EFFECTS OF MEDIUM CHAIN FATTY ACIDS AND KETONES ON LEUCINE METABOLISM IN ASTROCYTES: TOWARDS AN UNDERSTANDING OF THE ANTI-EPILEPTIC EFFICACY OF THE KETOGENIC DIET

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B.Sc. (Nutr. Sci.) The University of British Columbia, 1997

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In THE FACULTY OF GRADUATE STUDIES (Human Nutrition)
Department of Food, Nutrition and Health

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
2001
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A high fat, low glucose diet, termed "ketogenic" because it results in elevations in circulating ketones, has been used for over 75 years as a treatment for pediatric epilepsy. The mechanism by which the ketogenic diet suppresses epileptic seizures is not understood. Fundamentally, the diet must involve an effect on brain metabolism but there is a lack of information about the metabolic impact of a change in fuel source at the level of the brain cell. This study examined the effect of medium chain fatty acids (MCFA) and ketones on the oxidation of leucine in astrocytes.

The first series of experiments measured the production of $^{14}$CO$_2$ from [U$^{14}$C]-leucine, in the presence of no additional substrate (control) and increasing concentrations of octanoate (an MCFA) and β-hydroxybutyrate. The second series of experiments measured $^{14}$CO$_2$ production from oxidative decarboxylation of [1-$^{14}$C]-leucine and $^{14}$CO$_2$ production from the chemical decarboxylation of [1-$^{14}$C]-leucine derived α-ketoisocaproate (α-KIC) in the presence and absence of β-hydroxybutyrate and octanoate.

Inclusion of β-hydroxybutyrate caused a 60-70% reduction in $^{14}$CO$_2$ production from [U-$^{14}$C]-leucine; with octanoate the inhibition was even more dramatic with 80% reduction compared to control. Experiments using [1-$^{14}$C]-leucine did not find a statistically significant change in $^{14}$CO$_2$ production when β-hydroxybutyrate was included, but did find an increased level of labelled α-KIC in the media, reflecting leucine that had been transaminated but had not proceeded through to the second step of metabolism. The amount of residual α-KIC was increased by up to 54%. Octanoate did inhibit oxidative decarboxylation of [1-
$^{14}\text{C}$]-leucine, with 5.0 mM octanoate reducing the production of $^{14}\text{CO}_2$ by 94%. In contrast to the accumulation of $\alpha$-KIC seen in experiments using $\beta$-hydroxybutyrate, incubation with octanoate resulted in a decreased production of $\alpha$-KIC. This finding suggests that octanoate and $\beta$-hydroxybutyrate may inhibit leucine metabolism by different mechanisms.

These findings support the hypothesis that MCFAs and ketones alter leucine metabolism in astrocytes. They may have implications for the understanding of integrated fuel metabolism within the brain and for the mechanism of action of the ketogenic diet.
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</tr>
<tr>
<td>AED</td>
<td>antiepileptic drug</td>
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</tr>
<tr>
<td>BCAA</td>
<td>branched chain amino acid</td>
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<td>BCKA</td>
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<td>DMEM</td>
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<td>DPM</td>
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<td>GABA</td>
<td>γ-amino butyric acid</td>
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<tr>
<td>GLU</td>
<td>glutamate</td>
<td></td>
</tr>
<tr>
<td>GLN</td>
<td>glutamine</td>
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<tr>
<td>α-KG</td>
<td>α-ketoglutarate</td>
<td></td>
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<tr>
<td>α-KIC</td>
<td>α-ketoisocaproylate</td>
<td></td>
</tr>
<tr>
<td>LCFA</td>
<td>long chain fatty acid</td>
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<tr>
<td>LEU</td>
<td>leucine</td>
<td></td>
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<tr>
<td>MCFA</td>
<td>medium chain fatty acid</td>
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</tr>
<tr>
<td>MCT</td>
<td>medium chain triacylglycerol</td>
<td></td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
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<tr>
<td>TPP</td>
<td>thiamin pyrophosphate</td>
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This thesis is dedicated to the memory of Marc Lafreniere. His love and support throughout our twelve years of friendship, and his unfailing belief in me have been critical to my success. Thank you Marc.
1 Introduction

Epilepsy is a condition characterized by recurrent brain seizures that occurs primarily in children. How and why seizures are produced is not well understood, but evidence suggests that high levels of glutamate, the major excitatory neurotransmitter, may be involved. The ketogenic diet is a high fat, low glucose diet that has been used as a treatment for pediatric epilepsy for over 75 years (Swink et al. 1997). The mechanism by which the ketogenic diet suppresses epileptic seizures is not known.

Ketogenic diets result in elevated levels of circulating ketones. When medium chain triacylglycerols (MCT) are used as the dietary fat, elevated circulating concentrations of both ketones and medium chain fatty acids (MCFAs) develop. Ketones, and perhaps MCFAs, pass from the cerebral capillaries into astrocytes where they become the major fuel source when glucose is limited (Auestad et al. 1991). It is possible that the change in primary fuel substrate in the astrocyte produces subsequent changes in brain metabolism, which ultimately lead to a reduction in seizures.

Changes in the metabolism of the excitatory neurotransmitter glutamate, occurring in response to a high fat, low glucose diet, could be responsible for the antiepileptic efficacy of the ketogenic diet. Brain glutamate uptake is negligible (Grill et al. 1992) and studies indicate that the branched chain amino acid leucine is an important source of brain glutamate nitrogen. Yudkoff et al. (1994a) demonstrated that up to 30% of astrocytic glutamate nitrogen is derived from leucine; suggesting that changes to leucine metabolism will have important consequences for the levels of this excitatory neurotransmitter.

There is very little information on integrated fuel metabolism in astrocytes in the literature. Ketone bodies have been shown to influence some aspects of amino acid
metabolism in astrocytes (Yudkoff et al. 1997), but the impact of different fuel substrates, such as fatty acids and ketones, on the metabolism of leucine in astrocytes has not been reported.

Animal studies have demonstrated that metabolic consequences of a ketogenic diet include elevated levels of acetyl CoA and a high ATP: ADP ratio (De Vivo et al., 1978). High levels of ATP are known to inhibit some enzymatic reactions including the rate-limiting step of leucine oxidation, which is catalyzed by branched chain ketoacid dehydrogenase (Lehninger et al., 1993). Increased acetyl CoA from fatty acid and ketone oxidation would also be expected to inhibit this step in leucine metabolism (Lehninger et al., 1993). Inhibition of branched chain ketoacid dehydrogenase should cause an accumulation of α-ketoisocaprate (α-KIC), the cognate keto-acid of leucine. Increased levels of α-KIC are known to cause a reversal of the reaction catalyzed by branched chain amino acid transaminase, with the transfer of an amino group from glutamate to α-KIC resulting in the formation of leucine and α-ketoglutarate (α-KG) (Yudkoff et al. 1994a). Ultimately, a reversal of the transaminase reaction results in decreased astrocytic glutamate and glutamine (Yudkoff et al. 1994a and Yudkoff et al. 1996b).

This study examines the hypothesis that fatty acid and ketone metabolism will inhibit complete oxidation of leucine in astrocytes by interfering with the rate-limiting step of leucine catabolism, catalyzed by branched chain ketoacid dehydrogenase.
2 LITERATURE REVIEW

2.1 Pediatric Epilepsy

Epilepsy is a condition characterized by recurrent brain seizures, which usually develops in childhood. Seizures are the result of abnormal and excessive discharging of the neurons and can be accompanied by alterations in sensation, behavior or consciousness (Freeman, 1995). Seizures can be classified into five major types: absence, myoclonic, generalized tonic-clonic, partial onset, and others. Childhood seizures can be precipitated by a neurological insult, such as infection or trauma, but in most cases they are idiopathic. Almost 1% of all children will develop epilepsy by the age of fifteen (Annegars, 1993). Children are believed to be more susceptible to seizures than adults because developing neurons tend to be more excitable (Johnston, 1996).

The mechanisms by which seizures are produced in the epileptic brain are not fully understood. The excitability of neurons may be related to the level of glutamate, the major excitatory neurotransmitter. The epileptic brain appears to have increased levels of this amino acid, which could be due to increased production, decreased catabolism, or both. Antiepileptic drugs often act at the level of glutamate by competing with it for its receptor or by mimicking the effects of the inhibitory counterpart γ-aminobutyric acid (GABA) (Chapman, 2000).

The word epilepsy is derived from the Latin word epilepsius which literally means “a taking hold” (Shafer and Salmanson 1997). This implies that the person affected by epilepsy is overwhelmed by a “mysterious, supernatural power” (Shafer and Salmanson, 1997). This literal meaning is embedded in the stigma faced throughout history by those affected with this disease. The impact of epilepsy on the child and
family can be severe and is related to the physical effects of seizures, treatment-related effects, and social implications. The standard mortality rate for people with epilepsy is two-four times higher than normal, with causes of mortality including the direct effects of seizures and status epilepticus, accidents occurring during a seizure, and suicide (Guberman and Bruni, 1999). Some additional risks associated with epilepsy are lower I.Q., learning disabilities, and mental retardation. Behavioral problems, such as anxiety and aggression are also common in epileptic children. Cognitive and behavioral problems are partially due to underlying pathology in the central nervous system, but are exacerbated by the effects of recurrent seizures (Prasad et al., 1996). Antiepileptic drugs may also contribute to cognitive and behavioral problems, such as an inability to concentrate (Dodson, 1993).

Conventional treatments for epilepsy include a variety of antiepileptic drugs (AEDs), and surgery. Despite the continued development of new drugs, it has been estimated that from 20-30% of children have seizures that are not fully responsive to any of the available AEDs, or experience intolerable side effects from drug therapy (Wheless, 1995). Alternative treatments for epilepsy include the ketogenic diet, immunoglobulins and steroids. Of these options, the ketogenic diet is the only one with proven efficacy for the treatment of pediatric epilepsy (Prasad et al. 1996).

2.2 Diet Therapy for the Treatment of Pediatric Epilepsy

Dietary interventions such as fasting were recommended for the control of seizures as far back in time as Hippocrates and Galen (Prasad et al., 1996). Various anecdotal reports throughout history have supported the notion that fasting inhibits epileptic seizures. The impracticality of using fasting as a long-term treatment for epilepsy led
physicians to try to understand how food restriction could inhibit seizures. In the early part of this century Geyelin (1921) suggested that beneficial effects of fasting might be related to the resulting acidosis. In the same year, Wilder (1921) noticed that fasting led to an increased level of circulating ketones (acetoacetate and \( \beta \)-hydroxybutyrate) and speculated that they might somehow be related to the seizure control. Both of these physicians were clever enough to realize that a diet high in fat and low in carbohydrate would provoke a physiological response similar to that induced by complete fasting. The classic ketogenic diet was thus designed to produce ketosis and acidosis, thereby mimicking the effects of fasting, while still providing adequate protein and calories for growth. It did so by providing a ratio of 3:1 or 4:1 fat to carbohydrate plus protein in the diet. In the period following the introduction of the ketogenic diet, and prior to the development of pharmacological therapies, many physicians used this diet with a high degree of success (Peterman, 1925; Helmholz, 1927).

The classic ketogenic diet is composed mainly of dairy fats, while carbohydrate is severely restricted and only the minimum amount of protein needed to support growth is included (Kinsman et al., 1992). Dairy fats contain some short and MCFAs, and large amounts of long chain saturated fatty acids (Table 2.1). Fatty acids are hydrocarbon chains varying in length from 4-36 carbons with a carboxyl group (COOH) at one end and a methyl group (\( \text{CH}_3 \)) at the other. Long chain fatty acids (LCFAs) have greater than twelve carbons and are insoluble in water. As a result of their insolubility, LCFAs must be transported through the circulation in lipoproteins, complexes in which triacylglycerols form an internal core that is solubilized by phospholipids and specific apoproteins. MCFAs have between six and twelve carbons. MCFAs have unique properties related to their short chain length and resultant water solubility. Their
digestion is simpler than for LCFAs because they do not require the complicated system to transport the water insoluble LCFAs. Unlike LCFAs, they easily pass through cell membranes and enter the mitochondria independent of the carnitine acyl transferase system (Bach and Babayan, 1982). It is also possible that, unlike LCFAs, MCFAs cross the blood brain barrier and enter astrocytes.

When it was discovered that a diet high in MCTs resulted in a greater elevation of plasma ketones than dairy fats, a MCT version of the diet was developed (Huttenlocher, 1976). The MCT diet provides fat as MCT oil (Table 2.2), composed of triacylglycerols containing mainly the MCFAs octanoate (8:0) and decanoate (10:0). Because these MCTs are more ketogenic than dairy fats, a higher amount of carbohydrate can be incorporated into the diet, thereby improving its palatability and acceptability. The success rates of the classic and MCT diets in controlling seizures, however, have been reported to be very similar (Schwartz et al., 1989).

Use of the ketogenic diet decreased following the development of anti-seizure medications such as phenytoin in the late 1930's. Research related to the diet, which had focused on discovering its mechanism of action also declined. Since that time, many advances have been made in the pharmacological and surgical treatment of epilepsy, but there are still a significant number of children who cannot be helped by these therapies. Between 25-30% of people with epilepsy in the United States have seizures that are unresponsive to pharmacological therapies and only a small proportion of patients with epilepsy are suitable for surgery (So, 1993; Huttenlocher and Hapke 1990). In addition, antiepileptic drugs (AEDs) may cause severe side effects such as drowsiness, headaches, cognitive impairment, ataxia and depression.
(Guberman and Bruni, 1999) that make these drugs intolerable to the child or their family.

