EFFECTS OF ORAL CARBAMAZEPINE ADMINISTRATION ON BIOTIN METABOLISM IN RATS

By

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This dissertation is dedicated to my parents, John and Susan Rathman

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LIST OF ABBREVIATIONS

ACC1	Acetyl CoA carboxylase 1
ACC2	Acetyl CoA carboxylase 2
AI	Adequate intake
AIN	American Institute of Nutrition
ANOVA	Analysis of variance
AED	Anti-epileptic drug
AP	Alkaline phosphatase
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BBSA	Biotinylated bovine serum albumin
BNB	Bisnorbiotin
BSO	Biotin sulfoxide
CBZ	Carbamazepine
CBZ-e	Carbamazepine 10, 11-epoxide
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
ECL-Plus	Enhanced chemiluminescence plus
ECF	Enhanced chemifluorescence
EDTA	Ethylenediaminetetraacetic acid

EEG	Electroencephalogram
HCS	Holocarboxylase synthase
HEM	HEPES, EDTA, Mannitol
HEPES	4-(2-hydroxyethyl)-1-piperarzineethanesulfonic acid
3-HIA	3-hydroxyisovaleric acid
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
i.p.	Intraperitoneal
Kda	Kilodalton
kg	Kilogram
MCC	Methylcrotonyl CoA carboxylase
mg	Milligram
MSUD	Maple syrup urine disease
NFDM	Nonfat dry milk
NHS-biotin	N-hydroxysuccinimide ester
OAA	Oxaloacetate
PBS	Phosphate buffered saline
PC	Pyruvate carboxylase
PCC	Proptionyl CoA carboxylase
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SMVT-1	Sodium-dependent multivitamin transporter
TBS-T	Tris buffered saline, tween-20

TCA	Tricarboxylic acid cycle
TEMED	N,N,N’,N’-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
μg	Microgram
YPD	Yeast extract, peptone, dextrose

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Clinical studies indicate that anti-epileptic drug therapy with carbamazepine often is associated with a 50% decrease in plasma biotin. It is estimated that over 80% of epileptics taking anti-epileptic drugs such as carbamazepine exhibit some degree of biotin deficiency. Decreased biotin status among epileptics is accompanied by metabolic alterations including lactic acidemia that imply loss of function of biotin-dependent enzymes as observed in other examples of biotin deficiency.

Although the biotin deficiency that accompanies carbamazepine therapy has been clinically characterized, it is poorly understood at the biochemical level in tissues, particularly in the brain. It was hypothesized that carbamazepine decreases brain biotin status and function of biotin-dependent enzymes, which could result in lactate accumulation. It was also hypothesized that pharmacological doses of biotin would maintain biotin status and function during carbamazepine administration and, therefore, reduce lactate accumulation. These hypotheses were tested using a rat model of oral

carbamazepine administration consuming a physiologically relevant level of biotin, while examining free and protein bound pools of biotin in the brain, liver, serum, and urine. Serum and liver free biotin decreased after long-term carbamazepine administration (>48 d), and pharmacological biotin supplementation completely prevented these decreases. Carbamazepine decreased specific enzymatic activity and biotinylated forms of pyruvate and acetyl CoA carboxylases in both liver and brain. Decreased biotinylated pyruvate carboxylase was due to decreased protein and mRNA expressions in carbamazepine-treated rats. Biotin supplementation prevented the decrease in biotinylation of hepatic and brain acetyl CoA carboxylase and specific activity of both acetyl CoA and pyruvate carboxylases.

Brain and serum lactate concentrations were elevated after 68 d of carbamazepine administration, and were reduced to concentrations similar to control animals with biotin supplementation. Conversion of lactate to pyruvate, with concurrent generation of NADH, during biotin supplementation may explain how increases in pyruvate carboxylase activity could occur without changes in biotinylated pyruvate carboxylase since NADH is an activator of pyruvate carboxylase activity *in vitro*. These results support the use of biotin supplementation as a concurrent strategy during carbamazepine administration to maintain biotin status, function of biotin-dependent enzymes, and decrease carbamazepine-induced lactate accumulation.

CHAPTER 1
LITERATURE REVIEW

Biotin

Chemistry and Function of Biotin

Biotin is a water-soluble, B-vitamin that exists freely or bound to proteins in one form, as d-(+)-biotin (1). Its structure consists of an ureido group bound to a tetrahydrothiophene ring with a five-carbon valeric acid side chain. The two rings in the molecule have a boat configuration with respect to each other (1). The free carboxyl group of the valeric acid side chain is required for binding to the ε-amino group on a specific lysine residue of biotin-dependent carboxylases, and the ureido group has an important role in binding with avidin, a glycoprotein found in egg-whites.

Biotin is a required cofactor for pyruvate carboxylase (PC), acetyl CoA carboxylase isoforms 1 and 2 (ACC1/2), propionyl CoA carboxylase (PCC), and β-methylcrotonyl CoA carboxylase (MCC). The active form of these enzymes requires biotin covalently bound, which occurs through a reaction catalyzed by holocarboxylase synthase (HCS). Biotin is covalently attached to the apocarboxylases and participates in two sequential reactions catalyzed by this enzyme. First, biotin is adenylated in a reaction that is thermodynamically favorable because of the concurrent hydrolysis of pyrophosphate. Then, the holocarboxylase is formed via an amide linkage of the carboxyl group of biotin to the ε-amino group of a specific lysine residue in a highly conserved motif (AMKM) located 35 amino acids from the C-terminus of biotin-dependent enzymes (2).

Biotin is hydrolyzed from the holocarboxylase to form biocytin, or biotinyl lysine. Biotinidase cleaves the lysine from biocytin to form free biotin (3).

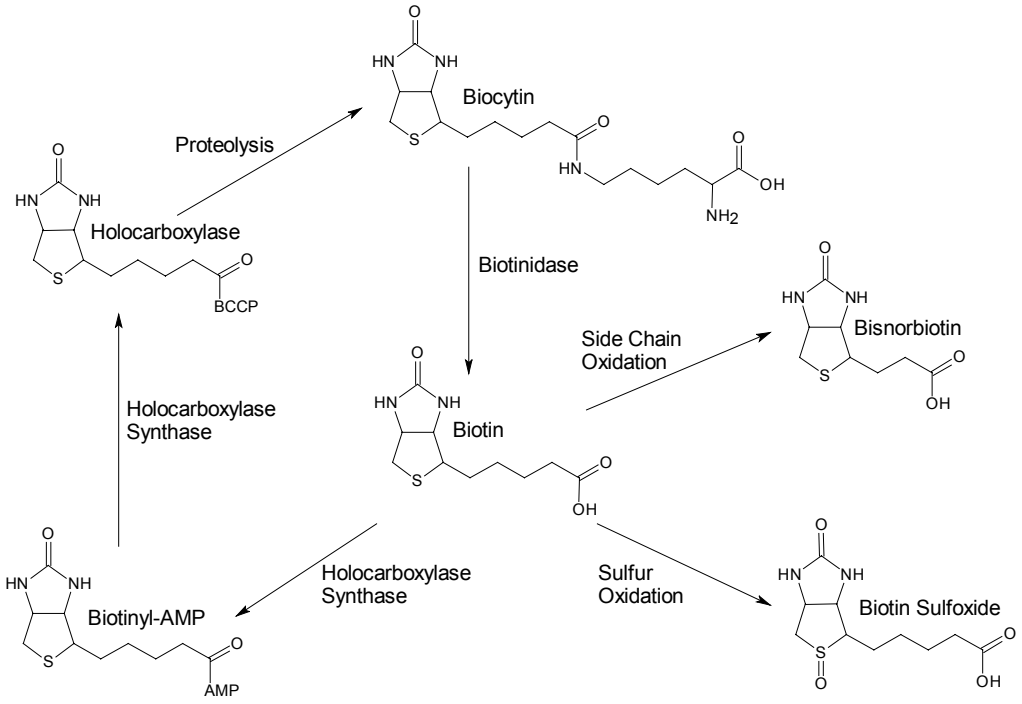

Figure 1-1. Metabolism of biotin.

L and d-biotin sulfoxides (BSO) and bisnorbiotin (BNB) are the major inactive metabolites of biotin (4) (Fig. 1-1). BNB is formed in microbes by β-oxidation of the valeric acid side chain of biotin; however, the exact mechanism in mammals is still not known (5). While BSO accounts for approximately 3% of avidin binding species in human serum, BNB accounts for approximately 38%, and biotin accounts for half of the total avidin binding species in the serum (6). In the urine, biotin, BSO, and BNB account for more than 80% of total avidin binding species (7).

The five biotin-dependent enzymes are involved in carbohydrate, fatty acid, and amino acid metabolism (Fig. 1-2). PC is located in the mitochondria and catalyzes the conversion of pyruvate to oxaloacetate (OAA), an intermediate of the tricarboxylic acid

(TCA) cycle and a precursor for gluconeogenesis. PCC, also located in the mitochondrial matrix, is involved in amino acid catabolism (8). Specifically, it catalyzes the carboxylation of propionyl CoA to D-methylmalonyl CoA. Propionyl CoA is a product formed from the catabolism of isoleucine, valine, methionine, and it is a product of odd-chain fatty acid oxidation. It is also formed during the synthesis of bile acids. The product of the reaction, D-methylmalonyl CoA, can then racemize to L-methylmalonyl CoA and then isomerize to succinyl CoA, which is also an intermediate of the TCA cycle. Propionyl CoA can be shunted to another pathway where 3-hydroxypropionate and methylcitrate are formed in the case of a biotin deficiency or PCC deficiency. Increased concentrations of these organic acids were found in the urine of patients with biotin deficiency (9,10). PCC consists of an α and β subunit, with the α chain containing the site for biotin binding (11). Methylcrotonyl CoA carboxylase is also a mitochondrial enzyme and is essential for the catabolism of leucine. It is responsible for catalyzing the conversion of 3-methylcrotonyl CoA to 3-methylglutaconyl CoA. In the case of biotin deficiency, 3-methylcrotonyl CoA is instead converted to 3-hydroxyisovaleric acid (3-HIA), which is also a validated early indicator of decreased biotin status (12). Both isoforms of ACC (ACC1 and ACC2) catalyze the carboxylation of acetyl CoA to form malonyl CoA, a substrate for fatty acid synthesis. The two isoforms are homologous, except for an additional 180 amino acids at the N terminus that target the protein to the outer mitochondrial membrane. ACC1 is highly expressed in the liver and adipose in the cytosol, whereas ACC2 is predominantly expressed in cardiac and skeletal muscle tissue (13).

