 1999; Yamada KA, Rensing N, and Thio LL, 2005; Nylen K, et al., 2005; Bough KJ, Yao SG, and Eagles DA, 2000; Bough KJ, et al 2002; Bough KJ and Eagles DA, 1999) and humans (Fuehrlein BS, et al., 2004; Musa-Veloso K, et al., 2005; Gilbert DL, Pyzik PL, and Freeman JM, 2000; Schwartz RM, Boyes S, and Aynsley-Green A, 1989; Huttenlocher, 1976; Seymour KJ, et al., 1999) have yielded much new data that is useful in the greater understanding of the diet’s properties, little of it is particularly relevant to the goal of this review, which is to determine whether or not the three major ketone bodies of the ketogenic diet possess intrinsic anticonvulsant properties. The next section highlights the most relevant controversy that has been added by nearly every KD study performed.

Most studies of the ketogenic diet have attempted to ascertain the correlation between ketosis, the amount of ketone bodies present in the blood, and seizure control. Unfortunately, no consensus has been reached. Bough and colleagues, in four studies published between 1999 and 2002, establish that, although ketosis appears vital for the anticonvulsant properties of the diet to emerge, ketonemia is not itself correlated with seizure threshold. More specifically, Bough and Eagles determined in their 1999 report that a direct correlation between ketonemia and seizure threshold does exist, but that
ketones cannot play a role in the anticonvulsant efficacy of the diet because the time courses of ketonemia and increased seizure threshold do not correlate. Ketonemia is required for seizure control but is not directly responsible for the mechanism of seizure control.

Of particular importance is the fact that beta-hydroxybutyrate is the ketone body measured when determining ketonemia in humans and animals. Recent *in vitro* and *in vivo* studies have established that this ketone body does not appear to possess intrinsic anticonvulsant properties of its own. Thus, the fact that high ketonemia levels, as measured by the BHB concentration, do not correlate with increasing seizure threshold is not surprising. In fact, these two conclusions support one another.

Nylen and colleagues further support these conclusions with a report published in 2005. They studied two different ketogenic diets in rats, each with a different ratio of fats to combined protein and carbohydrate intake, in an attempt to determine which had greater success in elevating seizure thresholds. Seizures were induced via the PTZ seizure model. Their experiments yielded mixed results. Young animals fed a 6.3:1 diet experienced significantly increased seizure thresholds, whereas other young animals fed a 4:1 diet did not. No adult animals experienced significant increases in seizure threshold on either diet. Seizures were induced using the PTZ seizure model. The investigators highlighted the fact that the animals on the 4:1 KD had higher BHB levels than those following the 6.3:1 regimen, supporting the notion that the level of ketosis, at least ketosis measured by BHB levels, is not related to seizure control.

Despite the findings of these researchers, no definitive conclusions regarding ketonemia and seizure control have been reached. BHB levels are still typically utilized
as a marker for ketosis, and ketosis is still used as an indicator for the success of a patient on the ketogenic diet. In either case, KD studies do not appear to indicate whether or not ketone bodies are the source of the anticonvulsant efficacy of the diet.

V. CONCLUSION

Despite its long tenure as a successful alternative treatment option for epilepsy, much is not understood about the ketogenic diet and its mechanism of action. The three major metabolites generated from the excessive fatty acid oxidation associated with the diet are acetone, acetoacetate, and beta-hydroxybutyrate. A review of relevant literature appears to indicate that, as hypothesized, acetoacetate and beta-hydroxybutyrate do not play a direct role in the anticonvulsant efficacy of the diet. This conclusion is supported by in vivo and in vitro studies which have indicated that these ketone bodies do not possess intrinsic anticonvulsant properties of their own.