Table 2.1: Fatty acid composition of bovine milk

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Weight %</th>
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<tr>
<td>4:0</td>
<td>3.32</td>
</tr>
<tr>
<td>6:0</td>
<td>2.34</td>
</tr>
<tr>
<td>Σ C≤6</td>
<td>5.66</td>
</tr>
<tr>
<td>* 8:0</td>
<td>1.19</td>
</tr>
<tr>
<td>* 10:0</td>
<td>2.81</td>
</tr>
<tr>
<td>12:0</td>
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<tr>
<td>14:0</td>
<td>11.41</td>
</tr>
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<td>14:1</td>
<td>2.63</td>
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<td>18:2</td>
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<tr>
<td>Σ C≥12</td>
<td>90.35</td>
</tr>
</tbody>
</table>

1 Adapted from Jensen and Newburg (1995)
Σ = sum of fatty acids of carbon chain length (C)
* Compare to Table 2.2

Table 2.2: Fatty acid distribution of medium chain triacylglycerol oil

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>% by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Σ ≤ C6</td>
<td>&lt; 6</td>
</tr>
<tr>
<td>8:0</td>
<td>60-80</td>
</tr>
<tr>
<td>10:0</td>
<td>18-32</td>
</tr>
<tr>
<td>Σ ≥C12</td>
<td>&lt; 4</td>
</tr>
</tbody>
</table>

1 As manufactured by Mead Johnson (Belleville, Ontario)
Σ, sum of fatty acids of carbon chain length (C)
Recently, there has been a resurgence of interest in the ketogenic diet, partly because of increased media attention to this form of treatment, and partly due to the increasing popularity of alternative medical therapies (Wheless, 1995). Despite the interest, the increasing clinical use of the ketogenic diet, and its long history of effectiveness, surprisingly little is known about how it works, or when it should be implemented. This is understandable considering the fact that research into the mechanism of action of the ketogenic diet was virtually non-existent between 1966 and 1990 (Stafstrom, 1999). It is clear that ketogenic diets provide an important treatment alternative in cases where epilepsy does not respond to AEDs, or when the side effects of AEDs are intolerable. Use of the ketogenic diet in less severe situations has yet to be evaluated. Further research is warranted to clarify the role of the ketogenic diet in the management of pediatric epilepsy.

### 2.3 Advantages and Disadvantages of the Ketogenic Diet

The ketogenic diet is thought to be beneficial for between 1/3 and 2/3 of children with intractable epilepsy. In 1972, Livingston reported that 52% of 1001 patients who were treated with the ketogenic diet had their seizures completely controlled, and 27% had a significant improvement in seizure frequency (Livingston, 1972). In December of 1998, researchers at the Johns Hopkins Medical Institute reported results of a prospective study of 150 children with intractable epilepsy (Freeman et al., 1998). The children were treated with a ketogenic diet and seizure frequency was measured. A significant number of children were able to successfully follow the ketogenic diet for a full year and 27% of these had at least a 90% reduction in seizure frequency. These
children had previously tried an average of six AEDs without success. Despite the prevailing opinion that the ketogenic diet should only be used as a last resort, it actually has a higher rate of success than many of the available drug therapies. An AED is considered effective if it reduces seizures by 50% in half of the recipients; studies of the recently developed AEDs indicate that none are able to achieve this level of seizure control (Chadwick, 1997; Faught, 1997). In the prospective study at Johns Hopkins, 75 of the children (50%) maintained a 50% reduction in their seizures after one year, and 41 of these (27%) achieved better than 90% reduction in seizures (Freeman et al., 1998). Recently, a systematic review of all published results regarding the efficacy of the ketogenic diet for the treatment of pediatric epilepsy was conducted (Leferre and Aronson, 2000). This analysis showed that the ketogenic diet results in complete cessation of seizures in 16% of cases, more than 90% reduction in seizure frequency in 32% of cases and more than 50% reduction in seizure frequency in 56% of cases. The authors concluded that despite the absence of controlled trials, the evidence to date strongly supports the efficacy of the ketogenic diet for intractable pediatric epilepsy.

If the ketogenic diet is so effective in inhibiting seizures, why is it not more widely used? Many textbooks on epilepsy do not even mention the ketogenic diet, and when it is mentioned its value is generally minimized. One reason for the hesitation to recognize it as a valid form of treatment could be the lack of knowledge about its mechanism of action. It must be noted, however, that very little is known about how some AEDs prevent seizures as well. Another reason may be that the diet has a reputation for being unpalatable and difficult to prepare. It is an extremely restrictive diet and can be difficult for parents or caregivers to manage. With proper support and training this difficulty can be minimized (Freeman et al., 1994) and some parents appreciate the opportunity to be
more involved in the management of their child's illness (Wheless, 1995). Freeman et al. (1998) found that effectiveness was the most important factor determining whether a child would remain on the diet. In their study, the probability of children remaining on the diet after one year was 80% for those whose seizures were reduced by more than 50%. Children who discontinued the diet did so not because it was unpalatable or difficult to prepare, but rather because it was not working.

As with any therapy, the ketogenic diet does have potential side effects that may limit its tolerability. Short-term complications, normally surfacing within a month of diet initiation may include dehydration, hypoglycemia, diarrhea, vomiting and refusal to eat (Freeman and Vining, 1994). Long-term complications may include urolithiasis, elevated cholesterol, irritability and metabolic disturbances such as acidosis (Vining et al., 1996; Schwartz et al, 1989; Herzberg et al., 1990). Vitamin supplements are required to prevent deficiencies. There have been reports of subjective improvements in the cognition and behavior of children on ketogenic diets, some of which may be due to a reduction in their AEDs (Nigro et al., 1995). Research is needed to properly clarify the effects of the diet on cognition and behavior (Prasad et al., 1996).

2.4 Potential Mechanisms of Action of the Ketogenic Diet

There is a serious lack of information about how the ketogenic diet works. Early hypotheses suggested effects related to ketosis, acidosis, hydration, elevation of serum lipids and electrolyte imbalance. Schwartzkroin (1999) recently summarized updated hypotheses including effects on the nature of brain metabolism, decreased excitability due to alterations in cell properties, effects on neurotransmitters and synaptic transmission, impact on neuromodulating "circulating factors" such as insulin, and
changes to the extracellular milieu. Whatever the exact mechanism, it is clear that the ketogenic diet must ultimately impact on brain neurotransmitter metabolism. It is known that when there is a shortage of glucose, fatty acids are rapidly oxidized in the liver and high levels of acetyl CoA are generated (Owen et al., 1967). Rapid production of acetyl CoA results in acetyl-CoA concentrations that exceed the capacity of the TCA cycle (Figure 2.1).

Figure 2.1 Hepatic ketone production due to increased fatty acid oxidation
As a result, acetyl-CoA condenses to form the ketone bodies, acetoacetate and β-hydroxybutyrate, which are released from the liver. During high fat diets, plasma ketone levels rise dramatically. During starvation, the decrease in plasma insulin and increase in glucagon results in the mobilization of adipose tissue fatty acids. The fatty acids are taken up and rapidly oxidized in the liver with the generation of ketones. It is well known that the brain is able to utilize ketones for energy and does so preferentially when glucose is in short supply (Auestad et al., 1991; Bixel and Hamprecht, 1995). In addition, the capacity to extract ketones from the cerebral capillaries and use them as a fuel source is particularly high in the young brain (Nehlig, 1999). The antiepileptic effect of the ketogenic diet is believed to be related to the switch from glucose to ketones and fatty acids as the primary energy fuel (Nordli and De Vivo, 1997 and Prasad et al., 1996).

One of the most popular theories predicts that a direct or indirect effect of the ketones, acetoacetate and β-hydroxybutyrate, is responsible for the anti-convulsant action of the ketogenic diet (Huttenlocher, 1976). Others have suggested that increased plasma levels of the MCFAs octanoate and decanoate may be directly involved (Sills et al., 1986a). Results of several studies have indicated that seizure control is not necessarily correlated with plasma ketone or MCFA concentrations (Schwartz et al., 1989; Sills et al., 1986a). It must be acknowledged that the very short half-life of these substances makes it difficult to accurately measure them, and plasma concentrations are not necessarily indicative of turnover. Further, the concentrations of ketones and MCFAs in the peripheral circulation may not reflect their levels in the central nervous
system. A recent study by Bough et al. (1999) demonstrated seizure control in calorie restricted, but non-ketotic rats, implying that ketosis is not necessary for seizure control.

Early animal studies showed that ingestion of a ketogenic diet resulted in elevated blood ketone levels and increased resistance to induced seizures (Uhlemann and Neims, 1972; Appleton and De Vivo, 1974). Using an animal model of the ketogenic diet, De Vivo et al. (1978) showed that rats fed high fat diets had an increased threshold for electroconvulsive shock. Their results indicated that something about the change to using fat as the major fuel source conferred upon the rats an increased resistance to seizures. They hypothesized that the increased ATP: ADP ratio, associated with chronic ketosis improves neuronal stability, thereby preventing seizures. Subsequent animal studies have confirmed that the ketogenic diet raises the seizure threshold (Nakazawa et al., 1983) and have demonstrated the effect to be particularly robust in young animals (Uhlemann and Neims, 1972; Bough et al, 1999).

2.5 The Excitatory Neurotransmitter System in Pediatric Epilepsy:

Most seizure problems begin in childhood and a greater variety of seizure types are seen in children, than in adults (Johnston, 1996). This raises the question of what it is about the immature brain that makes it particularly susceptible to seizures. Experimental evidence suggests that the development of childhood epilepsy may be related to unique aspects of the excitatory neurotransmitter system during brain development. Glutamate is the major excitatory neurotransmitter in the mammalian brain (Ericinska and Silver, 1990). There are two types of receptors for glutamate, the metabotropic receptors, which are linked to second messenger systems, and ionotropic receptors which are associated with ion channels (Johnston, 1996). Enhanced activity
of both types of glutamate receptors has been demonstrated in the developing brain as compared to the adult brain (Blue and Johnston, 1995; Nicoletti et al., 1986). For instance, the N-methyl-D-aspartate (NMDA) receptor is more responsive to glutamate during the post-natal period than it is later in life. It has also been shown that glutamate binding sites on the receptors are more numerous and they are less easily inhibited in young animals (McDonald and Johnston, 1990; Tremblay et al., 1988). This enhanced activity of glutamate receptors in the young brain may explain why epilepsy is most common in childhood. It may also provide clues as to why some types of therapies, including the ketogenic diet, are more effective in children than in adults. As discussed previously, animal studies have demonstrated a higher degree of seizure protection and higher blood ketone levels in young animals ingesting a ketogenic diet than in older animals (Uhlemann and Neims, 1972 and Bough et al. 1999).

Evidence to support the theory that epileptic seizures are related to the excitatory neurotransmitter system has been found in both animal and human studies. Experiments using young rats have demonstrated that seizures and excitotoxic brain injury can be induced by injecting analogues of glutamate (McDonald et al., 1992). Similarly, overstimulation of glutamate receptors has been shown to produce seizures and even to lead to long-term changes that resemble those seen in chronic epilepsy. Significantly elevated concentrations of glutamate have been found in brain tissue removed from humans during surgical treatment of epilepsy (Sherwin et al., 1988). Measurement of brain tissue removed from children with epilepsy revealed spontaneous bursts of electrical activity (Wuarin et al., 1990; Avoli and Olivier 1987). These spontaneous bursts of activity could be inhibited by using a competitive inhibitor of the NMDA glutamate receptor (Wuarin et al., 1990). Pharmacological therapies have
been developed based on the results of these and other experiments. The cumulative evidence suggests that the excitatory neurotransmitter system, and in particular glutamate, is involved in the production of seizures. Because it is known that the excitatory neurotransmitter system is more active in children and experience has shown that children respond best to a ketogenic diet, we can hypothesize that the ketogenic diet is acting at the level of glutamate metabolism.

2.6 Brain Metabolism and the Ketogenic Diet

The following summary of the metabolic consequences of a diet high in fat and low in carbohydrate and protein provides the necessary background to support a hypothesis that the ketogenic diet influences brain glutamate metabolism. During consumption of a high fat diet, there is a high level of oxidation of the dietary fatty acids in the liver. Rapid oxidation of fatty acids results in the generation of acetyl-CoA in amounts that exceed the capacity of the TCA cycle for metabolism (Figure 2.1). When this occurs, the excess acetyl CoA condenses to form the ketone bodies, acetoacetate and β-hydroxybutyrate, which are released from liver resulting in a rise in plasma ketones. In the case of the MCT diet, serum octanoate and decanoate levels also rise (Sills et al., 1986b), reflecting at least in part the transport of these MCFAs from the gastrointestinal tract to the liver via the portal vein. In the liver, MCFAs have multiple fates. They can be oxidized to acetyl-CoA which is converted to ketones, used for de novo synthesis of longer chain fatty acids, or oxidized in the TCA cycle to CO₂. Some unesterified MCFAs bypass the liver and consequently a significant amount of octanoic and decanoic acid is present in the peripheral blood, and potentially available for uptake by the brain (Fernando-Warnakulasuriya et al., 1981).
Energy substrates, including ketones and perhaps MCFAs, enter the brain by passing from the cerebral capillaries to the astrocytes. Astrocytes are star-shaped cells positioned between the cerebral capillaries and the neurons. They can be thought of as processing plants, which take up a variety of nutrients from the blood and convert them to substrates that can be used by neurons (Bixel and Hamprecht, 1995). Some metabolic steps such as the conversion of glutamate to glutamine, are located exclusively in astrocytes (Norenberg, 1979). This division of metabolic processes is referred to as compartmentation and it means that diet-induced changes in the uptake and metabolism of nutrient substrates by astrocytes can have subsequent effects on neuronal metabolism (Daikhin and Yudkoff, 2000). In the case of the ketogenic diet, the nutrients being taken up by the astrocytes are primarily ketone bodies and potentially MCFAs. Astrocytes in culture can oxidize the MCFAs with the generation of ketones that are exported to the neurons (Auestad et al., 1991). The ability of astrocytes in culture to oxidize octanoate is unique among the cells of the brain (Edmond et al., 1987). Within astrocytes, ketone bodies can be reconverted to acetyl CoA and enter the TCA cycle for the production of energy. Alternatively, ketones can be exported to meet neuronal energy needs. The most important metabolic result of the switch to fats from glucose as the primary fuel source is that rapid oxidation of fat produces large amounts of acetyl CoA and ketones. The impact of the increased availability of acetyl CoA and ketones on the metabolism of other fuels, particularly amino acids, has not been fully established.