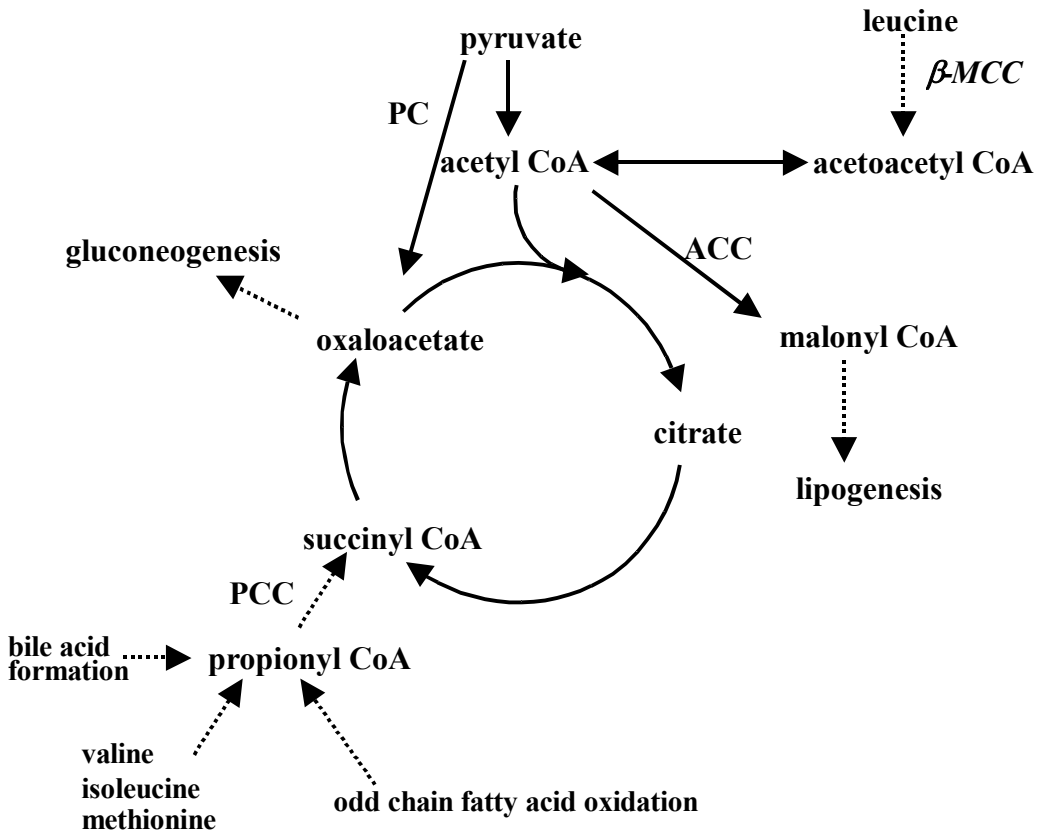

Figure 1-2. Diagram of pathways with biotin-dependent enzymes. PC, pyruvate carboxylase; ACC, acetyl CoA carboxylase isoforms 1 and 2; b-MCC, b-methylcrotonyl CoA carboxylase; PCC, propionyl CoA carboxylase.

Each of the carboxylases has two catalytic sites that are involved in the carboxylation of the substrate. The first site, the biotin carboxylase site, is where the carboxylation of biotin occurs using bicarbonate as the carboxyl donor. Carboxylated biotin is physically moved to the second site, the carboxyl transferase site, by rotating one of the 10 single bonds of the valeryl-lysyl side chain. This catalytic site transfers the carboxyl group from biotin to the substrate (3).

Food Sources, Bioavailability, and Requirement of Biotin

Biotin is widely distributed among foods. Foods sources with the highest concentrations of biotin include royal jelly (400 μg/100 g), brewer's yeast (80 μg/100 g),

egg yolk (20 μg/100 g), calf kidney (100 μg/100 g), soybeans (60 μg/100 g), cauliflower (17 μg/100 g), and peanuts (34 μg/100 g) (3,14). Most biotin in meats and cereals is protein bound and the bioavailability is highly variable, depending on the food source (15). Biotin from wheat is poorly available but it is nearly 100% available in corn (16). Protein bound biotin is only absorbed after non-enzymatic proteolysis and cleavage of lysine by biotinidase; therefore, factors that affect protein digestion also affect biotin uptake.

The dietary reference intake for biotin has been determined based on adequate intake (AI) data only (17). To date, there is no recommended dietary allowance reported for biotin. This is based on lack of data and a general consensus that colonic bacteria synthesize biotin that contributes to the daily supply (17). Microbial synthesis of biotin takes place in the lower part of the intestine where there is limited nutrient absorption, so it is controversial as to the amount of available biotin that colonic bacteria contribute that is available for host metabolism. One study done in minipigs concluded that only approximately 1.7% of the biotin requirement is satisfied by bacterial synthesis (18). When considering that frank nutritional biotin deficiencies are rare, the AI was considered to be adequate until further research proves otherwise. Epileptic patients taking certain anticonvulsants (19,20) and pregnant women (21) have been reported to have marginal biotin deficiencies but the effects of such a status are still not fully understood. The AI for adults is extrapolated from the AI for healthy infants consuming breast milk and has been determined to be 30 μg/day for men and women >19 years. (17).

There is no upper limit (UL) reported for biotin based on lack of symptoms of biotin toxicity when administered at high doses. Patients receive up to 200 mg biotin orally for treatment of inborn errors of metabolism without any toxicity symptoms (1).

Indices of Biotin Status

There is no one set indicator of biotin status. In older literature, plasma or serum biotin alone was used to indicate biotin status. In an effort to determine if there is a more sensitive indicator of biotin status, biotin intake was decreased in 10 healthy subjects who also consumed enough avidin to bind seven times the amount of biotin found in the diet (12). Avidin is a glycoprotein found in egg-whites that can bind biotin very tightly (K_d = 10^{-15} M) and, thus, render it unavailable. Urinary excretion of biotin and its metabolites decreased significantly after 3 d (7 d for BSO) and urinary excretion of 3-HIA increased significantly after 3 d; however, these decreases were not out of the normal range until days 7 and 17, respectively. Serum biotin, on the other hand, did not decrease significantly throughout the study. This study showed that serum biotin concentration alone should not be used as an indicator of biotin status (12). The method for measuring biotin also must be considered when reviewing literature stating biotin status. Microbiological methods are not highly accurate, since other nutrients such as fatty acids in the sample preparation may promote certain microorganisms' growth and interfere with assay results (22). These assays underestimate total avidin-binding species because they do not detect biotin metabolites. Avidin-binding assays without HPLC separation also underestimate total avidin-binding species because they do not account for the slightly lower affinities of the metabolites for avidin. Avidin-binding assays not complemented with HPLC separation overestimate biotin concentration, since biotin only accounts for half of the total avidin-binding species in serum and urine (6,7). The most

accurate and accepted measurement of biotin and biotin metabolites includes a combined HPLC/competitive assay with standard curves for all species measured.

Absorption and Transport

Biotin in the form of free biocytin is the predominant form of biotin found in foods (15). Biotinidase activity is necessary for breaking down biocytin to yield biotin and lysine and is found in pancreatic juice and intestinal mucosal secretions (15). Absorption of biotin is highest in the jejunum, followed by the ileum with 50% of the absorptive capacity of the jejunum, and the lowest is in the colon with 12% of the absorptive capacity of the jejunum (23). Biotin uptake occurs by both simple diffusion and carrier-mediated transport. Studies show that there is saturable uptake of biotin on the brush border of the jejunum at low biotin concentrations and a linear uptake at higher concentrations (23). The saturable process in these studies is inhibited by structural analogs of biotin and is sodium and energy dependent. Similar to other vitamins during deficiency states, transport of biotin across jejunal and ileal brush border membranes was higher in biotin-depleted rats (24). The observed increase in transport during biotin depletion was due to an increase in the number of transporters since there was an increase in V_{max} with little change in apparent K_m. This same study showed that upon supplementation with biotin, there was a decrease in V_{max} compared to controls. These studies suggest that biotin transport is regulated by the concentration of biotin in the diet or body stores.

Similar to the intestinal brush border transporter for biotin, the basolateral transporter in the intestine is saturable as a function of concentration, and it is inhibited by structural analogs of biotin. Unlike the transporter on the brush border membrane, the transporter on the basolateral membrane is sodium independent (25). Recently, a

sodium-dependent multivitamin transporter (SMVT) has also been identified (26) and determined to be an electrogenic process, where there is a 2:1 coupling ratio of Na^+:substrate (27). This transporter transports both biotin and pantothenate. The K_m for biotin is 8.1 ± 1.6 mmol/L, which highly exceeds the concentration of biotin normally found in the serum (~0.5 nmol/L). Another biotin transporter has recently been found in human peripheral blood mononuclear cells that has a K_m of 2.6 ± 0.4 nmol/L, which is much closer to the physiological concentration of biotin in the blood (28).

Biotin exists in the blood both freely and covalently bound to protein. Mock and Malik (29) estimate that 12% of biotin in human serum is covalently bound to protein, 7% is reversibly bound, and 81% is free biotin. Biotinidase can act as a specific human serum biotin binding protein (30) that is possibly the major carrier of biotin in plasma. This group also confirmed that human serum albumin and α and β-globulins can bind biotin nonspecifically.

Biotin Transport in the Central Nervous System

To protect the brain from fluctuations in blood composition, two barriers are present. There is a blood-cerebral spinal fluid (CSF) barrier that is formed by tight junctions between the choroid plexus epithelial cells, the arachnoid membrane cells, and the circumventricular organs (31). The second is the blood-brain barrier also formed of tight junctions between the cells of the cerebral capillary endothelium. Only water and some salts are permitted to freely pass through these barriers. There is no tight barrier between the CSF and the extracellular space of the brain. Certain observations show that brain biotin stores are preferentially maintained during biotin deficiency (32,33), which suggests a role of transport in the regulation of biotin concentration in the CSF and brain.

Other water-soluble vitamins including ascorbic acid, folate, and thiamin do not enter the brain through the blood-brain barrier (34), but through the choroid plexus (35), which is the interface of the blood-CSF barrier. Unlike these other water-soluble vitamins, there is no evidence in rat brain for a concentrating mechanism in the choroid plexus involved in transporting biotin between the blood and CSF (36). Spector and Mock (37) demonstrate that there is very little passive transport of biotin across the blood-brain barrier. There is evidence for sodium-dependent, carrier-mediated transport across the blood-brain barrier that is inhibited by pantothenic acid and nonanoic acid, but not biocytin or biotin methyl ester. Desthiobiotin and diaminobiotin have altered ureido ring structures and were also shown to inhibit transport of biotin (38). This is evidence that a free carboxyl end is required for transport across the blood-brain barrier by this specific biotin transporter that has a K_m well above the physiological range of normal serum biotin (38). In other studies with cultured primary cell bovine brain microvessel endothelial cells from the blood-brain barrier, biocytin but not pantothenic acid or nonanoic acid was shown to compete with the saturable biotin uptake (39), which suggests there is probably another transporter in which the free carboxyl group of biotin is not required for binding.

Biotin Excretion

Biotin is excreted in both the urine and feces, but the majority is excreted in the urine. In tracer studies where carbonyl-^{14}C biotin was given intravenously to rats, 60% of the label appeared in the urine 24 h after infusion while only 2% was detected in the feces (40). This could either mean that biliary excretion is quantitatively low or it could be evidence for enterohepatic circulation where biotin excreted into the bile was then reabsorbed or metabolized by microbial organisms in the colon; however, it has been shown that there is very little biliary biotin excretion (40).