In contrast, however, acetone has demonstrated, in several in vivo seizure models, its ability to prevent seizures, as well as to increase seizure threshold and latency to seizure. Acetone is not frequently measured in humans on the ketogenic diet, resulting in a lack of human data to correlate with that found in animal studies. However, a recent paper from Musa-Veloso and colleagues attempted to establish a connection between ketosis and the effectiveness of seizure control, using breath acetone as a marker of ketosis. The study, with only 13 enrolled children as subjects, did not yield dramatic results, but it is an important step in determining acetone’s potential anticonvulsant role in the human body (Musa-Veloso, et al., 2005). Another study performed in 1999
measured cerebral acetone levels, via magnetic resonance spectroscopy, in epileptic patients on the ketogenic diet and determined that acetone was present in all spectra of those patients, and further noted that all patients on the KD had well-controlled seizures (Seymour, et al., 1999). These two studies are important reminders of the need to continue the study of acetone’s potential role in the KD.

In conclusion, acetone alone appears to be the ketone body with the greatest potential for intrinsic anticonvulsant properties. A great deal more research, particularly in human beings, is needed to confirm and advance this theory, as it holds much promise for the development of future epilepsy treatments.
VI. REFERENCES


32. Gasior M, et al. (2006). The anticonvulsant activity of acetone, the major ketone body in the ketogenic diet, is not dependent on its metabolites acetal, 1,2-propanediol, methylglyoxal or pyruvic acid. Unpublished data.

**VII. FIGURES AND CAPTIONS**

**Figure 1 Caption** (Gasior M, et al., 2006): Metabolic pathways of production and degradation of the three ketone bodies (acetone, acetoacetate, and β-hydroxybutyrate) elevated in the KD. Fatty acids undergo a sequential removal of 2-carbon moieties in a process called β-oxidation (a). Under normal conditions, the end product of this reaction, acetyl-CoA, would be further oxidized in the tricarboxylic acid (TCA) cycle to CO₂ (not shown). Generation of large amounts of acetyl-CoA during high rates of fatty acid oxidation during the KD exceeds the metabolic capacity of the TCA cycle. As a result, acetyl-CoA is converted to acetoacetyl-CoA by thiolase (b), which if further metabolized to acetoacetate by hydroxymethylglutaryl CoA synthetase and lyase (c). Acetoacetate can be converted to β-hydroxybutyrate by β-hydroxybutyrate dehydrogenase (d) or spontaneously decarboxylated to acetone (e). Acetone is further metabolized a series of interconnected enzymatic reaction. The following enzymes are involved in acetone degradation: acetone monooxygenase (A), acetal monooxygenase (B), methylglyoxal glyoxalase I (C), glyoxalase II (D), D-2hydroxyacid dehydrogenase (E), alpha-
oxoaldehyde dehydrogenase (F), methylglyoxal reductase (G), acetol kinase (H), L-1,2-propanediol-1-P dehydrogenase (I), glycerol-1-P-phosphatase (J), alcohol dehydrogenase and lactaldehyde reductase (K), aldehyde dehydrogenase and L-lactaldehyde dehydrogenase (L), L-lactate dehydrogenase (M), not identified (N), acyl coA synthase (thiokinase) (O), pyruvate dehydrogenase complex (P). Compounds marked in bold were tested in the present study.
Figure 1: Metabolic pathway of fatty acids (Gasior M, et al., 2006)
Fatty acids $\rightarrow$ acetyl-CoA $\rightarrow$ acetoacetyl-CoA $\rightarrow$ acetoacetate $\rightarrow$ acetone $\rightarrow$ acetoacetate $\rightarrow$ \(-\text{hydroxybutyrate}\)

\text{A} \rightarrow \text{H} \rightarrow \text{I} \rightarrow \text{J} \rightarrow \text{N} \rightarrow \text{formic acid}

\text{B} \rightarrow \text{G} \rightarrow \text{L-Lactaldehyde} $\rightarrow$ L-Lactic acid $\rightarrow$ Lactic acid $\rightarrow$ oxaloacetic acid $\rightarrow$ citric acid $\rightarrow$ acetyl-CoA $\rightarrow$ glucose $\rightarrow$ phosphoenol-pyruvic acid $\rightarrow$ TCA cycle