Animal studies have provided some insight into the metabolic consequences of ingesting a ketogenic diet. De Vivo et al. (1978) fed rats a high fat, low glucose diet and measured levels of cerebral metabolites. Chronically ketotic rats had increased cerebral
levels of glucose-6-phosphate, lactate, pyruvate, β-hydroxybutyrate, α-ketoglutarate, alanine and citrate, and decreased levels of fructose 1,6-diphosphate and aspartate. Cerebral energy reserves were significantly higher in the ketotic rats, as reflected by a high ATP:ADP ratio. It was suggested that the high levels of ATP inhibited key enzymes, including pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase with subsequent accumulation of pyruvate and α-ketoglutarate. Higher levels of lactate probably reflected a diversion of pyruvate through lactate dehydrogenase, explained by the inability of pyruvate to proceed to acetyl CoA and enter the TCA cycle. Increased levels of pyruvate can be expected to shift the following reaction to the right: pyruvate + glutamate → α-KG + alanine, consistent with the elevated levels of alanine and α-KG. Their results indicated that glycolysis was inhibited and normal activity of the TCA cycle was maintained. The most significant finding was increased cerebral energy reserves in the ketotic rats, as reflected by a high ATP: ADP ratio. It was suggested that the high levels of ATP inhibited key enzymes, leading to significant alterations in the levels of TCA cycle intermediates (De Vivo et al., 1978).

The metabolic changes observed in the study by De Vivo et al. (1978) reflect biochemical changes in brain that result from a diet with fat as the major fuel source. The increased amounts of acetyl CoA from oxidation of ketone bodies in the brain inhibited glycolysis, and changes in the concentrations of TCA cycle intermediates were the result of the increased ATP: ADP ratio. The results of this study support the hypothesis that utilization of ketones as an energy substrate alters brain intermediary metabolism. However, because these studies involved analysis of whole brain, no information on the metabolic changes at the level of the astrocyte is provided.
Research into the metabolic consequences of a ketogenic diet, which incorporate current knowledge of the interactions between astrocytes and neurons is needed.

Yudkoff et al. (1997) have demonstrated that ketone bodies can influence amino acid metabolism in cultured astrocytes. Astrocytic transamination of glutamate to aspartate was inhibited, glutamine levels decreased and citrate concentrations increased in response to 5 mM acetoacetate or β-hydroxybutyrate (Yudkoff et al., 1997). The latter findings provide support for the hypothesis that the oxidation of ketones ultimately affects neurotransmitter levels via alterations in cell metabolism. However, Yudkoff et al. (1997) used a single concentration of ketone bodies (5.0 mM) likely to be several fold higher than the physiological concentration achieved in the brain, even during ketogenic diet therapy. No information on the possible effects of fatty acids, including MCFAs and LCFAs, on the oxidation of amino acids in astrocytes has been published.

The relationship between glutamate and the branched chain amino acid leucine is extremely important in brain metabolism and does not appear to have been considered in regards to the metabolic effect of the ketogenic diet. The following section describes the interaction between the metabolism of leucine and glutamate. It provides the background needed to rationalize the need for research to determine the impact of different fuel substrates, such as ketones and MCFAs on leucine metabolism in the brain.

2.7 The Relationship of Leucine and Glutamate Metabolism in the Brain

As discussed previously, glutamate is the major excitatory neurotransmitter in the brain. Glutamate in the brain has one of the following fates, incorporation into
protein, oxidation or use as a neurotransmitter. The brain derives very little, if any glutamate or glutamine directly from the bloodstream, in fact a net efflux of glutamine from astrocytes to the cerebral capillaries has been demonstrated (Grill et al., 1992 Smith et al., 1987). This is believed to be a protective mechanism designed to keep intracellular glutamate concentrations low, thereby preventing cellular damage and facilitating synaptic transmission (Huang et al., 1997). Glutamine, synthesized in the astrocytes from glutamate by the action of glutamine synthetase, is released to the neurons where it serves as the precursor to the neurotransmitter glutamate (Figure 2.2). Following neurotransmission, glutamate is taken up by the astrocytes again, completing the “glutamine-glutamate cycle” (Martinez-Hernandez et al., 1977). Because glutamate transport into the brain is negligible, a means of replenishing glutamate lost to oxidation and protein synthesis is required.
Figure 2.2 Schematic Representation of the Glutamate-Glutamine Cycle
Yudkoff et al. (1990) have demonstrated that a significant proportion of the glutamate in neurons is the result of transamination of leucine and the other branched chain amino acids, isoleucine and valine within astrocytes. In the brain, glutamate carbon is derived from glucose, and nitrogen is primarily derived from the branched chain amino acids (Daikhin and Yudkoff, 2000). Leucine uptake from the cerebral capillaries into astrocytes exceeds that rate of uptake of all other amino acids (Smith et al. 1987). The metabolism of leucine results in the formation of glutamate, and ketones which are then oxidized in the TCA cycle (Figure 2.3). Yudkoff et al. (1994a) estimated that 25-30% of astrocytic glutamate/glutamine nitrogen is derived from leucine alone. In these experiments, other amino acids that could have provided nitrogen for glutamate synthesis were available to the astrocytes, demonstrating that the cells used leucine preferentially. This suggests that alterations in the metabolism of leucine will have important consequences for the levels of glutamate/glutamine in brain.

The metabolism of leucine occurs in three steps (Figure 2.3). The first step is transamination with \( \alpha \)-ketoglutarate (\( \alpha \)-KG) to yield glutamate and \( \alpha \)-ketoisocaproic acid (\( \alpha \)-KIC). This transamination is reversible but under normal conditions the production of glutamate is favored. The second step involves the oxidative decarboxylation of \( \alpha \)-KIC to isovaleryl CoA. This step, catalyzed by the multienzyme complex "branched chain ketoacid dehydrogenase" (BCKA dehydrogenase), is the rate-limiting step in leucine oxidation and is irreversible. The final phase of leucine metabolism involves a series of reactions that ultimately result in the production of the ketone acetoacetate. The acetoacetate is then converted to acetyl CoA which can enter the TCA cycle, with complete oxidation of leucine resulting in the production of \( \text{CO}_2 \).
Figure 2.3 Schematic representation of leucine metabolism
The direction of the transamination reaction between leucine and $\alpha$-KG is controlled by the levels of the substrates and the energy needs of the cell (Lehninger et al., 1993). In the reverse direction ($\alpha$-KIC + Glutamate $\rightarrow \alpha$-KG + Leucine) glutamate is consumed, raising the possibility that a reversal of this reaction may be an important way of modulating the level of glutamate in the brain (Yudkoff, 1997). It is possible that the changes in brain metabolism, occurring in response to the ketogenic diet, affect one or more of the steps in leucine metabolism, with the end result being a reduction in brain glutamate. The enzyme complex BCKA dehydrogenase, which catalyzes the rate-limiting step of leucine oxidation, is strikingly similar to the pyruvate dehydrogenase complex, which catalyzes the decarboxylation of pyruvate (Yudkoff, 1997). Pyruvate dehydrogenase is known to be inhibited by acetyl-CoA and a high ATP (Lehninger et al., 1993). Further, the metabolic changes demonstrated in the brain of ketotic rats (De Vivo et al., 1978) suggest inhibition of this enzyme. It is possible that the closely-homologous BCKA dehydrogenase complex may also be inhibited by the high ATP:ADP ratio and high acetyl-CoA levels generated from ketone and MCFA oxidation. If so, consumption of a ketogenic diet with subsequent elevation of acetyl CoA and ATP:ADP ratio can be expected to inhibit the rate-limiting BCKA dehydrogenase step in leucine metabolism. This inhibition would cause $\alpha$-KIC and glutamate to accumulate, driving the reaction in the reverse direction, with subsequent consumption of glutamate. Yudkoff et al. (1994b) have demonstrated that providing astrocytes with a high concentration of $\alpha$-KIC results in a reversal of the transamination reaction, and increased oxidation of the $\alpha$-KG formed. The net result of this is a significant reduction in astrocytic glutamate and glutamine (Yudkoff et al., 1994b; Yudkoff et al., 1996b). Zielke and colleagues (1997) have also shown an increased rate of glutamate oxidation in response to elevated
extracellular $\alpha$-KIC concentrations *in vivo* using microdialysis. Ultimately, a decrease in export of glutamine to the neurons will lead to a reduction in neuronal synthesis of the excitatory neurotransmitter glutamate. This series of events could provide the key explanation for the mechanism of action of the ketogenic diet (Figure 2.4).

Previous studies have demonstrated the important link between glutamate and leucine metabolism in astrocytes, but the impact of dietary fat-derived fuel substrates (ketones, octanoate) on this relationship has yet to be determined. In particular, the impact of using fatty acids or ketones as the primary fuel substrate in astrocytes on the metabolism of leucine has not been examined.
Figure 2.4: Schematic representation of the research hypothesis

High fat, low glucose diet → Fatty acid oxidation in liver & generation of ketones

↑ MCFAs and ketones in circulation

Ketone and MCFA oxidation

↑Acetyl CoA, ↑ATP: ADP

Inhibition of BCKA dehydrogenase

Accumulation of α-KIC

Reversal of leucine transamination reaction

↓ glutamate/glutamine
3 STUDY OVERVIEW

The establishment of primary astrocyte cultures and subsequent metabolic experiments were conducted at the BC Research Institute for Children's and Women's Health. The metabolic studies were done in two phases. The first experiments involved measuring $^{14}$CO$_2$ produced in astrocytes from uniformly $^{14}$C labelled leucine ([U-$^{14}$C]-leucine) and comparing the effects of two different fuel substrates, $\beta$-hydroxybutyrate and octanoate, on the rate of oxidation. A second series of experiments utilized [1-$^{14}$C]-leucine to specifically address the effect of $\beta$-hydroxybutyrate and octanoate on the first two steps of leucine metabolism during which the number one carbon is released as $^{14}$CO$_2$.

4 PURPOSE

The purpose of this study was to determine whether or not octanoate and $\beta$-hydroxybutyrate inhibit astrocytic leucine metabolism to CO$_2$.

4.1 Objectives

- To establish primary cultures of cerebral cortical astrocytes for use in metabolic studies.
- To establish a method for the collection of labelled products of leucine metabolism in astrocyte cell cultures.
- To compare the oxidation of [U-$^{14}$C]-leucine to $^{14}$CO$_2$ in astrocytes cultured with the MCFA octanoate (8:0), the ketone $\beta$-hydroxybutyrate, or glucose (control) as the primary energy substrate.
• To determine the effect of β-hydroxybutyrate on the production of α-KIC from [1-\textsuperscript{14}C]-leucine in astrocytes.
• To determine the effect of octanoate on the production of α-KIC from [1-\textsuperscript{14}C]-leucine in astrocytes.
• To determine the effect of β-hydroxybutyrate on oxidative decarboxylation of [1-\textsuperscript{14}C]-leucine in astrocytes.
• To determine the effect of octanoate on oxidative decarboxylation of [1-\textsuperscript{14}C]-leucine in astrocytes.

4.2 Hypothesis

A change in primary fuel substrate from glucose to MCFAs or ketones will inhibit astrocytic leucine metabolism. Specifically, use of octanoate or β-hydroxybutyrate as the primary fuel source will inhibit the rate-limiting step of leucine metabolism catalyzed by the BCKA dehydrogenase complex. Inhibition will be reflected in decreased \textsuperscript{14}CO\textsubscript{2} production from [1-\textsuperscript{14}C]-leucine concurrent with increased rate of production of leucine-derived α-ketoisocaproate. Glucose, in contrast, will not effect the metabolism of leucine.

5 ETHICS

The study protocol and procedures were approved by The University of British Columbia Committee on Animal Care.
6 METHODS

6.1 Materials

*Tissue culture supplies:* Falcon tubes (Blue Max™ 50 ml and Blue Max™ Jr., 15 ml); flasks (250 ml) and plates (Multiwell™ 6-well) were purchased from Becton Dickinson Labware (Franklin Lakes, NJ). Serological pipettes (2 ml, 10 ml and 25 ml) were purchased from VWR (West Chester, PA), and Nalgene filters used in media preparation were from Nalge Nunc International (Rochester, NY).

*Tissue Culture Media:* Media was purchased from Gibco BRL/Life Technologies (Grand Island, NY). Media used in the preparation and maintenance of astrocyte cultures was Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (Ham) 1:1 #12400, (DMEM/F12). It was purchased as a powder, which was prepared in purified water with 1.2 g of sodium bicarbonate added per litre, and sterilized by filtration using Nalgene filters. The media used in experiments was Dulbecco’s Modified Eagle Medium #10317, which has a lower concentration of glucose than DMEM/F12 (5 mM vs 25 mM) and contains no glutamine. The ready-to-use liquid preparation was purchased.

*Chemicals and Reagents:* Sterile reagents such as fetal calf serum, antibiotics, trypsin, and versene were purchased from Gibco BRL/Life Technologies (Grand Island, NY). Trichloroacetic acid and toluene were from Fisher Scientific (Nepean, Ontario) and OSC liquid scintillation fluid was from Amersham/Searle (Arlington Heights, Illinois). All other chemicals and media including [U-14C]-
leucine were purchased from Sigma (St. Louis, MO), with the exception of [1-\(^{14}\)C]-leucine, which was from ICN Pharmaceuticals (Aurora, Ohio).

6.2 Animals

Male and female Sprague Dawley rats were purchased from UBC Animal Care and maintained in the animal care unit at the BC Research Institute for Children’s and Women’s Health. The animals were housed under standard conditions in a temperature and humidity controlled animal room, with ad libitum access to Laboratory Rodent Diet #5001 (PMI Feeds, Inc., Richmond, IN) and water. The animals were bred and newborn rats taken for preparation of astrocytes within 48 hours of birth. The timing of this was chosen because at this stage of brain development the rat brain is particularly enriched in astrocytic cells (Cole and de Vellis, 1989).