Biotin Deficiency: Symptoms and Metabolic Bases

There is evidence that women become marginally biotin deficient during pregnancy (21). Biotin deficiencies have also been reported in individuals with inborn errors of metabolism, including multiple carboxylase deficiencies. There are two types of multiple carboxylase deficiencies: an early onset type with HCS deficiency, or a late onset type characterized by a biotinidase deficiency. A HCS deficiency results in the inability to biotinylate apocarboxylases, which yields inactivity of the five biotin-dependent carboxylases. Biotinidase deficiency is associated with low biotin status since biotin remains in an unavailable biotinyl-lysine form. In a biotinidase deficiency, seizures unresponsive to anti-epileptic drug (AED) therapy are reported in 50-75% of affected children, along with symptoms of developmental delay, dermatitis, ataxia, hearing loss, optic atrophy, and sensorineural hearing loss (41). In one study of 78 symptomatic children with a biotinidase deficiency, 55% had generalized tonic-clonic, generalized clonic partial seizures, infantile spasms, or myoclonic seizures (42). Biotin supplementation (5-10 mg/d) was effective in stopping the seizures within 24 h in 75% of the children. A small number of patients developed irreversible neurological damage and did not respond to biotin therapy. In other studies of biotinidase deficiencies, reversal of brain atrophy with biotin treatment was reported (43). At the cellular level, the effects of a biotin deficiency result in death of neurons in contact with astroglial cells (44). This same study showed that a biotin deficiency in rats resulted in a latent condition of altered neuronal excitability. Biotin deficient rats experienced a greater duration and more severe seizures after kindling stimulations compared to controls.

Loss of function of biotin-dependent carboxylases accompanies a biotin deficiency and also results in adverse neurological effects. ACC is the rate limiting enzyme for *de*

novo long-chain fatty acid synthesis from glucose or ketone body precursors in the brain (45). Fatty acids in the brain serve as either structural components of cells or as components of the complex lipids of myelin, which include galactosphingolipids and phospholipids. As expected, the highest specific enzymatic activities of ACC in the brain occur during peak myelination (14-29 days postnatal) periods and in the oligodendrocytes, which are the myelinating cells of the central nervous system (46). Biotin depletion in the brain during myelination periods could be expected to have detrimental effects on myelination due to decreased ACC activity.

PC is also a biotin-dependent enzyme crucial for neurological health. There are two types of PC deficiencies characterized differently depending on clinical symptoms. In the neonatal type, there is severe lactic acidemia and death within 1-2 months after birth. In the less serious, juvenile type, there are reported episodes of lactic acidemia along with seizures, developmental delay, and failure to thrive (47). During biotin-responsive multiple carboxylase, biotinidase, and PC deficiencies, lactate and pyruvate build up in the body (33,48). Lactate accumulation in the brain is of particular concern because lactate does not easily cross the blood-brain barrier (49,50) and can have adverse effects in other tissues (51-53).

MCC is involved with the catabolism of leucine, which also has an important role in maintaining neurological health. Branched-chain amino acids rapidly cross the blood-brain barrier, and the influx of leucine into the brain exceeds that of any other amino acid (54). Leucine plays a significant role in buffering brain glutamate concentration. It is rapidly converted to glutamate and α-ketoisocaproate (α-KIA) in the astrocytes. α-KIA is then shunted to the extracellular space, where neurons take up α-KIA and consume

glutamate for the production of leucine to make a complete cycle (55). MCC catalyzes the conversion of β-methylcrotonyl CoA, a catabolite of α-KIA, to β-methylglutaconyl CoA. Intraperitoneal (i.p.) administration of α-KIA and leucine in rats leads to increased absence seizure activity speculated to be a result of altered glutamate concentration (55). Leucine is an allosteric activator of glutamate dehydrogenase, which catalyzes the reversible oxidation of glutamate and subsequently produces ammonia (56), a well documented neurotoxin (57). Hyperammonemia alters the balance between inhibitory and excitatory neurotransmission (57). MCC deficiency in humans results in convulsions or infantile spasm seizure activity that may be the result of a buildup of organic acids or ammonia (41).

Other organic acids, such as propionic acidemia present in a PCC deficiency, also induce seizure activity. Newborns with propionic acidemia have seizures as the primary symptom (47). Maple Syrup Urine Disease (MSUD) is characterized by a defect in the branched-chain α-ketoacid dehydrogenase complex that results in an accumulation of branched-chain amino acids and their ketoacids. This can be related to a biotin-deficient state because in both cases there is decreased catabolism of branched-chain amino acids. In MSUD, clinical symptoms include diffuse myelin loss in the brain, degeneration of the white matter in the brain and, if left untreated, myoclonic jerks and partial seizures (58).

Preferential Maintenance of Biotin in the Brain During Biotin Deficiency

There is less turnover of free biotin from biotinylated carboxylases in the brain compared to other tissues (36). Biotinidase activity is also normally low in human brain (59). In biotin-depleted rats, brain PC activity decreased to 53% of the control values, whereas hepatic activity decreased to 3% of controls (33). In another study, activities of

PC and PCC in rat brain, liver, kidney, and brain were compared during biotin deficiency (60). In this experiment, both PC and PCC activities dropped to only 65% and 60% of the control values after 40 days while hepatic and kidney carboxylase activities dropped to less than 5% and 25% of the control values. These studies were also in agreement with observations of Bhagavan and Coursin in which rats were depleted of biotin for 8 weeks, and liver biotin decreased 90% while brain biotin only decreased 50% (32). In another study, ACC activity and protein expression were decreased in liver and adipose during fasting conditions in rats, while there were no changes observed in brain tissue (45). All of these studies report that the brain is one of the last tissues in the body to be depleted of its biotin stores and that there may be unique regulation of biotin status in the brain. This suggests the importance of biotin for normal brain function.

Epilepsy

Epilepsy is a condition characterized by recurrent seizures that affects 0.5-1% of the population, with the highest incidence among neonates and elderly (61). It is defined as a condition in which a person is prone to epileptic seizures. There are many different types of seizures but generally they are defined as abnormal or excessive discharges of a set of neurons (61). Seizures are commonly classified according to the International Classification of Epileptic Seizures founded in 1981 by the International League Against Epilepsy into groups that include two criteria: the clinical symptoms and the electroencephalogram (EEG) pattern (62). Seizures are also classified according to their origin in the brain. Generalized seizures arise from the cortex in both hemispheres of the brain while partial seizures arise from a small locus in one hemisphere of the brain. Partial seizures can spread to become a generalized seizure and are then secondarily generalized.

Seizures can arise from a number of events including alterations in blood-brain barrier permeability or altered neuronal excitability (61). Acute cerebral insults including stroke, trauma, and infection are also seizure provoking. Other conditions that are associated with seizures include metabolic disorders or inborn errors of metabolism (63). In 70% of cases, however, the cause of seizures is unknown (64). Medical management of epilepsy is primarily performed through pharmacotherapy, although other treatment options include surgery, vagus nerve stimulation, and a ketogenic diet (64,65). In the United States, approximately 1.6 million people have inadequate relief of seizures or experience seizures unresponsive to anti-epileptic drug (AED) therapy (65).

The goal of AED therapy is to stop seizures without incurring side effects that would reduce quality of life among patients. AED therapy is accompanied by adverse side effects in over 50% of patients (64). One side effect that will be discussed further is the alteration of nutrient status, particularly with respect to the vitamin biotin. As previously stated, clinical investigations have documented decreased biotin status in epileptics undergoing long-term AED therapy (19,20).

Effects of Anti-Epileptic Drugs on Biotin Status

In a study of 404 epileptics treated with primidone, phenytoin, carbamazepine (CBZ), or phenobarbital, but not sodium valproate, plasma biotin concentration was significantly lower than controls not taking AEDs (0.9 ± 0.29 and 1.63 ± 0.49 nmol/L, respectively, $p < 0.0005$) (19). Patients with partial epilepsy who received higher daily intakes of AEDs had lower plasma biotin concentration than epileptics with generalized seizures prescribed lower AED doses. AEDs that induce a reduction of plasma biotin include primidone, CBZ, phenytoin, and phenobarbital. Patients receiving sodium valproate had a significantly higher plasma biotin concentration than those on primidone,

CBZ, phenytoin, or phenobarbital, but still lower than controls not taking AEDs. In this same study, three patients were followed before and after AED therapy with primidone or phenytoin. In all three patients, plasma biotin increased during the first week of therapy and then decreased below the initial plasma biotin concentration in the following weeks (19). In another study, Krause and colleagues examined 264 epileptics undergoing long-term AED therapy with phenytoin, primidone, CBZ, or sodium valproate (20). Plasma biotin concentration was significantly lower in AED-treated patients compared to controls (227 ± 80 ng/L vs. 448 ± 201 ng/L for the epileptics and controls, respectively). Patients taking valproate also had significantly lower plasma biotin than controls but significantly higher plasma biotin than those taking primidone, phenytoin, or CBZ. Urine and plasma lactate concentration was elevated in AED-treated epileptics (19); however, no information is known about lactate concentration at the tissue level. While elevated organic acids are suggestive of decreased biotin-dependent enzyme function, this phenomenon has not been investigated during AED therapy.

Mechanism of Altered Biotin Status During AED Therapy

Although decreased biotin status after AED therapy is not completely understood, several potential mechanisms include competition for uptake in the intestine and increased biotin metabolism. CBZ and primidone, but not valproate, competitively inhibit biotin transport across the intestinal brush border membrane in cell culture studies (66). The affected sodium-dependent transporter is also inhibited by biocytin, which suggests that there is no free carboxyl group required for transporter binding. Biotin transporters in the brush border of human kidney cortex and blood brain barrier apparently require a free carboxyl group because they are not inhibited by biocytin nor are they inhibited by primidone or CBZ (38,67). A number of AEDs, including CBZ and

primidone, have an ureido group that is structurally similar to biotin (seen in bold in Fig. 1-3), which suggests this could be the functional group competed against for the intestinal transporter binding site. Valproate does not have an ureido group and, therefore, it is reasonable that it does not compete for biotin uptake.

Another potential mechanism includes induction of sulfur oxidation and β-oxidation processes that catabolize biotin. CBZ and/or phenytoin treatment in children (68) and adults (69) yielded increased excretion of the inactive biotin catabolites, BNB and BSO. In contrast, valproate is a known inhibitor of mitochondrial β-oxidation (70); therefore, it would be expected that if BNB is a metabolite of biotin formed from β-oxidation in the mitochondria, then more biotin and less BNB would be present. This phenomenon has not been investigated, but these mechanisms may explain why plasma biotin concentrations are not as depressed in epileptics undergoing valproate therapy.