6.3 Astrocyte Preparation and Culture

Primary cultures of cerebral cortical astrocytes were prepared from the forebrains of newborn Sprague Dawley rat pups (less than 48 hours old) based on the method of McCarthy and DeVellis (1980). This procedure can be divided into three phases: tissue dissociation, release and separation of oligodendrocytes, and purification. All instruments and solutions used in these procedures were pre-sterilized. Efforts were made to work aseptically and quickly in order to maximize cell harvest and viability, and prevent contamination. The media used in the preparation and maintenance of astrocyte cultures was
METHODS

Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12(Ham) 1:1, (DMEM/F12).

6.3.1 Tissue dissociation:

Following cervical dislocation, the brains were removed from the pups. A cut was made from the base of the skull to the mid-eye area and the skin flaps folded back, revealing the underlying skull. An incision was then made through the midline fissure of the skull by lifting slightly upwards while cutting with the scissors. Brains were removed from the skull cavity with a spatula and placed in a 60 mm petri dish containing sterile DMEM/F12 media with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin, and kept warm with a heating pad. When all the brains had been removed, the dish was moved to a laminar flow hood for micro-dissection in a sterile environment. Using forceps, the meninges were carefully removed from each brain and discarded. The cerebral hemispheres were separated and the cortices peeled off and transferred to a sterile petri dish containing fresh media. Any remaining meninges were removed from the cortices. The cortices were then poured into a sterile nytex bag and the media and cells collected in a 100 mm petri dish containing 20 ml of media. While holding the bag closed with forceps, and keeping the bag immersed in the media, light strokes of a glass rod were used to gently push the tissue through the mesh bag. A syringe filled with media was used to carefully rinse free cells adhering to the bag. The cell/media suspension was then poured through a #60 sieve into a sterile cup and a syringe filled with media used to wash over the cells as they
filtered by gravity. The filtrate from the #60 sieve was then poured through a #100 sieve into a second sterile cup and washed with 10 ml of FCS, which helped to loosen any adhering cells. The cells with the media and FCS were then transferred to 15 ml sterile plastic tubes and centrifuged in an IEC Centra-4B centrifuge (Needham Heights, MA) at 800 rpm for 5 minutes. The supernatant was poured off and the cells resuspended in DMEM/F12 media containing 10% FCS and 1% penicillin/streptomycin. The cells were counted using a hemacytometer and plated in 75 cm² tissue culture flasks, at a concentration of 1.5 x 10⁷ cells per flask (in 9-10 ml of media). The approximate yield was one flask per brain. Cells were incubated at 37°C for 72 hours without moving the media to allow time for the astrocytes to adhere to the bottom of the flask. Following this, the media was changed every 48-72 hours.

6.3.2 Release and Separation of Oligodendrocytes:

Seven to nine days after the initial plating, specific procedures were used to remove oligodendrocytes and selectively retain astrocytes. First, the media was changed and then the flasks were secured in the horizontal position to a Lab-Line Junior Orbit Shaker placed in the incubator. The cells were shaken at 200 rpm for 6 hours, the media containing loose cells (oligodendrocytes, astrocytes and macrophages) was poured off, 9-10 ml of fresh media added, and shaking continued for 18 hours. At the end of this 18-hour period, the media was changed again and the cells were shaken continuously for a further 24 hours at 200 rpm.
6.3.3 Purification of Astrocytes

After 48 hours of shaking, the astrocytes remained in a confluent monolayer on the bottom of the flask, and the majority of oligodendrocytes and macrophages were released. Enhanced purity of the astrocyte cultures was then achieved by further shaking, followed by a change to the nutrient media. The media was replaced, the cells were shaken at 100 rpm for a further 48 hours, and then the media was replaced with media containing 5%, rather than 10%, fetal calf serum. The cells were then maintained at 37°C and the media changed every 48-72 hours. One week prior to the metabolic studies, the cells were replated in 6-well tissue culture plates with DMEM/F12 media containing 5% FCS, but without antibiotics. Passaging was performed by versene-trypsin treatment as described by Cole and de Vellis (1989). Cells were briefly washed with versene solution (2 ml per flask), followed by washing with a 0.25% trypsin solution (1.5 ml per flask). The trypsin was poured off and the cells incubated at 37°C for 5-10 minutes until the confluent layer ran freely upon inversion of the flask, indicating that the cells had dissociated. The cells were transferred to a 15 ml conical tube with 10 ml of DMEM/F12 media and centrifuged at 800 rpm for 5 minutes. The media was then poured off and the cells resuspended in antibiotic-free media, and plated in 6-well tissue culture plates with approximately 2 x 10^5 cells per well (one 75 cm^2 flask was passaged and replated into one 6-well plate). Viability of cells used in metabolic studies was confirmed with Trypan Blue staining and light microscopy.
6.4 Collection of $^{14}\text{CO}_2$ from $[^{14}\text{C}]$-Labelled Substrates

Methods for assay of astrocytic leucine metabolism were developed based on published procedures of Auestad et al. (1991) and Yudkoff et al. (1994a). The two-step method used by Auestad et al. (1991) to collect $^{14}\text{CO}_2$ from the metabolism of labelled fatty acids in astrocytes was initially tested. The method was successfully used to collect $^{14}\text{CO}_2$ from $[\text{U}^{-^{14}\text{C}}]$-octanoate (8:0) and leucine. The inter-assay variability was high, so reagents were modified until variability was minimized. The procedure of Auestad et al. (1991) used methylbenzethonium hydroxide (hyamine hydroxide) for the collection of $\text{CO}_2$, however, only methylbenzethonium chloride but not the hydroxide is currently available. Several methods of preparing methylbenzethonium hydroxide were attempted and eventually, a 0.5 M solution in 1 M sodium hydroxide was found to be ideal for the purpose of collecting $\text{CO}_2$. A disadvantage of the method of Auestad et al. (1991) is that it involves passaging cells with the use of versene and trypsin immediately prior to the metabolic experiments. The step is necessary to transfer cells to glass vials for the metabolic studies, but potentially damages cell integrity, and microscopic evaluation of cells did suggest changes to the cell morphology following passaging. To avoid the potentially cell damaging effects of passaging and unknown effects on metabolism, the metabolic studies were attempted in 6-well tissue culture plates as described by Yudkoff et al. (1994a). The cells were passaged and then replated in 6-well tissue culture plates where they were allowed 1 week to adapt to their new environment prior to the metabolic studies. Each well served as a separate trial in
the experiments. To minimize background counts, additional procedures used by Auestad et al. (1991) were also followed, as described in detail in section 6.4. Preliminary studies using octanoate and leucine as the substrates, found that this combination of methods provided the greatest recovery of $^{14}$C$_2$O$_2$, with the least inter-assay variability, and the lowest background counts. An additional advantage of using 6-well plates was that the cells were undisturbed, adhered to the bottom of the tissue culture plate, rather than released and resuspended in media.

6.4.1 Measurement of [U-$^{14}$C]-Leucine Oxidation

The first series of experiments measured astrocytic $^{14}$CO$_2$ production from [U-$^{14}$C]-leucine in the presence of octanoate (0.0, 0.5, 1.0 and 5.0 mM), β-hydroxybutyrate (0.0, 0.5, 1.0 and 5.0 mM), and glucose (additional 5.0 mM). The media used in these experiments was Dulbecco's Modified Eagle Medium (DMEM), which contains approximately 5 mM glucose, and no glutamine. An incubation time of 90 minutes and a leucine concentration of 0.2 mM were selected for the experiments based on previous studies by Yudkoff and colleagues (1994a). The appropriateness of these was confirmed with time course and concentration studies, the results of which are contained in the appendix (Figures A.1 and A.2).

Preparation and Preincubation: In preparation for the experiments, the media was removed and 0.9 ml of DMEM media was added to each well. Then, 20 µl of
the substrate mix, containing leucine, 10.0 mM; \( \alpha \)-ketoglutarate, 10.0 mM; thiamin pyrophosphate (TPP), 5.0 mM; coenzyme A, 5.0 mM; carnitine, 6.2 mM; was added to each well. Therefore, the final concentrations of these metabolites in the 1 ml reaction were 0.2 mM leucine, 0.2 mM \( \alpha \)-KG, 0.1 mM coenzyme A, 0.1 mM TPP and 0.12 mM carnitine. Then, the potentially competing substrates octanoate, \( \beta \)-hydroxybutyrate were added to produce final concentrations of 0.5 mM, 1.0 mM or 5.0 mM in the 1 ml volume. To test whether additional glucose would effect leucine oxidation, glucose was added to increase the baseline concentration by 5 mM, again in a final volume of 1 ml. Following substrate and competitor addition, the tissue culture plate lids, with balls of glass wool (weighing 0.05 g) glued to the underside of the lids such that a ball of glass wool was suspended over each well, were replaced. The cells were then preincubated for 15 minutes at 37\(^0\)C.

First Incubation: At the end of the preincubation, 8 \( \mu \)l of [U-\( ^{14} \)C]-leucine (approximately 0.9 \( \mu \)Ci of [U-\( ^{14} \)C]-leucine) was added to each well, providing a final volume of 1.0 ml in each well. Using a micropipetter, 0.20 ml of 1M NaOH was added to each ball of glass wool, then the lids were placed on the plates and the cells were incubated at 37\(^0\)C for 90 minutes.

Second Incubation: At the end of the 90-minute incubation, 0.25 ml of 0.5 M H\( _2 \)SO\(_4 \) was added to each well to stop cell metabolism and release CO\(_2 \). The tissue culture dish lids were returned to their original position and plates
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incubated for another 90 minutes at 4°C. The release of the $^{14}\text{CO}_2$ product from the acidified media at 4°C rather than room temperature reduced the chemical breakdown of acetoacetate, and resulted in lower background counts (Auestad et al., 1991). During this incubation, CO$_2$ produced from the oxidative decarboxylation of leucine was released from the media and collected in the sodium hydroxide-moistened glass wool.

**Third Incubation**: At the end of the second incubation, each ball of glass wool was removed from the lids and transferred to a glass vial containing 5 ml of H$_2$O. The vials were then sealed with a rubber cap fitted with a suspended centre well containing fluted filter paper. By inserting a needle through the rubber cap using a 1-ml hypodermic syringe, 0.3 ml of 0.5 M hyamine hydroxide was added to the filter paper and 0.5 ml of 5.0 M H$_2$SO$_4$ was added to the water. The vials were then incubated at 37°C for 30 minutes.

**Quantification of $^{14}\text{CO}_2$**: The centre well and its contents were then transferred to a 10-ml plastic scintillation vial containing 8 ml of OCS liquid scintillation fluid and 2 ml of toluene. The $^{14}\text{CO}_2$ collected in the centre wells was then quantified using a Beckman Liquid Scintillation Counter (Fullerton, CA). Controls (blank reactions), containing no cells, were carried out concurrently in all experiments. Labeled CO$_2$ produced in the control reactions reflected chemical breakdown of leucine, and was therefore subtracted from the value for CO$_2$ release by the cells. Each sample was counted at least twice and where the dpm values varied
by more than 5%, the sample was counted a third time. The average dpm for each sample was used in the analysis of the data, excluding erroneous results identified by repeated counting.

6.4.2 Measurement of Oxidative Decarboxylation of [1-\(^{14}\)C]-leucine

The next series of experiments measured astrocytic \(^{14}\)CO\(_2\) production from [1-\(^{14}\)C]-leucine in the presence of \(\beta\)-hydroxybutyrate (0.0, 1.0 and 5.0 mM) and octanoate (0.0, 0.5, 1.0 and 5.0 mM), and quantified [1-\(^{14}\)C]-leucine-derived \(\alpha\)-ketoisocaproate by chemical decarboxylation and measurement of \(^{14}\)CO\(_2\) from the same trials. In the case of \(\beta\)-hydroxybutyrate two sets of experiments were performed, the first in low glucose DMEM/F12 media and the second in phosphate buffered saline (PBS). Preliminary studies using low glucose media indicated that \(^{14}\)CO\(_2\) collection from [1-\(^{14}\)C]-leucine was quite low and significant inhibition of oxidation by \(\beta\)-hydroxybutyrate was not evident. However, glucose concentrations in low glucose media are not actually limiting, as 5.0 mM is normal physiological level. The presence of physiological levels of glucose may alter the utilization of \(\beta\)-hydroxybutyrate, or the effects of its metabolism on leucine. Therefore, additional studies with \(\beta\)-hydroxybutyrate and octanoate were done in phosphate buffered saline (PBS).

Preparation and Preincubation: As previously, the media was removed from the wells and replaced with 0.9 ml of PBS or standard media. The leucine substrate mix (20 \(\mu\)l) and the substrate (\(\beta\)-hydroxybutyrate or octanoate) were added as
METHODS

described in section 6.4 to give a final volume of 1.0 ml. Additional preparatory procedures were as described in section 6.4.

_**Incubations:**_ The incubations were conducted exactly as described for experiments with [U-\(^{14}\)C]-leucine in section 6.4 with the exception that the labeled substrate was [\(^{1}\)\(^{14}\)C]-leucine, 0.9 μCi in 0.9 μl.

6.4.3 **Measurement of the Production of [\(^{1}\)\(^{14}\)C]-Leucine-Derived α-Ketoisocaproate**

Leucine-derived α-ketoisocaproate (α-KIC) was measured in the same trials by chemical decarboxylation of α-KIC using H\(_2\)O\(_2\) and subsequent collection of \(^{14}\)CO\(_2\). Following the second incubation and removal of glass wool to vials (see above), 0.25 ml of H\(_2\)O\(_2\) was added to each well and new lids with fresh glass wool containing 0.30 ml of hyamine hydroxide were added. The plates were incubated at 37°C for 30 minutes.

**Quantification of \(^{14}\)CO\(_2\):** Labeled CO\(_2\) from the oxidation of [\(^{1}\)\(^{14}\)C]-leucine and chemical decarboxylation of [\(^{1}\)\(^{14}\)C]-α-KIC was quantified by liquid scintillation counting. The filter paper and glass wool were each transferred to 10-ml plastic scintillation vials with 8 ml of OCS liquid scintillation fluid and 2 ml of toluene. The \(^{14}\)CO\(_2\) collected in the hyamine hydroxide-soaked filter paper and glass wool was then quantified by liquid scintillation counting. Controls (blanks), containing no
cells, were run concurrently in all experiments and used to correct for non-specific chemical breakdown of the leucine substrate or leucine-derived α-KIC.

6.5 Cell Protein Determination

Cell protein was determined for all experiments. Immediately following the removal of the glass wool from the lids, the media was removed from the plates and the cells frozen at −20°C until analyzed. For analysis of protein the cells were thawed and recovered from the wells by scraping. Potential interfering substances were removed by addition of 1 ml sodium deoxycholate to each sample, followed by the addition of 1 ml trichloroacetic acid (12%), and the cell protein recovered by centrifugation at 3000 rpm in a Sorvall T6000B Centrifuge (Newton, CT) at 4°C for 30 minutes. The filtrate (sodium deoxycholate, trichloroacetic acid and any interfering substances) was removed and the cell protein assayed by the method of Lowry (Lowry et al., 1951) at an absorbance of 660 nm in a Beckman DU640 Spectrophotometer (Fullerton, CA).
7 DATA ANALYSES

7.1 Data Handling and Calculations

The rate of CO₂ production was calculated as dpm/hr based on the incubation time of 90 minutes in all experiments. Calculation of specific radioactivity in the incubation is required to convert dpm values to the amount of CO₂. The calculation of specific radioactivity was based on the actual amount of leucine in the system (0.2 μmol/1ml total incubation) equivalent to 2 \times 10^5 \text{ pmol}, and the amount of radioactive leucine added in each experiment. The amount of radioactivity added in each experiment was 0.9 μCi for [U⁻¹⁴C]-leucine, and 0.8 μCi for [₁⁻¹⁴C]-leucine. Radioactivity was converted to dpm values, 1 μCi = 2.2 \times 10^6 \text{ dpm}, thus 1.998 \times 10^6 \text{ dpm} and 1.760 \times 10^6 \text{ dpm} were added in the experiments with [U¹⁴C]-leucine and [₁⁻¹⁴C]-leucine, respectively. The specific radioactivity of the labelled leucine in the reactions was then calculated by dividing the dpm value by the amount of leucine in pmoles.

\[
\text{Specific Radioactivity} = \frac{\text{radioactivity (dpm)}}{\text{amount of leucine (pmol)}}
\]

The rate of oxidation of [U⁻¹⁴C]-leucine and oxidative decarboxylation of [₁⁻¹⁴C]-leucine was then calculated by dividing the rate of CO₂ production (dpm/hr) by the specific activity of radioactive leucine (dpm/pmol). In the case of uniformly labeled leucine ([U⁻¹⁴C]-leucine), the oxidation rate was then divided by six because each leucine molecule can potentially form six molecules of ^{14}\text{CO}_2. Rates of leucine oxidation and oxidative decarboxylation (pmol/hr) were then expressed per mg of protein, using the results of the analysis of cell protein.
**DATA ANALYSES**

Oxidation rate (pmol/hr) = \(^{14}\text{CO}_2\) production (dpm/hr) / specific activity (dpm/pmol)

The amount of \(^{14}\text{CO}_2\) released by the chemical decarboxylation of \(\alpha\)-KIC (Step 1, Figure 7.1) represents the amount of \([1-^{14}\text{C}]\)-leucine that had been transaminated, but had not proceeded through subsequent steps of leucine metabolism via branched chain ketoacid dehydrogenase. The amount of \(\alpha\)-KIC (pmol) and rate of \(\alpha\)-KIC production (pmol/hr) were calculated as explained above. The rate of net transamination of leucine to \(\alpha\)-KIC is equal to the sum of the rate of production of \(^{14}\text{CO}_2\) from branched chain ketoacid dehydrogenase (Step 2, Figure 7.1) plus the rate of production of \(^{14}\text{CO}_2\) from chemical decarboxylation of \(\alpha\)-ketoisocaproate (Step 1, Figure 7.1).

**Figure 7.1 Schematic representation of the first two steps of leucine metabolism**
7.2 Statistical Analyses

All data were analyzed using the Statistical Package for the Social Science (SPSS Inc. version 7.5 for Windows, Chicago, Illinois). For experiments using [U-\(^{14}\)C]-leucine, means and standard deviations were calculated for the oxidation of leucine to \(^{14}\)CO\(_2\) (pmol/mg protein/hr). For experiments with [1-\(^{14}\)C]-leucine, means and standard deviations were calculated for oxidation of leucine to \(^{14}\)CO\(_2\) (nmol/mg protein/hr), rate of production of \(\alpha\)-KIC from leucine (nmol/mg protein/hr) and rate of net transamination of leucine (nmol/mg protein/hr). One-way analysis of variance was used to determine significant differences in outcome variables between experiments with differing amounts of substrates. When significant differences were found, the Post Hoc Least Significant Difference Test was used to determine which of the means were different. The level of significance was \(P = 0.05\) in all tests.
8 RESULTS

8.1 Metabolism of [U-14C]-Leucine by Astrocytes

The production of $^{14}$CO$_2$ from [U-14C]-leucine in astrocytes was significantly inhibited when 0.5, 1.0 and 5.0 mM octanoate or 1.0 and 5.0 mM β-hydroxybutyrate (BHB) were included as additional substrates (Table 8.1). In contrast, the inclusion of an additional 5 mM of glucose in the reaction mix had no effect on leucine oxidation ($P=0.612$).

Table 8.1: Effect of additional substrates on the production of $^{14}$CO$_2$ from [U-14C]-leucine in astrocytes

<table>
<thead>
<tr>
<th>Additional Substrate</th>
<th>% of control</th>
<th>n</th>
<th>P-value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>5.0mM glucose</td>
<td>106</td>
<td>14</td>
<td>0.612</td>
</tr>
<tr>
<td>0.5mM BHB</td>
<td>73</td>
<td>9</td>
<td>0.067</td>
</tr>
<tr>
<td>1.0mM BHB</td>
<td>40</td>
<td>9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5.0mM BHB</td>
<td>31</td>
<td>9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>0.5mM octanoate</td>
<td>50</td>
<td>11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1.0mM octanoate</td>
<td>21</td>
<td>12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5.0mM octanoate</td>
<td>35</td>
<td>12</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

$^1$ rate of $^{14}$CO$_2$ production compared to control (no additional substrate)
8.1.1 Effect of β-Hydroxybutyrate on [U-14C]-Leucine Oxidation

The mean rate of oxidation of [U¹⁴C]-leucine to ¹⁴CO₂ was 14.44 pmol/mg protein/hr (Table 8.2). The addition of 0.5 mM β-hydroxybutyrate decreased the oxidation of leucine to CO₂ by 27%, however, this was not of statistical significance (P=0.067). A probable reason for this is the interassay variation resulting in the high standard error. Addition of 1.0 mM and 5.0 mM β-hydroxybutyrate decreased the rate of leucine oxidation CO₂ by 60% (P<0.001) and 66% (P<0.001), respectively (Table 8.2). Increasing concentrations of β-hydroxybutyrate caused correspondingly greater inhibition of leucine oxidation, but differences between the concentrations were only significant between 0.5 mM and 1.0 mM β-hydroxybutyrate (P=0.041) and 0.5 mM and 5.0 mM β-hydroxybutyrate (P=0.011).
Table 8.2: Effect of β-hydroxybutyrate on the production of $^{14}$CO$_2$ from [U-$^{14}$C]-leucine in astrocytes

<table>
<thead>
<tr>
<th>Concentration of β-hydroxybutyrate (mM)</th>
<th>Trials</th>
<th>DPM</th>
<th>Cell protein (mg)</th>
<th>Oxidation rate (pmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>14</td>
<td>$2.82 \times 10^2 \pm 30.2$</td>
<td>0.222 ± 0.012</td>
<td>14.44 ± 1.60</td>
</tr>
<tr>
<td>0.5</td>
<td>11</td>
<td>$2.22 \times 10^2 \pm 39.4$</td>
<td>0.236 ± 0.016</td>
<td>10.57 ± 1.76</td>
</tr>
<tr>
<td>1.0</td>
<td>12</td>
<td>$1.23 \times 10^2 \pm 32.4$</td>
<td>0.227 ± 0.011</td>
<td>5.77 ± 1.30 *</td>
</tr>
<tr>
<td>5.0</td>
<td>12</td>
<td>94.3</td>
<td>0.244 ± 0.017</td>
<td>4.84 ± 0.790 *</td>
</tr>
</tbody>
</table>

* values expressed as mean ± standard error of 11-14 separate trials.

* values statistically different from control (0.0 mM β-hydroxybutyrate) in same column ($P<0.05$)

8.1.2 Effect of Octanoate on [U-$^{14}$C]-Leucine Oxidation

The addition of 0.5 mM, 1.0 mM and 5.0 mM octanoate decreased the rate of leucine oxidation to CO$_2$ by 50% ($P<0.001$), 79% ($P<0.001$) and 65% ($P<0.001$), respectively (Table 8.3). The results show maximal inhibition of leucine oxidation occurred with the addition of 1.0 mM octanoate, with no further effect of increasing octanoate concentration. The dose response noted with β-hydroxybutyrate was not evident with octanoate and significant differences between concentrations were only found when comparing 0.5 mM octanoate with 1.0 mM octanoate ($P=0.036$).
RESULTS

Table 8.3: Effect of octanoate on the production of $^{14}$CO$_2$ from [U-$^{14}$C]-leucine in astrocytes

<table>
<thead>
<tr>
<th>Concentration of octanoate (mM)</th>
<th>Trials</th>
<th>DPM</th>
<th>Cell protein (mg)</th>
<th>Oxidation rate (pmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>14</td>
<td>$2.82 \times 10^2$</td>
<td>0.222</td>
<td>14.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\pm 30.2$</td>
<td>$\pm 0.012$</td>
<td>$\pm 1.60$</td>
</tr>
<tr>
<td>0.5</td>
<td>9</td>
<td>$2.04 \times 10^2$</td>
<td>0.256</td>
<td>7.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\pm 59.2$</td>
<td>$\pm 0.040$</td>
<td>$\pm 1.85^*$</td>
</tr>
<tr>
<td>1.0</td>
<td>9</td>
<td>55.5</td>
<td>0.223</td>
<td>3.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\pm 8.74^*$</td>
<td>$\pm 0.014$</td>
<td>$\pm 0.652^*$</td>
</tr>
<tr>
<td>5.0</td>
<td>9</td>
<td>96.8</td>
<td>0.223</td>
<td>5.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\pm 20.3^*$</td>
<td>$\pm 0.011$</td>
<td>$\pm 0.799^*$</td>
</tr>
</tbody>
</table>

$^1$ values expressed as mean ± standard error of 9-14 separate trials
* values statistically different from control (0.0 mM octanoate) in same column ($P<0.05$)

8.2 Metabolism of [1-$^{14}$C]-Leucine by Astrocytes

Initially, experiments with [1-$^{14}$C]-leucine were conducted in DMEM media containing about 5 mM glucose. Recovery of $^{14}$CO$_2$ was low in some experiments, interassay variability was high and statistically significant differences with addition of BHB were not detected, either in rates of leucine oxidation to CO$_2$ or rates of $\alpha$-KIC production from leucine. The dpm obtained in some experiments were not sufficiently above those of the blanks to allow for data analysis. When assayed in PBS, rates of oxidation of leucine to CO$_2$ and production of $\alpha$-KIC from leucine were 7-8 and 4-5 fold higher than in DMEM media containing 5 mM glucose. Thus the effects of BHB on the oxidative
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decarboxylation of [1-14C]-leucine were also quantitated in PBS. For comparison, results are shown for experiments in DMEM media and PBS. Further experiments, conducted only in PBS, tested the effect of octanoate on the oxidative decarboxylation of [1-14C]-leucine.