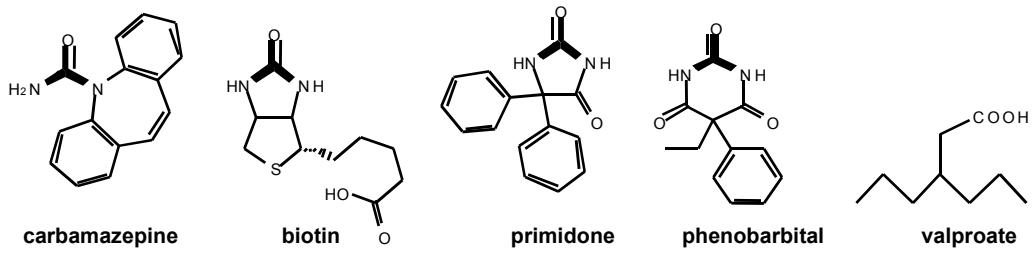

Figure 1-3. Structures of biotin and anti-epileptic drugs with common ureido groups.

Carbamazepine

CBZ, originally synthesized for treatment of trigeminal neuralgia, is now one of the AEDs of choice for secondarily generalized tonic-clonic seizures (71). Its mechanism of action is not completely understood, but there is evidence that it may enhance sodium channel inactivation (71,72). This stabilizes neuronal membranes pre- and postsynaptically by reducing high-frequency repetitive firing of action potentials or by

slowing synaptic transmission (71). Another proposed mechanism involves the blockage of N-methyl-D-aspartate (NMDA) receptor mediated events (73). NMDA is a glutamate receptor with involvement in seizure generation. CBZ is also known to be involved in the induction of hepatic enzymes such as multiple cytochrome P450 subfamilies (74).

Approximately 70-80% of orally administered CBZ is absorbed (75) with no differences observed when the drug is taken with or without food. The body rapidly metabolizes CBZ and, therefore, less than 1% is excreted as CBZ in urine (76). CBZ is capable of inducing its own metabolism to CBZ 10,11-epoxide (CBZ-E), which also has anticonvulsant properties (71). As the duration of CBZ therapy increases, autoinduction becomes more efficient and consequently shortens the half-life of plasma CBZ (77).

Effects of Anti-Epileptic Drugs on Other Vitamins

Megaloblastic anemia observed in patients undergoing AED therapy prompted a further look at folate and B12 status of these patients. Reports of folate deficiency in non-anemic epileptics treated with AEDs followed (78). Intestinal absorption of supplemental folic acid during AED therapy was not affected; however, the absorption of food folates was inhibited (79). Within one week of treatment, primidone (100 mg/kg, twice daily) caused a decrease in pteroylpentaglutamates in the liver to less than half of that of the controls and total liver folate concentration decreased by 30% in rats (80). This same study found no effect on brain folate concentration. Disturbances of folate metabolism have also been observed after CBZ, phenytoin, phenobarbital, and valproate. The effects, however, were different depending on the type of AED. Red cell folate concentrations were significantly decreased in patients receiving phenytoin or CBZ but not with valproate (81). Folic acid supplementation in humans and rats restored plasma folate that was decreased by phenytoin treatment but the decreased brain folate was not

restored (82). Results from this same study showed that phenytoin increased methylation capacity (S-adenylmethionine: S-adenylhomocysteine ratio) in brain but decreased the capacity in the liver. Supplementation with folic acid restored methylation capacity in the liver with no effect on the brain.

There are potential confounding problems with the rat studies of oral AED administration described above in that none measured daily food intake compared to controls (80,82,83). It was noted in these studies that AED-treated rats gained significantly less weight when administered either phenytoin or CBZ but there was no record of food intake. This presents a major flaw in the design of the study because any decrease in folate concentration in the body could have been due to less food intake and, therefore, less folate intake.

Vitamin B6 status was not altered significantly in the whole brain of rats that were genetically epilepsy prone (84); however, there were differences in individual regions of the brain. The concentration of pyridoxal phosphate was lower in the cerebral cortex in the seizure prone rat compared to controls but the opposite was true in the cerebellum. No changes were noted in the hippocampus or the basal ganglion. Vitamin status in 146 epileptics on chronic AED therapy was determined for a number of vitamins and there was a reported deficiency for vitamins B6 and B2 (85). There was no significant change for vitamins B1, B12, A, C, E, or beta-carotene. The most markedly reduced vitamins included folate, biotin, and vitamin D. In this study, 40% of epileptics were folate deficient, 35-43% had decreased plasma vitamin D concentration, and 80% had reduced plasma biotin concentration. In other studies, vitamin B12 concentration in the serum of

AED-treated epileptics was lower than controls (86). Valproate-treated individuals had higher serum vitamin B12 than controls (87).

A vitamin K deficiency is prevalent among neonates due to low placental transport and is especially prevalent in neonates exposed to AEDs in utero (88). It is hypothesized that CBZ, phenytoin, and phenobarbital induce microsomal mixed-function oxidase enzymes in fetal liver and these enzymes increase degradation of vitamin K. In one clinical study, antenatal vitamin K therapy (10 mg/d) administered during the last days of pregnancy of AED-treated mothers resulted in normal vitamin K status in neonates (89).

Overall Rationale

Although it is established that chronic treatment with AEDs including CBZ results in some degree of biotin deficiency in over 80% of patients, the impact on brain biotin and biotin-dependent enzymes involved in carbohydrate, lipid, and protein metabolism is unknown. This research project investigated the metabolic changes of biotin in the brain and the potential protective role of biotin supplementation during CBZ administration. This characterization has important clinical significance as biotin may have future use in medical management of epilepsy. The following hypotheses were tested using an *in vivo* rat model with a physiologically relevant level of dietary biotin using the experimental strategy outlined in the specific aims.

Hypothesis #1: Chronic oral CBZ administration in rats decreases biotin status.

Specific aims

1 To determine dose-dependent effects of chronic CBZ administration on biotin status in brain, serum, and liver.

2 To determine time-dependent effects of oral CBZ administration on biotin status in brain, serum, and liver.

Hypothesis #2: Chronic oral CBZ administration in rats reduces biotin-dependent enzyme expression and activity.

Specific aims

1 To measure both apo and holoenzyme forms of the biotin-dependent enzymes, acetyl CoA, pyruvate, propionyl CoA, and methylcrotonyl CoA carboxylases after oral CBZ administration in brain and liver.

2 To measure specific enzyme activities of acetyl CoA, pyruvate, propionyl CoA, and methylcrotonyl CoA carboxylases after oral CBZ administration in brain and liver.

Hypothesis #3: Chronic oral CBZ administration in rats and subsequent reduction of biotin-dependent enzyme activity generates potentially neurotoxic intermediates.

Specific aims

1 To determine biotin status dependence of lactate and ammonia in liver, serum, and brain after oral CBZ administration.

Hypothesis #4: Supplementation with a pharmacological dose of biotin restores biotin status and function in rats receiving chronic oral CBZ administration.

Specific aims

1 To determine effects of pharmacological biotin supplementation on serum, brain, and liver biotin after oral CBZ administration.

2 To determine effects of pharmacological biotin supplementation on activity and expression of biotin-dependent enzymes after oral CBZ administration.

3 To determine effects of pharmacological biotin supplementation on serum, brain, and liver lactate concentration following oral CBZ administration.

Hypothesis #5: While investigating hypothesis #4, we found that biotin supplementation increased pyruvate carboxylase activity without altering the biotinylated form of the enzyme; consequently, it was hypothesized that pyruvate carboxylase can be activated by other compounds including NADH.

Specific aims

1 To determine whether NADH is an activator of pyruvate carboxylase *in vitro* using both physiological and pharmacological concentrations of NADH.

2 To determine whether kinetic constants of pyruvate carboxylase are altered by NADH.

CHAPTER 2
THE ABUNDANCE AND FUNCTION OF BIOTIN DEPENDENT ENZYMES ARE REDUCED IN A RATS CHRONICALLY ADMINISTERED CARBAMAZEPINE

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Pharmacological intervention remains the most common therapeutic approach for the treatment of epilepsy. Several compounds exhibiting anti-epileptic properties have been developed over the last 50 years, each with a characteristic mode of action and efficacy. As with many other types of medications, these AEDs have the potential to induce nutrient-drug interactions that affect nutritional status. Among the nutrient-drug interactions known to occur with AEDs are reductions in folic acid, vitamin D, and vitamin B_{12} (80,85,86,90).

Although less well appreciated and understood, one of the most prominent nutrient-drug interactions that occur with AED therapy, in terms of both frequency and magnitude, is that with the water-soluble vitamin biotin. Along with decreased serum biotin concentrations (9,19,91), CBZ increased urinary excretion of the organic acids 3-hydroxyisovaleric acid (3-HIA) and lactic acid (associated with insufficient MCC activity PC activity, respectively). Although lending substantial weight to the evidence of an AED-biotin interaction, these studies were potentially limited by the technique used to measure biotin, which lacks chemical specificity (92). Additionally, serum biotin in humans may be limited as a useful indicator of biotin status (93). A later study, however, confirmed these earlier clinical observations by demonstrating an increase in the urinary

excretion of 3-HIA, a marker of MCC activity and a validated index of biotin status, in AED treated individuals (94).

Although increased excretion of organic acids has been demonstrated during AED therapy, its association with insufficient or reduced activity of biotin-dependent enzymes during AED therapy has not been investigated, particularly at the tissue level. In this report, we have established a non-toxic rat model of chronic CBZ administration and evaluated its effects on the abundance and activity of biotin-dependent enzymes in both the liver and brain.

Materials and Methods

Materials

Male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Purified biotin-free rat diet based on a modified AIN 76A formulation described previously (95,96) was obtained from Research Diets (New Brunswick, NJ). CBZ, 5-ethyl-5-p-tolybarbituric acid, CBZ 10, 11-epoxide (CBZ-e), D-biotin, protease inhibitor cocktail and ortho-phenylenediamine dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO); avidin-horseradish peroxidase was purchased from Pierce Chemical Company (Birmingham, AL); 96-well microtiter plates (Nunc Maxisorb) and bovine serum albumin (BSA) were purchased from Fisher Scientific (Pittsburgh, PA). Enhanced chemiluminescence reagent (ECL-Plus) was purchased from Amersham Pharmacia (Picastaway, NJ). Biotinylated BSA was synthesized by mixing 50 mL of 10 g/L BSA in ice-cold 0.1 mol/L $NaHCO_3$ (pH 7.5) with 5 mL of a 12 g/L N-hydroxysuccinimide ester (NHS-biotin) in dimethyl sulfoxide overnight at 4°C. The mixture was dialyzed for 48 h with gentle stirring at 4°C.

Animals and Dietary Treatments

Male Sprague-Dawley rats (n=20), 50-74 g initial weight, were housed individually in hanging wire-bottom cages in an environmentally controlled room with constant temperature (22^{o}C) and a 12-h light:dark cycle. The animals were fed a modified AIN 76A diet containing 0.06 mg biotin/kg diet for 5 days prior to the study to standardize their biotin status as previously described (97). The composition of the diet was spray-dried egg white (20%), corn starch (15%), sucrose (50%), cellulose (5%), corn oil (5%), AIN 76A mineral and vitamin mix (with 0.06 mg/kg biotin), and choline bitartrate (0.2%). The spray-dried egg white was its sole protein source. The protein avidin, contained within the egg white, binds ~1.44 mg biotin/kg purified diet (98). The amount of dietary biotin reported in these experiments represents that in excess of the biotin binding capacity of avidin as determined by HPLC-avidin binding assay as previously described (97). On day 5, rats were randomly divided into 3 dietary treatment groups receiving either 0 g CBZ/kg diet (n=10), 1.5 g CBZ/kg diet (n=5), or 2.9 g CBZ/kg diet (n=5) and remained on these diets for the next 19 d. On days 12, 19, and 23, animals were placed in metabolic cages for 3 h to allow the discrete collection of urine. On day 24, animals were anesthetized with halothane vapor and killed by exsanguination. Brain, liver, and serum were collected and prepared as described below. All procedures were approved by the University of Florida Animal Care and Use Committee.

Sample Preparation

After anesthesia, whole blood was withdrawn, allowed to coagulate for 30 min, and centrifuged at 10,000 x g for 5 min to collect serum. Whole brain and liver (~500 mg) were removed and homogenized in 10 volumes of ice-cold homogenization buffer [300 mmol/L mannitol, 10 mmol/L HEPES (pH 7.2), 1 mmol/L EDTA and protease inhibitor

cocktail]. A portion of the homogenate was set aside for protein biotinylation analysis and the remainder was centrifuged at 200,000 x g for 30 min at 4°C to collect the soluble fraction. For the measurement of biotin and biotin metabolites, serum and tissue soluble fractions were ultrafiltered using a 5000 nominal molecular weight cutoff filter (Millipore, Bedford, MA). All samples were immediately frozen in a mixture of dry ice and isopropanol and stored at -80°C until needed.

Competitive Binding Assay of Biotin

The measurement of biotin in urine, serum, and liver was performed with a coupled HPLC/competitive binding assay (99) with minor modifications. The reversed-phase column used was a Sphereclone 250 x 4.6 mm (Phenomenex, Torrance, CA), and the biotin-containing chromatography fractions were dried under a stream of nitrogen before the assay. A filtered solution (200 μL, 0.45 micron filter, Fisher) of biotinylated bovine serum albumin (BBSA) (50 mmol/L bicarbonate, pH 9.0, 2.5 mg/mL BSA, 1:100 BBSA) was added to flat-bottom 96 well plates and incubated at 4ºC overnight. Biotin, BNB, and BSO standards were prepared from a 3000 pmol/L stock and 100 μL of standard or sample was added to a U-bottom 96 well plate. Avidin solution (50 μL, 100 mg/mL BSA, 0.1 mol/L HEPES, 1 mol/L NaCl, pH 7.0, 1:25,000 NeutrAvidin) was added to each well with either standard or sample and thoroughly mixed. The sample or standard and avidin were incubated for 1 h at room temperature. The coated flat-bottom plate was washed three times with an automatic washer using 0.5% Tween-20, and then 50 μL of the contents from the U-bottom plate were transferred to the coated wells in the flat-bottom plate and allowed to incubate 1-4 h at room temperature. The plate was washed 3 times with 0.5% Tween-20, and 50 μL of a chemiluminescent substrate was added for

detection [0.08% ortho-phenylenediamine dihydrochloride (OPD), 0.1 mol/L citric acid, 0.2 mol/L sodium phosphate, pH 5.0, 0.04% (v/v) H_2O_2]. Before the absorbance was recorded, the reaction was stopped by adding 100 μL of 2 N H_2SO_4 to each well. The plate was read after 45 min at 490 nm and subtracted from the reading at 650 nm.

Measurement of CBZ and CBZ-e

Urine, serum, and tissue CBZ and CBZ-e were measured using a method described previously (100) with minor modifications. Briefly, one gram of diet was mixed with 5 mL of acetone containing 10 mg of 5-ethyl-5-p-tolybarbituric acid/L and vortexed 10-15 min. This mixture was centrifuged at 13,000 x g for 10 min and the soluble fraction was recovered for analysis. Urine and serum were prepared by mixing an equal volume of acetone containing tolylbarbituric acid (10 mg/L) and centrifugation at 13,000 x g for 5 min. Tissues were prepared by homogenizing in 10 volumes ice-cold homogenization buffer (10 mmol/L HEPES, 300 mmol/L mannitol, 1 mmol/L EDTA and protease inhibitor cocktail, pH 7.2) using a Polytron homogenizer and centrifugation at 200,000 x g for 30 min at 4°C. The soluble fraction was recovered and mixed with an equal volume of acetone containing 10 mg tolylbarbituric acid/L, and then the sample was centrifuged at 13,000 x g for 5 min at room temperature. The soluble fractions from these samples were then mixed with two volumes of dichloromethane, vortexed, and the aqueous phase was removed. The organic layer was dried at 65°C and resuspended in methanol. All samples were filtered using a 0.5 μm filter immediately before HPLC injection. A reversed phase column was used [Luna 5 μm C18, 250 x 2 mm (Phenomenex, Torrance, CA)] with a mobile phase of 17% acetonitrile, 55% methanol, and 28% 0.4 mmol/L potassium phosphate (pH 6). Eluted compounds were detected by ultraviolet absorption

at 195 nm. Known amounts of CBZ or CBZ-e were separated and a peak area ratio was determined to generate a standard curve.

Synthesis of Avidin-AlexaFluor 430

NeutrAvidin, an isoelectrically neutral and deglycosylated form of avidin, was conjugated to the succinimidyl ester form of AlexaFluor 430. NeutrAvidin, 10 g/L in 50 mmol/L sodium bicarbonate, pH 8.3, was mixed on a vortexer for 1 h at room temperature. Unconjugated dye was removed by size exclusion chromatography over a DG-10 column (Bio-Rad, Hercules, CA) equilibrated in PBS (20 mmol/L sodium phosphate, pH 7.2, 150 mmol/L NaCl). Equal fractions (1 mL) were collected and the peaks with the highest absorbance at 280 nm combined. Sodium azide (0.2 g/L) was added for preservation, and the conjugate was stored at 4°C protected from light.

Detection and Quantification of Biotinylated Proteins

Using the avidin-blotting technique, we specifically detected five distinct proteins in the liver and brain, corresponding to the five carboxylase enzymes ACC1, ACC2, PC, PCC, and MCC. The specificity of this detection was confirmed through competition with excess biotin. Two slightly different techniques were used in this analysis, i.e., direct fluorescent avidin blotting for PC, PCC, and MCC, and enzyme-linked horseradish peroxidase-avidin conjugate for detection of ACC isoforms 1 and 2. The enzyme-linked method was used for the latter due to the sensitivity limit of the direct fluorescent avidin blotting.

The concentrations of protein in liver and brain samples were determined using a Markwell protein assay (101). Equal amounts of liver total membrane fractions (0.1 mg for the analysis of ACC1 and ACC2, 0.08 mg for all other carboxylases) were resolved by 5 % SDS-PAGE for ACC1 and ACC2 [0.375 M Tris-HCl, pH 8.8, 0.1% SDS (Fisher),

5% acrylamide (1:29 bisacrylamide:acrylamide), 0.05% ammonium persulfate (Sigma, St. Louis), 30 μl TEMED (Fisher)] and an 8% gel with separating gel buffer pH 8.0 for PC, PCC, and MCC [0.375 M Tris-HCl, pH 6.8, 0.23% SDS, 35% (v/v) glycerol, 0.035 mg/mL bromophenol blue, 1.43 mmol/L β-mercaptoethanol (Sigma, St. Louis)], while diluting the sample in 20 uL sample dilution buffer. Proteins were run overnight with running buffer [0.025 M Tris base, 0.2 M glycine (Sigma, St. Louis), 0.1% SDS] at 47 volts. MCC and PCC are very close in molecular weight (79.8 kDa for MCC and 79.3 kDa for the α chain of PCC) but can be separated using an 8% (w/v total acrylamide) SDS-PAGE gel with a separating gel buffer pH 8.0.

The resolved gel was equilibrated in cold transfer buffer [0.03 mol/L Tris base, 0.2 mol/L glycine, 60% (v/v) methanol] for 10 min and was then electroblotted to PVDF (Immobilon-P, Millipore, Bedford, MA) for 2 h at 12 V. The blot was stained with amido black stain [50% (v/v) methanol, 10% (v/v) glacial acetic acid, napthol blue black] and destained [50% (v/v) methanol, 10% (v/v) glacial acetic acid], and then washed with 2 changes of methanol before allowing it to air dry.

For the detection of ACC1 and ACC2, the blot was incubated in 0.5 % non-fat dry milk (NFDM) in Tris-buffered saline (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl) with 0.05% (v/v) tween-20 (TBS-T) and a 1:6000 dilution of Neutralite avidin-horseradish peroxidase conjugate for 40 min. The PVDF was washed 3 times in TBST without NFDM. A chemiluminescent substrate that exhibits fluorescent properties (ECL-Plus) was then applied to the blot and allowed to stand for 5 min. Placing the blot back into TBS-T stopped the reaction. For the detection of PC, PCC, and MCC, the blot was incubated in TBS-T/0.5% NFDM containing avidin-AlexaFluor 430 conjugate at a 1:750

dilution for 45 min at room temperature on an orbital shaker. Biotinylated proteins on both blots were then detected and emitted fluorescence quantified on a Storm fluorescent optical scanner as described by the manufacturer (Amersham-Pharmacia).

Resolving 80 μg of tissue homogenate by 10% (w/v total acrylamide) SDS-PAGE has been determined to be within the linear range of the detection limits of avidin AlexaFluor-430 to detect PC, MCC, and PCC. The specificity of this detection was confirmed through competition with excess biotin.

Lactate and Ammonia Measurements

Ammonia and lactate were measured in serum and brain and liver homogenates using an ultraviolet endpoint method. 2-Oxoglutarate, ammonia, and NADPH were converted to glutamate and $NADP^+$ by glutamate dehydrogenase and the production of $NADP^+$ was measured (Sigma Diagnostic Kit, St. Louis, MO). Lactate concentration was measured in tissue homogenates or serum using an ultraviolet measurement of NADH production from lactic acid and conversion of NAD^+ to pyruvic acid and NADH by the enzymatic activity of lactate dehydrogenase (Sigma Diagnostics Kit, St. Louis, MO).

Measurement of Pyruvate Carboxylase Activity

PC activity was measured by a modification of the method previously described (102). The assay mixture contained in a final volume of 0.1 mL 100 mmol/L Tris-HCl (pH 8.0), 3.8 mmol/L $MgCl_2$, 3.14 mmol/L ATP, 0.32 mmol/L acetyl CoA, 0.5% triton X-100, freshly prepared 7.5 mmol/L pyruvate, and 4 mmol/L [^{14}C]$NaHCO_3$ (specific activity 0.9 mCi/mmol). Blanks were prepared by omitting pyruvate from the reaction mixture. Equal aliquots of the crude homogenate (0.1 mg) were added and allowed to incubate at 37°C for 15 min. PC activity was linear for at least the first 25 min. Adding

0.05 ml 200 g/L trichloroacetic acid stopped the reaction and the precipitated protein was centrifuged at 5,000 x g for 5 min. The supernatant was transferred to a scintillation vial and 0.1 ml of 100 g/L TCA was added to wash the protein pellet. The pellet was centrifuged at 5,000 x g for 5 min and the supernatant added to the corresponding scintillation vial. The pooled supernatants were dried under a stream of nitrogen at 65°C for 30 min and resuspended in 0.5 mL distilled water. Five milliliters scintillation cocktail were added to each vial and the samples were counted by liquid scintillation counting. Specific activities were expressed as pmol OAA formed·mg protein^{-1}·min^{-1}.