8.2.1 Effect of β-Hydroxybutyrate on the Oxidative Decarboxylation of [1-14C]-Leucine

The mean rate of oxidation of [1-14C]-leucine to 14CO2 was 0.132 nmol/mg/hr in DMEM media and 0.583 nmol/mg/hr in PBS. The addition of 1.0 mM and 5.0 mM β-hydroxybutyrate did not produce a statistically significant effect on the rate of oxidative decarboxylation of leucine (Table 8.4 and 8.5).
### Table 8.4: Effect of β-hydroxybutyrate on the rate of oxidative decarboxylation of [1-14C]-leucine in astrocytes cultured in DMEM media

<table>
<thead>
<tr>
<th>Concentration of β-hydroxybutyrate (mM)</th>
<th>Trials</th>
<th>DPM</th>
<th>Cell protein (mg)</th>
<th>Rate of 14CO₂ production (nmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>4</td>
<td>2.60 x 10² ± 61.9</td>
<td>0.171 ± 0.024</td>
<td>0.132 ± 0.044</td>
</tr>
<tr>
<td>1.0</td>
<td>3</td>
<td>2.51 x 10² ± 89.6</td>
<td>0.183 ± 0.019</td>
<td>0.112 ± 0.045</td>
</tr>
<tr>
<td>5.0</td>
<td>6</td>
<td>2.89 x 10² ± 47.6</td>
<td>0.179 ± 0.014</td>
<td>0.130 ± 0.011</td>
</tr>
</tbody>
</table>

1 values expressed as mean ± standard error of 3-6 separate trials
2 no statistically significant effects of addition of β-hydroxybutyrate

### Table 8.5: Effect of β-hydroxybutyrate on the rate of oxidative decarboxylation of [1-14C]-leucine in astrocytes cultured in PBS

<table>
<thead>
<tr>
<th>Concentration of β-hydroxybutyrate (mM)</th>
<th>Trials</th>
<th>DPM</th>
<th>Cell protein (mg)</th>
<th>Rate of 14CO₂ production (nmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6</td>
<td>1.08 x 10³ ± 1.93 x 10²</td>
<td>0.138 ± 0.005</td>
<td>0.583 ± 0.103</td>
</tr>
<tr>
<td>1.0</td>
<td>6</td>
<td>0.887 x 10³ ± 2.44 x 10²</td>
<td>0.110 ± 0.008 *</td>
<td>0.629 ± 0.186</td>
</tr>
<tr>
<td>5.0</td>
<td>6</td>
<td>1.33 x 10³ ± 3.79 x 10²</td>
<td>0.106 ± 0.005 *</td>
<td>0.936 ± 0.267</td>
</tr>
</tbody>
</table>

1 values expressed as mean ± standard error of 6 separate trials
2 no statistically significant effects of addition of β-hydroxybutyrate
8.2.2 Effect of β-Hydroxybutyrate on the Rate of Production of α-Ketoisocaprate from [1-14C]-Leucine

The amount of $^{14}\text{CO}_2$ produced by the chemical decarboxylation of [1-14C]-α-KIC remaining in experiments after the 90-minute incubation, reflects the amount of leucine that had been transaminated to α-KIC, but had not proceeded through the subsequent step of oxidative decarboxylation. The rate of production of $^{14}\text{CO}_2$ from chemical decarboxylation of leucine-derived α-KIC in experiments in DMEM media was 16.2 nmol/mg/hr. The addition of 1.0 mM and 5.0 mM β-hydroxybutyrate did not cause a statistically significant change in the rate of production of α-KIC (Table 8.6). In PBS, the rate of production of leucine-derived α-KIC was determined to be 60.3 nmol/mg/hr. The addition of 1.0 mM and 5.0 mM β-hydroxybutyrate increased the rate of α-KIC production by 54% ($P=0.008$), and 36% ($P=0.062$), respectively (Table 8.7). Thus, the increase in production of α-KIC by 1.0 mM β-hydroxybutyrate was statistically significant, whereas the apparent increase by 5.0 mM β-hydroxybutyrate was not. However, there was no statistically significant difference between the rate of production of α-KIC in the presence of 1.0 mM β-hydroxybutyrate compared with 5.0 mM β-hydroxybutyrate ($P=0.329$).
Table 8.6 Effect of β-hydroxybutyrate on the rate of production of $^{14}$CO$_2$ derived from chemical decarboxylation of α-ketoisocaproate in astrocytes cultured in DMEM media

<table>
<thead>
<tr>
<th>Concentration of β-hydroxybutyrate (mM)</th>
<th>Trials</th>
<th>DPM</th>
<th>Cell protein (mg)</th>
<th>Rate of $^{14}$CO$_2$ production (nmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>6</td>
<td>$3.19 \times 10^4 \pm 2.17 \times 10^3$</td>
<td>0.155 ± 0.018</td>
<td>16.2 ± 1.61</td>
</tr>
<tr>
<td>1.0</td>
<td>4</td>
<td>$4.48 \times 10^4 \pm 1.48 \times 10^3$*</td>
<td>0.191 ± 0.016</td>
<td>17.8 ± 1.88</td>
</tr>
<tr>
<td>5.0</td>
<td>6</td>
<td>$4.21 \times 10^4 \pm 3.59 \times 10^3$*</td>
<td>0.179 ± 0.014</td>
<td>17.7 ± 0.622</td>
</tr>
</tbody>
</table>

\* values statistically different from 0.0 mM β-hydroxybutyrate in same column ($P<0.05$)

Table 8.7 Effect of β-hydroxybutyrate on the rate of production of $^{14}$CO$_2$ derived from chemical decarboxylation of α-ketoisocaproate in astrocytes cultured in PBS

<table>
<thead>
<tr>
<th>Concentration of β-hydroxybutyrate (mM)</th>
<th>Trials</th>
<th>DPM</th>
<th>Cell protein (mg)</th>
<th>Rate of $^{14}$CO$_2$ production (nmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6</td>
<td>$1.08 \times 10^5 \pm 1.33 \times 10^4$</td>
<td>0.138 ± 0.005</td>
<td>60.3 ± 8.61</td>
</tr>
<tr>
<td>1.0</td>
<td>6</td>
<td>$1.35 \times 10^5 \pm 8.62 \times 10^3$*</td>
<td>0.110 ± 0.008*</td>
<td>93.1 ± 5.79*</td>
</tr>
<tr>
<td>5.0</td>
<td>6</td>
<td>$1.16 \times 10^5 \pm 1.15 \times 10^4$*</td>
<td>0.106 ± 0.005*</td>
<td>82.2 ± 8.29</td>
</tr>
</tbody>
</table>

\* values statistically different from 0.0 mM β-hydroxybutyrate in same column ($P<0.05$)
8.2.3 Effect of β-hydroxybutyrate on the Rate of Net Transamination of [1-\(^{14}\)C]-Leucine

The net rate of [1-\(^{14}\)C]-leucine transamination by branched chain amino acid transaminase is equal to the rate of \(^{14}\)CO\(_2\) production from oxidative decarboxylation, plus the rate of production of \(^{14}\)CO\(_2\) from the chemical decarboxylation of [1-\(^{14}\)C]-leucine-derived \(\alpha\)-ketoisocaproate. No significant differences were found in the rate of leucine transamination for experiments conducted with increasing concentrations of β-hydroxybutyrate in DMEM media (Table 8.8 and 8.9). The rate of net leucine transamination was 3-4 fold higher in PBS compared to DMEM media. The rate of net leucine transamination in PBS was 61.0 nmol/mg cell protein/hr and it increased significantly when 1.0 mM and 5.0 mM β-hydroxybutyrate were included (\(P=0.008\) and \(P= 0.05\), respectively).

Table 8.8 Effect of β-hydroxybutyrate on the rate of net transamination of [1-\(^{14}\)C]-leucine in astrocytes cultured in DMEM\(^1\)

<table>
<thead>
<tr>
<th>Concentration of β-hydroxybutyrate (mM)</th>
<th>Trials</th>
<th>DPM</th>
<th>Cell protein</th>
<th>Net transamination rate (nmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>4</td>
<td>3.47 x 10^4 ± 1.87 x 10^3</td>
<td>0.171 ± 0.024</td>
<td>16.3 ± 2.41</td>
</tr>
<tr>
<td>1.0</td>
<td>3</td>
<td>4.40 x 10^4 ± 1.36 x 10^3</td>
<td>0.183 ± 0.019</td>
<td>18.3 ± 1.53</td>
</tr>
<tr>
<td>5.0</td>
<td>6</td>
<td>4.24 x 10^4 ± 3.61 x 10^3</td>
<td>0.179 ± 0.014</td>
<td>17.7 ± 0.676</td>
</tr>
</tbody>
</table>

\(^1\) values expressed as mean ± standard error of 3-6 separate trials
* values statistically different from control (0.0 mM β-hydroxybutyrate) in same column (\(P<0.05\))
### Table 8.9 Effect of β-hydroxybutyrate on the rate of net transamination of [1-14C]-leucine in astrocytes cultured in PBS

<table>
<thead>
<tr>
<th>Concentration of β-hydroxybutyrate (mM)</th>
<th>Trials</th>
<th>DPM</th>
<th>Cell protein (mg)</th>
<th>Net transamination rate (nmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>6</td>
<td>1.10 x 10^5 ± 1.32 x 10^4</td>
<td>0.138 ± 0.005</td>
<td>61.0 ± 8.69</td>
</tr>
<tr>
<td>1.0</td>
<td>6</td>
<td>1.36 x 10^5 ± 8.49 x 10^3</td>
<td>0.110 ± 0.008 *</td>
<td>92.1 ± 5.18 *</td>
</tr>
<tr>
<td>5.0</td>
<td>6</td>
<td>1.17 x 10^5 ± 1.13 x 10^4</td>
<td>0.106 ± 0.004 *</td>
<td>83.0 ± 8.51</td>
</tr>
</tbody>
</table>

1 values expressed as mean ± standard error of 6 separate trials
* values statistically different from control (0.0 mM β-hydroxybutyrate) in same column (P<0.05)

8.2.4 Effect of Octanoate on the Oxidative Decarboxylation of [1-14C]-Leucine

In these trials, the mean rate of oxidative decarboxylation of [1-14C]-leucine was 2.35 nmol/mg/hr. Addition of octanoate to the incubation media resulted in significant reductions in 14CO2 production, with 0.5 mM octanoate causing a 54% reduction to 1.09 nmol/mg/hr (P<0.001), 1.0 mM octanoate causing a 77% reduction (P<0.001) to 0.527 nmol/mg/hr, and 5.0 mM octanoate causing a 94% reduction to 0.120 nmol/mg/hr (P<0.001) (Table 8.10).
Table 8.10  Effect of octanoate on the rate of oxidative
decarboxylation of [1-\(^{14}\)C]-leucine in astrocytes cultured in PBS\(^1\)

<table>
<thead>
<tr>
<th>Concentration of Octanoate (mM)</th>
<th>Trials</th>
<th>DPM</th>
<th>Cell protein (mg)</th>
<th>Rate of (^{14})CO(_2) production (nmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>3</td>
<td>7.62 x 10(^3) ± 1.20 x 10(^3)</td>
<td>0.240 ± 0.017</td>
<td>2.35 ± 0.250</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>3.59 x 10(^3) ± 1.20 x 10(^3)*</td>
<td>0.214 ± 0.041</td>
<td>1.09 ± 0.219 *</td>
</tr>
<tr>
<td>1.0</td>
<td>5</td>
<td>8.09 x 10(^2) ± 1.98 x 10(^2)*</td>
<td>0.144 ± 0.008</td>
<td>0.527 ± 0.029 *</td>
</tr>
<tr>
<td>5.0</td>
<td>5</td>
<td>2.16 x 10(^2) ± 84.2*</td>
<td>0.121 ± 0.045</td>
<td>0.120 ± 0.011 *</td>
</tr>
</tbody>
</table>

\(^1\) values expressed as mean ± standard error of 3-5 trials (# of trials in brackets)

* values statistically different from control (0.0 mM octanoate) in same column (P<0.05)

8.2.5 Effect of Octanoate on the Production of \(\alpha\)-Ketoisocaproate from [1-\(^{14}\)C]-Leucine

As stated previously, the amount of CO\(_2\) produced by the chemical
decarboxylation of [1-\(^{14}\)C]-\(\alpha\)-KIC remaining in experiments after the 90-minute
incubation, reflects the amount of leucine that has been transaminated to \(\alpha\)-KIC,
but has not proceeded through the subsequent steps of leucine metabolism. The
rate of production of leucine-derived \(\alpha\)-KIC occurring in trials with no additional
substrate, conducted in PBS was 30.3 nmol/mg/hr (Table 8.11). When 0.5 mM of
octanoate was added the rate of production decreased to 28.4 nmol/mg/hr (6%
decrease, P=0.81). With 1.0 mM octanoate, production of leucine-derived \(\alpha\)-KIC
RESULTS

rose to 46.3 nmol/mg/hr (53% increase, $P=0.10$) while with 5.0 mM octanoate it was 5.31 nmol/mg/hr (83% decrease, $P=0.008$).

Table 8.11 Effect of octanoate on the rate of production of $^{14}$CO$_2$ derived from chemical decarboxylation of $\alpha$-ketoisocaproate in astrocytes cultured in PBS

<table>
<thead>
<tr>
<th>Concentration of octanoate (mM)</th>
<th>Trials</th>
<th>DPM</th>
<th>Cell protein (mg)</th>
<th>Rate of $^{14}$CO$_2$ production (nmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>3</td>
<td>$9.72 \times 10^4 \pm 6.74 \times 10^3$</td>
<td>$0.240 \pm 0.017$</td>
<td>$30.3 \pm 2.96$</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>$7.05 \times 10^4 \pm 9.34 \times 10^3*$</td>
<td>$0.214 \pm 0.004$</td>
<td>$28.4 \pm 7.26$</td>
</tr>
<tr>
<td>1.0</td>
<td>3</td>
<td>$8.34 \times 10^4 \pm 1.14 \times 10^4$</td>
<td>$0.133 \pm 0.007$</td>
<td>$46.3 \pm 4.84$</td>
</tr>
<tr>
<td>5.0</td>
<td>5</td>
<td>$3.06 \times 10^3 \pm 8.90 \times 10^2*$</td>
<td>$0.128 \pm 0.045$</td>
<td>$5.31 \pm 2.94*$</td>
</tr>
</tbody>
</table>

* values statistically different from control (0.0 mM octanoate) in same column

1 values expressed as mean ± standard error of 3-5 trials (# of trials in brackets)

8.2.6 Effect of Octanoate on the Net Transamination of [1-$^{14}$C]-Leucine

As discussed previously, the rate of net transamination of [1-$^{14}$C]-leucine is equal to the rate of production of $^{14}$CO$_2$ collected from oxidative decarboxylation plus the rate of $^{14}$CO$_2$ production from the chemical decarboxylation of [1-$^{14}$C]-leucine-derived $\alpha$-ketoisocaproate. The transamination rate calculated in this manner was 33.0 nmol/mg/hr in trials with no additional substrate. Addition of 0.5 mM and 1.0 mM octanoate resulted in non-significant changes to the transamination rate with 0.5 mM octanoate causing a 9.7% decrease to 29.5
RESULTS

nmol/mg/hr ($P=0.69$) and 1.0 mM octanoate causing a 43.5% increase to 42.3 nmol/mg/hr ($P=0.13$) (Table 8.12). Inclusion of 5 mM octanoate resulted in 83.4% decrease in the transamination rate to 5.36 nmol/mg/hr ($P=0.004$).

Table 8.12 Effect of octanoate on the rate of net transamination of [1-$^{14}$C]-leucine in astrocytes cultured in PBS$^1$

<table>
<thead>
<tr>
<th>Concentration of octanoate (mM)</th>
<th>Trials</th>
<th>DPM</th>
<th>Cell protein (mg)</th>
<th>Net transamination rate (nmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>3</td>
<td>$1.05 \times 10^5 \pm 6.58 \times 10^3$</td>
<td>$0.241 \pm 0.018$</td>
<td>$33.0 \pm 2.57$</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>$7.41 \times 10^4 \pm 9.95 \times 10^3^*$</td>
<td>$0.214 \pm 0.041$</td>
<td>$29.5 \pm 7.04$</td>
</tr>
<tr>
<td>1.0</td>
<td>3</td>
<td>$8.44 \times 10^4 \pm 1.15 \times 10^4$</td>
<td>$0.133 \pm 0.007$</td>
<td>$42.3 \pm 4.85$</td>
</tr>
<tr>
<td>5.0</td>
<td>5</td>
<td>$3.28 \times 10^3 \pm 8.53 \times 10^2^*$</td>
<td>$0.128 \pm 0.045$</td>
<td>$5.36 \pm 3.02^*$</td>
</tr>
</tbody>
</table>

$^1$ values expressed as mean ± standard error of 3-5 trials (# of trials in brackets)

* values statistically different from 0.0 mM octanoate in same column ($P<0.05$)
9 DISCUSSION

The ketogenic diet has been used to treat pediatric epilepsy since the 1920s and the knowledge that fasting inhibits seizures dates back much further. Despite the large number of anti-epileptic drugs available for epilepsy treatment, many children do not respond to drug therapy or experience intolerable side effects. The ketogenic diet provides an alternative and highly successful form of treatment in such cases. Reports in the literature indicate a marked reduction in seizure frequency for up to two thirds of children using a ketogenic diet. The lack of understanding of the mechanism of action of the ketogenic diet has impeded the acceptance of it as a valid form of therapy. In view of the fact that epilepsy has been linked to an overactive excitatory neurotransmitter system, it is important to explore ways in which a high fat, low glucose diet can potentially impact on neurotransmitter metabolism. One such way might be through an impact on leucine metabolism, which is an important source of the α-amino group for the production of brain glutamate. This study was undertaken to determine the effect of medium chain fatty acid and ketone metabolism on the oxidation of leucine in astrocytes.

9.1 Inhibition of Astrocytic Leucine Metabolism by Octanoate and β-Hydroxybutyrate

The results of this study demonstrate that astrocytic leucine metabolism is significantly altered when MCFAs or ketones replace glucose as the primary fuel source. Octanoate and β-hydroxybutyrate significantly inhibited the production of
DISCUSSION

$^{14}$CO$_2$ from [U-$^{14}$C]-leucine (Table 8.1). Production of $^{14}$CO$_2$ from the oxidation of [U-$^{14}$C]-leucine was reduced by as much as 66% with 5.0 mM β-hydroxybutyrate ($P<0.001$) and 79% with 1.0 mM octanoate ($P<0.001$) (Tables 8.2 and 8.3). The $^{14}$CO$_2$ produced from [U-$^{14}$C]-leucine includes $^{14}$CO$_2$ from the decarboxylation of $\alpha$-KIC and $^{14}$CO$_2$ from oxidation of the remaining carbon skeleton in the TCA cycle. Thus the decrease could be related to decreased transamination of leucine, decreased flux through BCKA dehydrogenase, inhibition of the entry of leucine carbon into the TCA cycle, or some combination of these possibilities.

While the results of experiments using [U-$^{14}$C]-leucine indicate that β-hydroxybutyrate and octanoate inhibit leucine oxidation, they provide no information about the particular step in the metabolism that is affected.

Additional experiments using [1-$^{14}$C]-leucine were conducted to elicit more specific information about the inhibition of leucine oxidation by MCFAs and ketones. Leucine labeled only on the first carbon was chosen for this purpose because $^{14}$CO$_2$ collected from metabolism of [1-$^{14}$C]-leucine is specifically derived from the decarboxylation of $\alpha$-KIC by BCKA dehydrogenase, whereas $^{14}$CO$_2$ from [U-$^{14}$C]-leucine is potentially from decarboxylation, and from oxidation of leucine-derived acetyl-CoA in the TCA cycle. Another advantage of using [1-$^{14}$C]-leucine was that [1-$^{14}$C]-$\alpha$-KIC could be chemically decarboxylated to produce $^{14}$CO$_2$, which could then be collected and used to estimate the level of leucine derived-$\alpha$-KIC in the media at the end of the 90 minute incubation. The amount of $\alpha$-KIC reflected leucine that had been transaminated to $\alpha$-KIC, but had not proceeded through the branched chain ketoacid dehydrogenase step, thus
providing information about the net transamination of [1-\(^{14}\)C]-leucine. For the trials with [1-\(^{14}\)C]-leucine, the results obtained using octanoate were quite different from those with \(\beta\)-hydroxybutyrate, thus they will be discussed separately.

### 9.11 Effect of \(\beta\)-Hydroxybutyrate on [1-\(^{14}\)C]-Leucine Metabolism in Astrocytes

Unlike experiments using [U-\(^{14}\)C]-leucine, the results of experiments with [1-\(^{14}\)C]-leucine indicate that \(\beta\)-hydroxybutyrate does not cause a decrease in \(^{14}\)CO\(_2\) production from leucine in astrocytes (Table 8.5). However, the high interassay variability, reflected by the high standard deviation, suggests interpretation of these data must be cautious. It is possible that there was a difference in \(^{14}\)CO\(_2\) production, but it was not detected. In light of the marked effect of \(\beta\)-hydroxybutyrate in inhibition of the production of \(^{14}\)CO\(_2\) from [U-\(^{14}\)C]-leucine, however, it seems likely that [1-\(^{14}\)C]-leucine oxidation should be similarly inhibited.

The results of the studies with [1-\(^{14}\)C]-leucine further demonstrate that \(\beta\)-hydroxybutyrate increased leucine transamination by brain astrocytes (Table 8.9). The net transamination rate is based on \(^{14}\)CO\(_2\) production from oxidative decarboxylation plus the rate of \(\alpha\)-KIC production as derived from chemical decarboxylation of labelled \(\alpha\)-KIC. In these studies, as discussed above, \(\alpha\)-KIC production was increased, but \(^{14}\)CO\(_2\) was not different. A higher amount of \(^{14}\)C in \(\alpha\)-KIC at the end of the 90-minute incubation could potentially be explained by a
higher transamination of leucine. Alternatively, it could be the result of an inhibition of the second step of leucine metabolism, which would result in accumulation of α-KIC, as reflected in the increased $^{14}$C in α-KIC at the end of the 90-minute incubation (Table 8.7). The rate of production of $^{14}$CO$_2$ from α-KIC increased by 54% ($P=0.012$) and 36% ($P=0.06$), respectively in experiments with 1.0 mM and 5.0 mM β-hydroxybutyrate. In light of the findings from experiments with [U-$^{14}$C]-leucine, it seems more likely that the higher amount of α-KIC, and subsequently higher rate of α-KIC production, reflected inhibition of the BCKA dehydrogenase enzyme, rather than an increased in BCAA transaminase activity (Figure 9.1).

Rates of net transamination were increased in the in vitro experiments with 1.0 or 5.0 mM β-hydroxybutyrate compared to no β-hydroxybutyrate, but the difference was statistically significant only at the 1 mM level (Table 8.9). If the increased α-KIC was due to increased transamination of leucine, without inhibition of BCKA dehydrogenase, then we would expect to see a corresponding increase in $^{14}$CO$_2$. On the other hand, if it were due to inhibition of BCKA dehydrogenase, the production of $^{14}$CO$_2$ should be decreased. The results of the experiments with [1-$^{14}$C]-leucine show that the increased levels of α-KIC were not accompanied by a change in $^{14}$CO$_2$ production. Again, it must be noted that interpretation of these results must be cautious because of the high variability in the data on $^{14}$CO$_2$ production. The net transamination rates calculated were more than 100 times the rate of production of $^{14}$CO$_2$ from [1-$^{14}$C]-leucine; this is much higher than the 17-fold difference reported by Yudkoff et al. (1994a). The reason
for this is not known but could relate to methodological problems in the collection of $^{14}$CO$_2$ from [1-$^{14}$C]-leucine in the current study. Possibly, further experiments involving higher concentrations of leucine and longer incubation times might identify changes in $^{14}$CO$_2$ production from [1-$^{14}$C]-leucine in response to β-hydroxybutyrate. However, the results of experiments done here with [1-$^{14}$C]-leucine and using [U-$^{14}$C]-leucine as the substrate suggest that if there was a change in $^{14}$CO$_2$ production this would be a reduction rather than an increase in $^{14}$CO$_2$. The experiments with [U-$^{14}$C]-leucine demonstrate a very significant reduction in $^{14}$CO$_2$ production when ketones are provided as the primary fuel for astrocyte metabolism. This suggests decreased oxidation of the carbon skeleton of leucine in the presence of fatty acids and ketones, consistent with entry of acetyl-CoA from these substrates into the TCA cycle. It is not clear why an inhibition of $^{14}$CO$_2$ production from leucine in the presence of β-hydroxybutyrate was demonstrated with [U-$^{14}$C]-leucine, but not with [1-$^{14}$C]-leucine. A possible explanation is that it was simply a limitation of the method used.

9.12 Effect of Octanoate on the Metabolism of [1-$^{14}$C]-Leucine in Astrocytes

The results of studies with [U-$^{14}$C]-leucine suggest that the impact of octanoate on the metabolism of leucine may be more profound than that of β-hydroxybutyrate (Table 8.1). Consistent with this possibility, results of experiments with [1-$^{14}$C]-leucine indicate that octanoate does cause a significant decrease in $^{14}$CO$_2$ production in astrocytes, whereas such an impact was not detected with β-hydroxybutyrate. All concentrations of octanoate resulted in...
significant decreases in $^{14}$CO$_2$ production with the most dramatic decrease, 94%, observed in trials with 5.0 mM octanoate (Table 8.10).

The effect of octanoate on the net transamination of leucine, calculated by adding $^{14}$CO$_2$ production from oxidative decarboxylation and the rate of $\alpha$-KIC production, was also different from what was observed with $\beta$-hydroxybutyrate. With $\beta$-hydroxybutyrate, an increase in residual $\alpha$-KIC was observed and thus an increase in net transamination was calculated. With octanoate, $\alpha$-KIC was slightly decreased with 0.5 mM, increased with 1.0 mM and decreased with 5.0 mM. The only effect that was statistically significant was the 82.5% decrease seen with the inclusion of 5.0 mM octanoate (Table 8.11). When the $\alpha$-KIC data are used to calculate a net transamination rate (Table 8.12), the trials with 5.0 mM octanoate are significantly reduced compared to those with no additional substrate (5.36 nmol/mg/hr vs. 33.0 nmol/mg/hr, $P=0.004$). Thus, unlike the situation with $\beta$-hydroxybutyrate, there does not appear to be an accumulation of $\alpha$-KIC and there is a dramatic decrease in the $^{14}$CO$_2$ production. This may suggest an inhibition at the level of the BCAA transaminase resulting in both decreased [1-$^{14}$C]-leucine-derived $\alpha$-KIC and decreased $^{14}$CO$_2$. It is therefore possible to speculate that octanoate may be acting via a different mechanism than $\beta$-hydroxybutyrate to inhibit leucine metabolism.
9.2 Hypothesis of the Mechanism of Inhibition of Leucine Oxidation by MCFAs and Ketones

The finding that fatty acids and ketones inhibit the production of CO$_2$ from leucine in astrocytes is consistent with the research hypothesis. The increased levels of $\alpha$-KIC seen in experiments with $\beta$-hydroxybutyrate supports the hypothesis that the inhibition occurs at the second step of leucine metabolism.
On the other hand, the decrease in α-KIC seen observed in experiments with octanoate is not consistent with this hypothesis and suggests that another explanation may be needed for those particular data. The following discussion will describe a hypothetical chain of events that could lead to inhibition of the decarboxylation of α-KIC by branched chain ketoacid dehydrogenase and result in the observed increase in leucine-derived α-KIC and decrease in CO₂ production from leucine seen in experiments with β-hydroxybutyrate.

The second step of leucine metabolism, decarboxylation of α-KIC catalyzed by branched chain ketoacid dehydrogenase, is sensitive to the energy state of the cell. This type of dehydrogenase enzyme is known to be inhibited when ATP, acetyl CoA, or fatty acids are high (Lehninger, 1993) Acetyl CoA levels were not determined in this study. However, because acetyl CoA is the product of fatty acid and ketone oxidation, we can assume that under the conditions of this experiment, with β-hydroxybutyrate or octanoate provided as fuel sources, the acetyl CoA level would be high. It is also possible that the ATP level was elevated by the oxidation of fatty acids and ketones. De Vivo et al. (1978) found an elevated ATP: ADP ratio in the brains of rats fed a high fat diet. Thus, high levels of acetyl CoA, and perhaps high ATP, could have an inhibitory effect on the branched chain ketoacid dehydrogenase enzyme, resulting in the observed accumulation of leucine-derived α-KIC. A block at this step would also explain the reduced CO₂ production in the presence of octanoate or β-hydroxybutyrate.
The transamination of leucine by BCAA transaminase is a reversible reaction, the direction of which is controlled by the concentrations of the reactants. The forward reaction in which glutamate and α-KIC are formed from leucine and α-KG is known to be favoured under normal circumstances. This reaction, however, is readily reversible when the concentrations of glutamate or α-KIC are increased (Yudkoff et al., 1994a). Even small increases in the level of α-KIC have been shown to dramatically affect the branched chain amino acid transamination reaction, resulting in the consumption of glutamate. Using astrocytes in culture, Yudkoff et al. (1994a) demonstrated that the transamination reaction rapidly responded to changes in α-KIC concentration, with 0.05 mM α-KIC causing a reduction in astrocytic glutamate within only 5 minutes of α-KIC addition to the media. At a level of 1.0 mM α-KIC, the intra-astrocytic glutamate concentration was decreased by 50% in the same period of time. A later study by the same group found that increasing extracellular α-KIC concentration resulted in increased transamination of α-KIC with glutamate (reverse transamination) and increased oxidation of α-ketoglutarate (α-KG) via the TCA cycle (Yudkoff et al., 1996b). The latter authors speculated that removal of α-KG would further pull the transamination to the left, thereby consuming even more glutamate. Flux through glutamine synthetase was also decreased due to the lower levels of glutamate. The end result was lower intracellular glutamine. Thus, it is reasonable to speculate that the increase in α-KIC observed in the present study may have caused a reversal of the transamination reaction, with subsequent consumption of glutamate and glutamine. In vivo, glutamine
produced in the astrocytes is released to neurons where it is precursor to neurotransmitter glutamate. Thus, if these metabolic events were to occur in vivo, increases in astrocytic \( \alpha \)-KIC could ultimately result in a reduction in the major excitatory neurotransmitter glutamate.