Statistical Analysis

Results were expressed as means ± SD. The significance of differences ($p<0.05$) between the two drug levels was assessed by a Student's t-test at each time point. Homogeneity of variance was routinely verified and no data transformation was necessary for these experiments.

Results

Dietary Carbamazepine Administration in a Rodent Model of Biotin Nutriture

After mixing crystalline CBZ with the semipurified diet, analysis indicated concentrations of 1.5 and 2.9 g CBZ/kg diet. Over the 19 d course of treatment, rats consuming the CBZ-containing diets exhibited growth rates (Fig. 2-1a) and food intakes (Fig. 2-1b) not different from control animals consuming the purified diet alone.

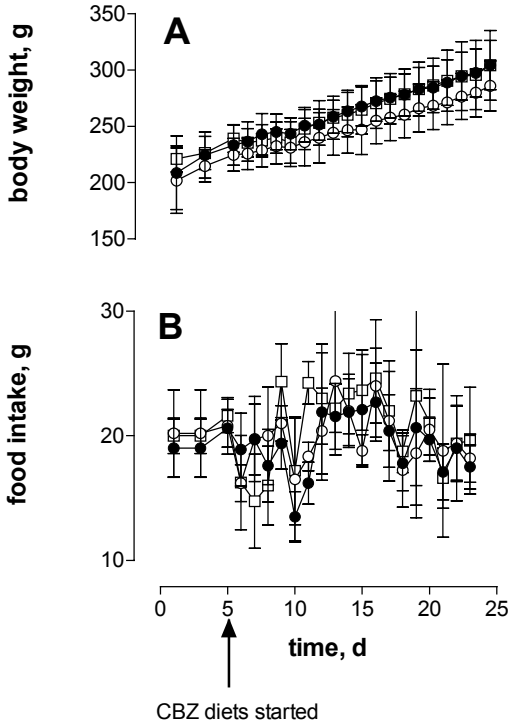


Figure 2-1. Effect of dietary carbamazepine (CBZ) on body weight (A) and food intake (B) in rats. Results are mean ± SD, n=10 (0 g CBZ/kg diet, solid black squares) and n=5 (1.5 and 2.9 g CBZ/kg diets, open squares and solid circles, respectively).

After 1 wk of consuming the CBZ-containing diets, urinary CBZ excretion in rats fed the 2.9 g/kg diet was significantly higher than in those fed the 1.5 g/kg diet. Urinary CBZ excretion was not different between dietary groups for the remainder of the study. (Fig 2-2a). The urinary excretion of CBZ-e was significantly greater for rats fed the 2.9 g/kg diet than for those fed the 1.5 g/kg diet at all time points (Fig. 2-2b). At the end of the study, serum CBZ concentration was not different between the two drug treatment groups (3.9 ± 0.72 μmol/L and 4.9 ± 0.1.6 μmol/L for the 1.5 g CBZ/kg and 2.9 g CBZ/kg groups, respectively). Brain CBZ concentration was significantly different between the 1.5 and 2.9 g/kg groups (Fig. 2-2c). Hepatic CBZ concentration was similar to that in brain and was not different between the two dietary levels (Fig. 2-2d).

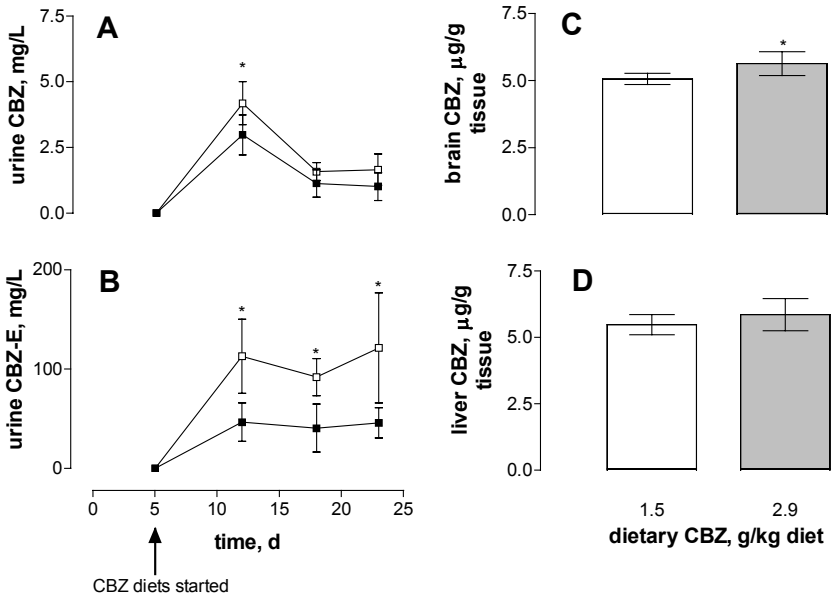


Figure 2-2. Effect of dietary carbamazepine (CBZ) on urinary CBZ (A) and CBZ 10,11-epoxide (CBZ-e) (B) excretions and brain (C) and liver (D) CBZ concentrations in rats. Results are means ± SD, n=10 (0 g CBZ/kg diet) and n=5 (1.5 and 2.9 g CBZ/kg diets). For A and B, means that differ ($p<0.05$) from the other dose at the same time point are indicated by an asterisk. For C and D, an asterisk above the bar indicates significant differences. Open symbols, 2.9 g CBZ/kg diet; closed symbols, 1.5 g CBZ/kg diet.

Effect of Carbamazepine on Organic Acids and Ammonia

In rats consuming the diet containing 2.9 g CBZ/kg, brain ammonia concentrations were significantly elevated compared with those consuming either no CBZ or 1.5 g/kg ($p<0.05$) (Fig. 2-3a). Brain lactate concentration was likewise elevated in rats consuming diet containing either 2.9 or 1.5 g CBZ/kg compared to control animals ($p<0.05$) (Fig. 2-3b). Consumption of diets containing CBZ did not affect hepatic lactate concentration (Fig. 2-3c).

Effect of Carbamazepine Administration on Urine, Serum, Liver, and Brain Biotin and Biotin Metabolites

Urinary biotin excretion was significantly reduced in rats consuming 1.5 g CBZ/kg diet but was elevated in those consuming 2.9 g CBZ/kg diet ($p<0.05$, Fig 2-4a).

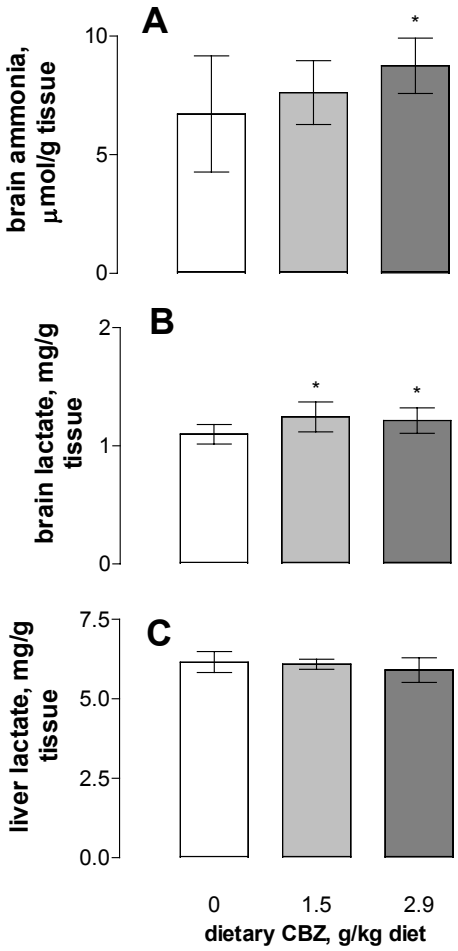


Figure 2-3. Effect of dietary carbamazepine (CBZ) on brain lactate (A) and ammonia (B) and liver lactate (C) concentrations in rats. Results are means ± SD, n=10 (0 g CBZ/kg diet), n=5 (1.5 and 2.9 g CBZ/kg diets). Means without a common letter differ, $p<0.05$.

Consumption of CBZ-containing diets had no effect on serum biotin concentration, But elevated the concentration of BSO and biocytin ($p<0.05$, Fig. 2-4b). The concentration of BNB and biocytin were reduced in the brain of rats consuming either 1.5 or 2.9 g CBZ/kg diet ($p<0.05$) (Fig. 2-4d). There was no effect of dietary CBZ on hepatic biotin, BSO, or biocytin at the two dietary CBZ concentrations tested (Fig. 2-4c).

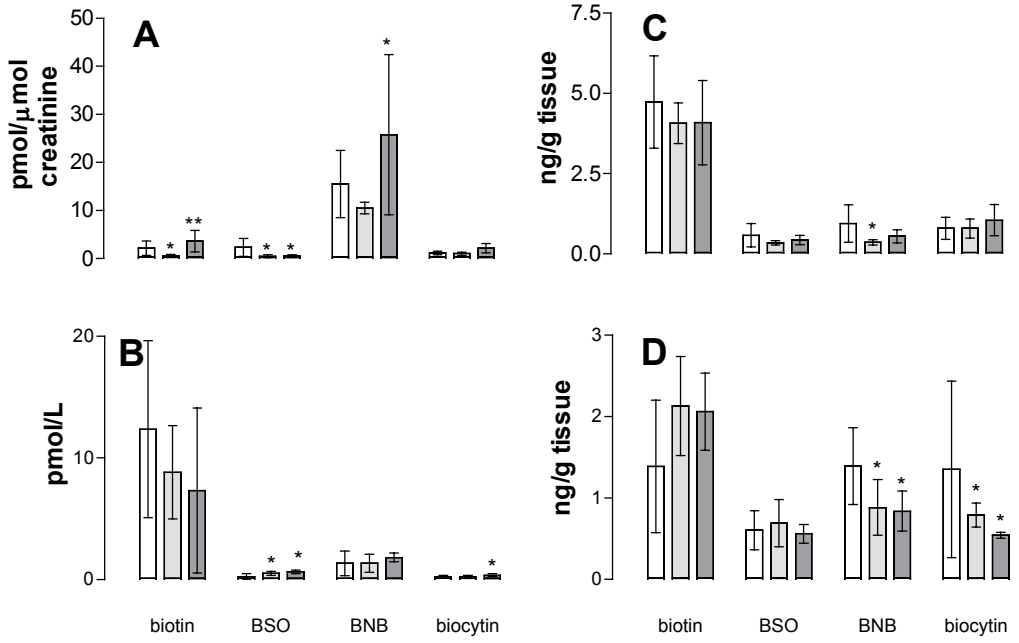


Figure 2-4. Effect of dietary carbamazepine (CBZ) on urine (A), serum (B), liver (C), and brain (D) biotin, biotin sulfoxides (BSO), bisnorbiotin (BNB), and biocytin concentrations in rats. Results are means ± SD, n=10 (0 g CBZ/kg diet), n=5 (1.5 and 2.9 g CBZ/kg diets). Means without a common letter differ, $p<0.05$.

Effect of Carbamazepine Administration on the Abundance of Biotin-Dependent Enzymes in Liver and Brain

As assessed by avidin-blotting, the abundance of biotinylated PC was reduced approximately 25% ($p<0.05$) in the brain of rats consuming either 1.5 or 2.9 g CBZ/kg diet, whereas the abundance of biotinylated PCC, MCC, or ACC was not affected (Fig. 2-5). The relative abundance of hepatic biotinylated PC was significantly reduced by 25 and 39% in rats consuming the 1.5 and 2.9 g/kg diets, respectively. Hepatic ACC 1 and 2 were reduced in the rats consuming both dietary concentrations of CBZ compared to controls (41 and 25% for ACC1 and 40 and 38%, for ACC2, $p<0.05$, for the 1.5 g/kg and 2.9 g/kg diets, respectively) (Fig. 2-6).

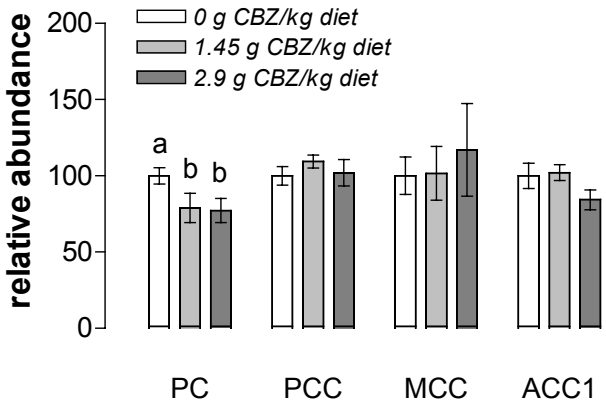


Figure 2-5. Effect of dietary carbamazepine (CBZ) on the relative abundance of biotinylated biotin-dependent enzymes in rat brain. Results are means ± SD, n=10 (0 g CBZ/kg diet), n=5 (1.5 and 2.9 g CBZ/kg diets). Means without a common letter differ, $p<0.05$.

Hepatic MCC and PCC abundances were reduced by 17 and 11% ($p<0.05$), respectively, in rats consuming 2.9 g CBZ/kg diet compared with controls (Fig. 2-6).

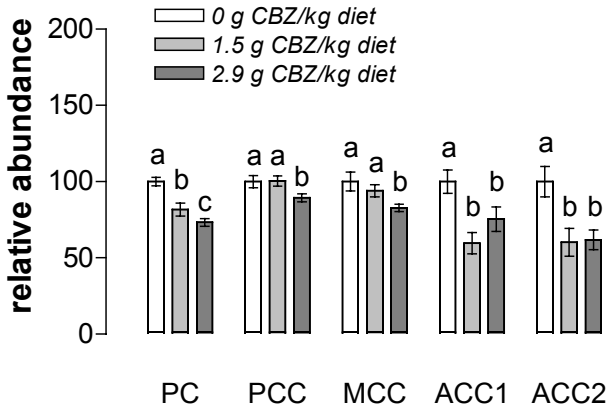


Figure 2-6. Effect of dietary carbamazepine (CBZ) on the relative abundance of biotinylated biotin-dependent enzymes in rat liver. Results are means ± SD, n=10 (0 g CBZ/kg diet), n=5 (1.5 and 2.9 g CBZ/kg diets). Means without a common letter differ, $p<0.05$.

Effect of Carbamazepine Administration on the Activity of Hepatic Pyruvate Carboxylase

To determine whether the observed reduction in carboxylase abundance was consistent with a reduction in enzymatic activity, the specific enzymatic activity of hepatic PC in rats consuming the 0 and 2.9 g CBZ/kg diets was measured. The activity

of hepatic pyruvate carboxylase was reduced approximately 32% in rats consuming 2.9 g CBZ/kg diet compared to controls (3.7 ± 0.23 versus 5.1 ± 0.91 nmol·mg^{-}1·min^{-1}, $p<0.05$).

Discussion

This report describes experiments to determine the effects of CBZ administration on the metabolism and function of the water-soluble vitamin biotin in rats. Although reduction in circulating biotin is well established in the clinical setting, the effect of these reductions on tissue-associated biotin and function of biotin-dependent enzymes is very poorly understood. The dietary model used here was useful for analyzing such effects for several reasons. First, the oral administration of CBZ through the diet more closely models the normal administration route in humans, in contrast to other studies that used injected drug (103). This was an important consideration in light of the results by Said et al. (104), who demonstrated that one mechanism by which CBZ might reduce biotin status is competitive inhibition of intestinal biotin absorption. Second, we observed no reductions in food intake or growth rate, demonstrating the tolerability of the drug when mixed with the AIN76A diet. Furthermore, we observed the well established phenomenon of autoinduction of catabolism, evidenced by the substantial reduction in urine drug concentration after the first week of consumption. One potentially important consideration of these studies is that the dietary level of CBZ used achieved circulating drug concentrations was somewhat lower than we intended, resulting in a model that could be considered subclinical. This could partially explain the lack of toxicity in our study compared to symptoms of toxicity that have been observed in other studies using dietary CBZ (83). This also suggests, however, that the effects of dietary CBZ observed

in this study should be viewed as conservative in relation to what could occur at higher dosages.

In agreement with earlier studies, we have observed changes in the concentration of biotin metabolites consistent with altered biotin catabolism. Oral consumption of CBZ elevated both BSO and BNB, suggesting that the two major pathways of biotin catabolism (sulfur oxidation and β-oxidation) were induced, which is in agreement with earlier reports (103).

Elevation of circulating organic acids, such as lactic acid, is a common clinical finding in individuals treated with various AEDs (9,105). Although we observed no such elevation in serum lactate, brain lactate was significantly increased. Independence of these two compartments of the body is in agreement with earlier observations (50). One likely explanation for the increase of brain lactate is that CBZ reduced the abundance of biotinylated PC, which led to a reduced enzymatic capacity to maintain the TCA cycle intermediates. This could lead to an increase in pyruvate, which might increase the flux to lactate through lactate dehydrogenase. Loss of PC activity has been previously associated with elevation of lactate (106,107). The loss of PC activity can be explained at least in part by a reduction in the abundance of brain biotinylated PC. This provides strong evidence that either the abundance of the PC polypeptide or the biotinylation of the polypeptide is reduced in CBZ-treated rats. Lactate accumulation in the brain is of particular concern, because lactate exhibits low permeability across the blood-brain barrier and can be detrimental in other tissues (49,52). Additionally, lactic acidemia is associated with impaired neurological function. Elevated lactic acid concentration is observed in the brain of piglets with experimentally induced convulsions and in the

extracellular fluid of the hippocampus of humans during spontaneous seizures (108,109). Lactate concentration was also higher in the cerebral spinal fluid of status epileptics than that of control individuals (110). Similarly, the elevation of 3-HIA that has been observed in CBZ therapy is explained by the reduction in the abundance of the biotinylated form of MCC, which is responsible for the catabolism of leucine. Reduction of MCC activity is associated with accumulation and excretion of the resulting 3-HIA (111-114).

The dietary administration of CBZ also elevated brain ammonia, which was somewhat unexpected because of the labile nature of this metabolite. Two mechanisms can be proposed to account for this elevation. The first is that biotin deficiency has been shown to reduce the expression and activity of ornithine transcarbamoylase, a critical enzyme in the urea cycle (115). This inhibition of the urea cycle has been proposed to account for the hyperammonemia observed during biotin deficiency. However, because we observed no marked reduction in biotin status, this may be an unlikely mechanism. A second plausible explanation is the observation that leucine is an allosteric activator of glutamate dehydrogenase, the enzyme that catalyzes production of ammonia (116). It is reasonable to predict that leucine metabolism would be impaired in hepatocytes with decreased abundance of biotinylated MCC, although this has not been directly determined.

Reductions in the abundance of biotinylated ACC isoforms and PCC have additional ramifications for metabolic alterations during CBZ therapy. Simultaneous reductions in ACC 1 and 2 would promote a shift in the hepatocytes from fatty acid synthesis to fatty acid oxidation. A reduction in the abundance of the cytosolic ACC1

limits the ability to synthesize fatty acids *de novo*, and is associated with elevations in odd-chain fatty acids (117-121). A concurrent reduction in the abundance of outer mitochondrial membrane protein ACC2 would promote fatty acid oxidation by lowering the barrier to entry of fatty acids into the mitochondrial matrix (13,122,123). Although the reduction in enzyme abundance observed in these experiments is consistent with this interpretation, direct functional evidence is needed. Hepatocytes also contained less biotinylated PCC (Fig. 2-6) in rats consuming CBZ, limiting the enzymatic capacity to catabolize select amino acids, cholesterol, and odd-chain fatty acids. Again, although these results are strongly suggestive that these other metabolic pathways would be affected, positive functional data are required to substantiate these findings.

Taken together, these data suggest that oral administration of CBZ reduces the abundance of the biotinylated, and therefore functional, form of biotin-dependent enzymes, even when the CBZ is administered with food. Furthermore, this effect is also evident in the rat brain, where the metabolic derangement results in accumulation of lactate and ammonia, both potentially neurotoxic intermediates. The reduction in abundance of the biotinylated carboxylases could arise from either reduction in the abundance of the polypeptide or reduced biotinylation of each carboxylase, and the mechanisms behind these observations await further investigation.

CHAPTER 3
DIETARY CARBAMAZEPINE ADMINISTRATION DECREASES LIVER PYRUVATE CARBOXYLASE ACTIVITY AND BIOTINYLATION BY DECREASING PROTEIN AND mRNA EXPRESSION IN RATS

(As published in J. Nutr. 133: 2119-2124, 2003)

We demonstrated in Chapter 2 that dietary CBZ administration to rats yielded decreased abundance of both brain and hepatic biotinylated PC (124). We also observed increased brain lactate concentration, which could be due to decreased function of brain PC. The mechanism by which CBZ decreased abundance of the biotinylated and, therefore, active PC is unknown and was investigated in the current study. Decreased abundance of biotinylated PC could be due to either decreased expression of the total protein or decreased abundance of the holocarboxylase form of the enzyme. This study examined PC protein abundance and the proportions of apo-PC and biotinylated PC using an *in vitro* biotinylation technique as well as expression of PC mRNA following dietary CBZ administration to rats.