This hypothetical series of events may explain the effect of \( \beta \)-hydroxybutyrate on leucine metabolism but the effect of octanoate appears to need another explanation. The dramatic effect of octanoate on astrocytic \(^{14}\text{CO}_2\) production was observed in both the experiments with [U-\(^{14}\text{C}\)]-leucine and those with [1-\(^{14}\text{C}\)]-leucine. The experiments with [1-\(^{14}\text{C}\)]-leucine did not indicate that \( \alpha \)-KIC had accumulated in the incubation media, in fact the only significant result suggests that \( \alpha \)-KIC was reduced. These findings are not consistent with the initial research hypothesis, and they may suggest that octanoate is exerting its influence at a separate step in the pathway of leucine metabolism, perhaps by inhibiting the initial transamination of leucine.

9.3 Alternative Hypotheses of the Mechanism of Inhibition of Leucine Oxidation by MCFAs and Ketones

As described previously, it is reasonable to believe that the inhibition of branched chain ketoacid dehydrogenase is related to high levels of acetyl CoA from fatty acid and ketone metabolism. Other explanations, however, are possible, including a direct effect of ketones or fatty acids on the enzyme, or inhibition secondary to changes in the concentration of other TCA cycle intermediates such as citrate, or changes to the internal CoA pool. Yudkoff et al.
(1997) found increased levels of citrate in astrocytes cultured with ketones. Increased citrate concentrations were associated with inhibition of glutamine synthetase. It is possible that citrate was similarly increased in this study, and this may have contributed to changes in leucine metabolism. Consistent with this possibility, De Vivo et al. (1978) found increased concentrations of citrate in the brains of rats fed a very high fat diet. Citrate is known to inhibit the α-ketoglutarate dehydrogenase complex, which is homologous to the BCKA dehydrogenase complex (Lehninger, 1993). Thus, if citrate levels were increased in astrocytes incubated with β-hydroxybutyrate, then this may have had an inhibitory effect on BCKA dehydrogenase, which would result in increased α-KIC and decreased $^{14}$CO$_2$ production from [1-$^{14}$C]-leucine. However, no specific information of the effects of increased citrate concentrations on BCKA dehydrogenase are available. Another possibility is that the oxidation of fatty acids and ketones may have affected the intra-mitochondrial pool of CoA, although inclusion of CoA in the reaction mix makes this unlikely.

It is also possible that the decreased production of CO$_2$ and increased α-KIC observed when astrocytes were provided with octanoate or β-hydroxybutyrate for fuel were not due to inhibition of BCKA dehydrogenase, but rather to some other effect on leucine oxidation. For example, the decrease in $^{14}$CO$_2$ production from [U-$^{14}$C]-leucine in experiments with octanoate or β-hydroxybutyrate could be due to an inability of the carbon skeleton of leucine to enter the TCA cycle. Acetyl CoA is the end product of leucine metabolism and it normally proceeds into the TCA cycle with the production of CO$_2$. The high levels
of acetyl CoA coming from the oxidation of fatty acids and ketones could conceivably inhibit \([U-^{14}C}\)-leucine-derived acetyl CoA from entering the TCA cycle and thus result in decreased production of \(^{14}CO_2\). This hypothesis would not explain the finding of increased \(\alpha\)-KIC unless it was acting in addition to the effect on BCKA dehydrogenase.

9.4 Towards an Understanding of the Antiepileptic Efficacy of the Ketogenic Diet

Children are more susceptible to epileptic seizures than adults (Johnston, 1996) and also have a better response to the ketogenic diet (Prasad et al., 1996). The developing nervous system, described by Johnston as "hyperexcitable", is more prone to seizures induced by fever or injury, and those of an idiopathic nature. Receptors of the excitatory neurotransmitter system are more easily opened, and more difficult to inhibit in the immature brain (Nicoletti et al., 1986). This enhanced activity probably contributes to the tendency of children rather than adults to develop epileptic seizures. The ability of ketogenic diets to suppress seizures is generally believed to be related in some way to the switch from glucose to fatty acids and ketones as the primary fuel source in the brain. The greater efficacy of the ketogenic diet in children, as compared to adults, may be related to unique aspects of brain and/or fatty acid metabolism during development. Children achieve a higher level of blood ketones than adults in response to fasting (Haymond et al., 1983) and younger children attain ketosis in response to a ketogenic diet more easily than older children (Schwartz et al.,
The uptake of ketones into the brain is also greater in young animals and children (Nehlig, 1999; Persson, et al., 1972). In addition, expression of the enzymes involved in ketone metabolism is higher in the young, and thus utilization of ketones is higher (Dahlquist, et al., 1972; Hawkins et al., 1971). The cumulative evidence suggests that the enhanced ability to extract and use ketones may be related to the greater efficacy of the ketogenic diet in children. Interestingly, the rate of brain leucine oxidation is also higher in children compared to adults (Shambaugh and Koehler, 1983); elevations in leucine metabolism may result in elevations in glutamate and thus may be related to the greater neuronal excitability seen in children. It is possible that the use of ketones and MCFAs as the primary fuel source alters brain metabolism via inhibition of leucine oxidation, which subsequently decreases the excitability of neurons through a reduction in brain glutamate.

The results of the two sets of experiments described here suggest leucine metabolism is significantly altered when astrocytes are provided with ketones or MCFAs as their major fuel source. An important relationship exists between the metabolism of leucine and that of the major excitatory neurotransmitter glutamate in the brain. Glutamate uptake at the blood brain barrier is negligible and leucine has been identified as a major source of nitrogen for the replenishment of glutamate lost to oxidation or anabolic processes. Thus changes to the metabolism of leucine will have important consequences for the synthesis of glutamate, and for neurotransmission. Data obtained from the oxidation of [U-14C]-leucine in the presence of different fuel sources suggest that the oxidation of
leucine is inhibited when fatty acids or ketones are provided as primary fuel sources for astrocytes. The reduction in leucine metabolism was observed with concentrations of β-hydroxybutyrate and octanoate as low as 0.5 mM a value that may be physiologically significant. Huttenlocher determined the concentration of β-hydroxybutyrate in the plasma and cerebral spinal fluid of children on a ketogenic diet and found them to be 2.5 mM and 0.4 mM respectively (Huttenlocher, 1976). Circulating levels of octanoate in children receiving an MCT diet have been reported to increase from a normal of 0.04 mM to 0.6 mM (Schwartz et al., 1989). If octanoate does indeed cross the blood brain barrier we would expect to see a corresponding increase in cerebral octanoate in children on MCT diets. It must be acknowledged that concentrations of β-hydroxybutyrate and octanoate within the astrocyte are not known and may differ from values reported in whole brain.

The data also suggest that octanoate inhibits the oxidation of [1-14C]-leucine although the mechanism of how this is accomplished is unclear and is not consistent with the hypothetical mechanism proposed in this thesis. Further, the results of the experiments with [1-14C]-leucine suggest that when β-hydroxybutyrate is used as the primary fuel source by astrocytes, the second step of leucine metabolism is inhibited and α-KIC accumulates. An accumulation of α-KIC should cause a reversal of the reaction catalyzed by BCAA transaminase, ultimately resulting in decreased astrocytic glutamate and glutamine. Astrocytes export glutamine to the neurons where it is converted back to glutamate and used in neurotransmission. If the level of glutamine reaching
the neurons is decreased secondary to inhibition and/or reversal of BCAA transaminase, then we can expect a decrease in the excitability of those neurons. Although not desirable in the normal brain, this would be efficacious in an epileptic brain where glutamate levels are high and receptors are extraordinarily sensitive. Antiepileptic drugs generally work by interfering with the excitatory neurotransmitter system. For example, they may compete for the glutamate receptor, or increase the conversion of glutamate to GABA, an inhibitory neurotransmitter (Chapman, 2000). It is possible that the ketogenic diet also exerts its effects at the level of glutamate metabolism. Glutamate levels were not measured in these experiments, but in view of the relationship between leucine and glutamate in brain, it is reasonable to hypothesize that the observed changes in astrocytic leucine metabolism would be accompanied by changes in astrocytic glutamate and glutamine. The finding that leucine oxidation is inhibited by fatty acids and ketones in astrocytes contributes to the current understanding of integrated fuel metabolism in brain, and may provide a clue to the mechanism of action of the ketogenic diet. Further research, however, is needed to confirm an effect on astrocytic and neuronal glutamate and glutamine.

9.5 Limitations of the Study

Several limitations must be acknowledged when interpreting the results of this study. The experiments were done in an in vitro, single cell system, the astrocyte culture, which is very different from the situation occurring in the whole brain. It is possible that events occurring in cultured astrocytes are different than
what would occur in the whole brain. While this must be recognized as a limitation, it is also clear that useful information can be obtained in such a simple system. The highly controlled experimental system allows manipulation of substrates and observation of the direct effects on leucine metabolism within astrocytes, without interference by the neuroendocrine system. Although the results must be interpreted with caution, studies can now be designed based on the results. The hypothesis can be tested in more complex systems such as cocultures of astrocytes and neurons, whole brain extracts or slices, whole animals and clinical studies in humans.

The results of the experiments conducted in PBS are limited by the fact that the only fuels available to the astrocytes were leucine and β-hydroxybutyrate. The cells did not have a choice of amino acids as they normally would, or as they did in experiments with DMEM, and likewise there was no glucose available to them. While this situation is not realistic, it did reveal an accumulation of α-KIC, which has been interpreted to reflect inhibition of BCKA dehydrogenase, when astrocytes were using β-hydroxybutyrate as the primary fuel source. This may in fact be a more accurate reflection of what occurs as a result of a high fat, low glucose diet, when glucose is limiting. When DMEM media was used, glucose was abundant (5 mM) and because astrocytes will use glucose in preference to other fuel substrates, this may have limited the ability to study the effect of β-hydroxybutyrate on leucine metabolism.

A further limitation of this study is that although the hypothesis links the efficacy of the ketogenic diet to its effect on glutamate and glutamine, these
neurotransmitters were not measured. Unfortunately, the methods used in this study did not allow for the determination of glutamate and glutamine in the astrocytes cultures. The results, however, clearly show an effect on the metabolism of leucine, and reasonable hypotheses can be made based on the data obtained and established knowledge of the relationship between brain leucine and glutamate metabolism. At the same time, studies to measure glutamate and glutamine concentrations are needed before definitive conclusion can be made that the metabolism of MCFAs and ketones ultimately lowers astrocytic glutamate/glutamine.

9.6 Future Research Directions

Cell Culture: Future research using cell cultures should aim to clarify the effect of MCFAs and ketones on the metabolism of leucine. The effect of octanoate on the oxidation of \([1^{-14}C]\)-leucine appears to differ from the effect of \(\beta\)-hydroxybutyrate. The results indicate that octanoate may actually be a more potent inhibitor of leucine metabolism and further studies are needed to determine the mechanism of its effect. As well, the effect of acetoacetate, and a combination of acetoacetate and \(\beta\)-hydroxybutyrate, on leucine oxidation should be tested to determine whether the effect is similar to that obtained with only \(\beta\)-hydroxybutyrate. Studies are necessary to determine whether or not astrocytic glutamate concentration is actually decreased by MCFA and ketone metabolism. The impact of fatty acid and ketone metabolism on the levels of leucine-derived glutamate and glutamine could be tested using the stable isotope \([^{15}N]\)-leucine.
Cell cultures could also be used to determine the levels of acetyl CoA, citrate and the ATP:ADP ratio in astrocytes using fatty acids and ketones as the primary fuel source.

Animal Studies: Future research in animals should determine whether the events occurring at the level of the astrocyte will also occur \textit{in vivo}. Microdialysis could be used to show that increased \(\alpha\)-KIC results in increased oxidation of glutamate/glutamine \textit{in vivo} (Zielke et al., 1997), and to test whether infusion of MCFAs and ketones will inhibit leucine metabolism and decrease glutamate concentration in brain \textit{in vivo}. Animal studies could also be used to explore whether or not chronic feeding of a high fat diet leads to the changes in brain leucine metabolism observed in short term metabolic incubations. Animals could be fed high fat diets such as those used by De Vivo et al. (1978) and metabolic studies done to trace the metabolism of labelled leucine and the production of \(\alpha\)-KIC and glutamate.

Human Studies: Experiments should be designed to measure the levels of leucine and \(\alpha\)-KIC in the circulation of children on the ketogenic diet. Stable isotope methods are safe for use in pediatric research (Koletzo et al., 1998) and could also be used to trace leucine metabolism to \(\alpha\)-KIC and glutamate.


BIBLIOGRAPHY


Figure A.1: Time course of the production of $^{14}$CO$_2$ production from [U-$^{14}$C]-leucine, as reflected by DPM production in astrocyte cell cultures
Figure A.2: Production of $^{14}$CO$_2$ as reflected by DPM production in astrocytes cultured with increasing concentrations of [U-$^{14}$C]-leucine.