Materials and Methods

Materials

Male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Purified biotin-free rodent diet based on a modified AIN 76A formulation described previously (125) was obtained from Research Diets (New Brunswick, NJ). Carbamazepine (CBZ), 5-ethyl-5-p-tolylbarbituric acid, carbamazepine 10,11-epoxide (CBZ-e), d-biotin, protease inhibitor cocktail, [^{14}C]$NaHCO_3$, and *o*-phenylenediamine dihydrochloride were purchased from Sigma (St. Louis, MO); avidin-horseradish peroxidase (Avidin-HRP) and

avidin-alkaline phosphatase (Avidin-AP) was purchased from Pierce Chemical Company (Birmingham, AL); 96-well microtiter plates (Nunc Maxisorb), ScintiSafe liquid scintillation cocktail, and bovine serum albumin (BSA) were purchased from Fisher Scientific (Pittsburgh, PA). Enhanced chemifluorescence (ECF) reagent was purchased from Amersham-Pharmacia (Picastaway, NJ). Biotinylated BSA and avidin-AlexaFluor 430 conjugate were synthesized as previously described (97).

Animals and Dietary Treatments

Male Sprague-Dawley rats (n=11), 50-75 g initial weight, were housed individually in hanging wire-bottom cages in an environmentally controlled room with constant temperature (22°C) and a 12 h light:dark cycle. The rats were acclimatized by feeding a powdered egg-white based modification of the AIN 76A diet containing 0.06 mg biotin/kg diet for 5 days to standardize their biotin status as described in Chapter 2 (97). After 5 days of acclimatization, the rats were randomly assigned to 2 groups: one group received 0 g CBZ/kg diet (n=5) and one group received 3.75 g CBZ/kg diet (n=6) fed for 28 days. Food intake and body weights were recorded daily for all rats. All procedures were approved by the University of Florida Animal Care and Use Committee.

Sample Preparation

On day 28, rats were anesthetized under halothane and killed via cardiac puncture and exsanguination. Whole blood was allowed to coagulate for 30 min, and then centrifuged at 13,000 x g for 5 min to separate serum. Whole brain was removed and minced before homogenizing in 10 volumes of ice-cold HEM homogenization buffer (30 mmol/L HEPES, pH 7.2, 1 mmol/L EDTA, 300 mmol/L mannitol, and protease inhibitor cocktail). The left lobe of the liver was divided into 10 sections and all samples were immediately frozen in liquid nitrogen and stored at –80°C until further use for enzymatic

activity assays or mRNA isolation. For liver protein extract preparation, a frozen liver portion was thawed and homogenized in 10 volumes of ice-cold HEM. A portion of each homogenate was saved for enzyme biotinylation assays and the remainder was centrifuged at 200,000 x g for 30 min at 4°C to collect the soluble fraction for determination of biotin and biotin metabolites. All homogenized samples were immediately frozen in liquid nitrogen and stored at –80°C until assayed.

Pyruvate Carboxylase Activity Assay

PC activity in brain and liver was determined as described by Suormala et al. (102) with the modifications presented in the Chapter 2 methods section (124). Specific enzymatic activities were expressed as nmol OAA formed·mg protein^{-1}·min^{-1}.

Detection of Biotinylated Biotin-Dependent Carboxylases

The five biotin-dependent carboxylases, ACC1, ACC2, PC, PCC, and MCC were detected using the avidin-blotting technique described previously (97) with slight modifications as presented in the Chapter 2 methods section.

***In Vitro* Biotinylation**

Total liver PC protein was determined as described by Rodriguez-Melendez et al. (126). Briefly, liver homogenate (approximately 500 μg) protein was added to 450 μL of a master mix providing a final concentration of 60 mmol/L Tris HCl, pH 7.5, 0.82 mmol/L biotin, 0.1 mmol/L EDTA, 0.6 g/L bovine serum albumin, 3 mmol/L reduced glutathione, 8 mmol/L $MgCl_2$, and 10 mmol/L adenosine triphosphate. The reaction mixture was incubated for 6 h at 37°C. At 2 and 4 h of incubation, an additional 26.75 μL of a solution containing 10 mmol/L adenosine triphosphate and 8 mmol/L $MgCl_2$ was added. Finally, the reaction was terminated by adding 500 μL sample dilution buffer

[0.375 mol/L Tris-HCl, pH 6.8, 2.3 g/L SDS, 350 mL/L glycerol, 0.035 mg/ml bromophenol blue, 1.43 mol/L β-mercaptoethanol]. A portion of each protein sample (40 μg) was loaded and resolved on a 10% SDS-PAGE gel, transferred to PVDF membrane, and detected by avidin-western blotting (125).

Measurement of Biotin, Biotin Sulfoxide, Bisnorbiotin, and Biocytin

The measurement of biotin and its metabolites in serum, liver, and brain was performed using a coupled HPLC/competitive binding assay (97,99).

Measurement of Carbamazepine

Serum, liver, brain, and dietary CBZ were measured using a method previously described (100) with slight modifications (124) presented in the Chapter 2 methods section.

RNA Isolation and Northern Blotting

Frozen liver was homogenized in Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) and RNA was isolated as directed by the manufacturer. RNA was quantified by absorbance at 260 nm and purity assessed by calculation of the ratio of the absorbance at 260 nm to absorbance at 280 nm. RNA integrity was analyzed by 1.2% agarose gel electrophoresis in 1 x TBE (89 mmol/L Tris base, 89 mmol/L boric acid, 2 mmol/L EDTA) with ethidium bromide staining (127).

After electrophoresis, RNA was transferred to a nylon membrane (Millipore, Bedford, MD) using an overnight downward capillary transfer technique (127). The nucleic acids were cross-linked to the membrane using 160,000 μjoules short wave UV light and allowed to dry completely before probing. The random priming labeling kit (Gibco BRL) was used to label a rat PC cDNA probe with ^{32}P. The PC cDNA probe was

hybridized to the homologous RNA and to normalize for lane-to-lane variation, a mouse beta-actin cDNA probe was used to detect beta-actin mRNA. Both PC and beta-actin were detected by phosphor imaging using a STORM840 fluorescent and phosphor imager (STORM, Sunnyvale, CA) following the manufacturer's instructions. Densitometry of the resulting image was performed using ImageQuant software (Amersham Pharmacia Biotech, Picastaway, NJ).

Real-Time Reverse Transcriptase PCR Conditions

Using cDNA sequences for PC retrieved from GenBank, real-time PCR primers and TaqMan probe were designed using Primer Express software version 1.0 (PE Applied Biosystems, Foster City, CA). The primers and TaqMan probe for PC were synthesized by Keystone Labs (Biosource International, Camarillo, CA) with a FAM reporter dye and quencher BHQ1 (Table 3-1). The primers and TaqMan probe for 18S rRNA gene were purchased from PE Applied Biosystems and used as the endogenous control for RNA.

Table 3-1. Primers and TaqMan probe for rat pyruvate carboxylase. Positions in the pyruvate carboxylase gene (GenBank accession no. NM 012744) are indicated. The TaqMan probe has the 5′ and 3′ ends labeled

Sequence of PC primers and probe

Primer/probe	Sequence (5' →3')	Positions (bp)
Forward primer	CCTGCTCGTCAAAGTCATTGC	1,306-1,326
(Antisense) Reverse primer	ACACCTCGGACACGGAACTC	1,379-1,398
TaqMan probe	FAM-CAAAGACCACCCTACAGCTGCCACCA-BHQ1	1,333-1,358

Real-Time Reverse Transcriptase PCR Conditions

All assays were performed using one-step RT-PCR reagents and a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA) in a 25 μL reaction volume as employed by Moore et al. (128). RT-PCR conditions were as follows: 30 min at 48°C, 10 min at 95°C, 50 cycles of 15 s at 95°C, and 1 min at 60°C. Relative quantification was determined from a 3-4 log range standard curve generated from

serially diluted RNA to final concentrations of 10, 1, 0.1, 0.01, and 0.001 ng/μl. Quantification was performed by interpolation using a standard regression curve of C_t values generated from RNA samples of these known concentrations. Total RNA (1 ng) isolated from individual rats was used for these experiments and all samples were run in duplicate. The reaction mixture contained 900 nmol/L each of the forward and reverse primers and 250 nmol/L of PC TaqMan probe, whereas the 18S rRNA TaqMan assay utilized 50 nmol/L forward and reverse primers and 50 nmol/L TaqMan probe. In order to ensure that the total RNA samples were not contaminated with DNA, RNA samples with no reverse transcriptase added were run.

Statistical Analysis

Results were expressed as means ± SD. The significance of differences ($P<0.05$) was tested by Student's t-test. Homogeneity of variance was routinely verified and no data transformation was necessary for these experiments.

Results

Effect of CBZ on Metabolites

Direct analysis of the diet by HPLC confirmed the intended concentration of 3.4 g CBZ/kg. There were no significant differences in food intake or body weight gain between treatment groups over the 28-d experiment (Fig. 3-1). At the end of the study, serum CBZ concentration of the drug-treated group was 11 ± 7.1 μmol/L, while liver and brain CBZ concentrations were 80 ± 4.0 and 38 ± 2.1 nmol/g tissue, respectively.

After consuming the 3.4 g CBZ/kg diet for 28 d, the rats had a significantly higher concentration of lactic acid in the brain compared to control rats (10 ± 2.8 versus 6.2 ± 2.1 μmol/g, $p<0.05$). Serum concentrations of lactic acid at 28 d were similar between

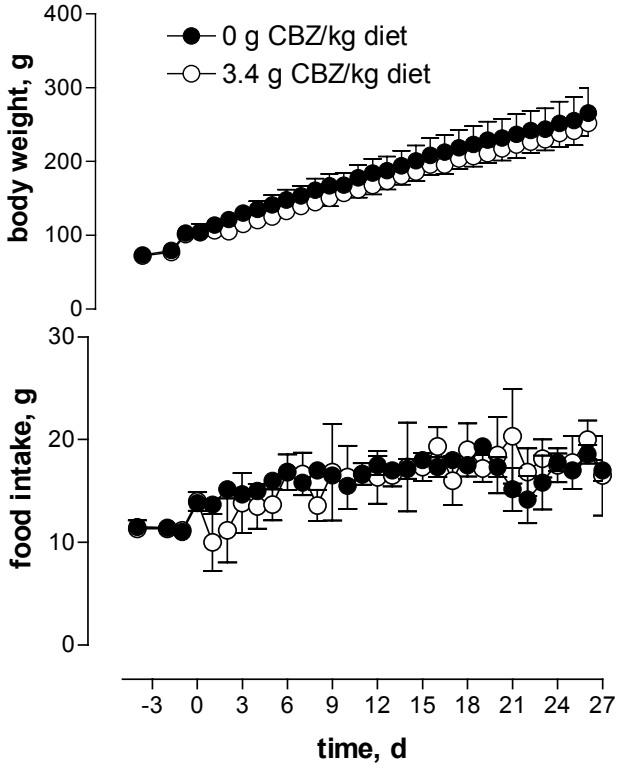


Figure 3-1. Body weights (top) and food intakes (bottom).

rats consuming the 3.4 g CBZ/kg diet (6.3 ± 1.0 mmol/L) and those consuming the control diet (7.2 ± 2.8 mmol/L).

The administration of 3.4 g CBZ/kg diet for 28-d did not have a significant effect on serum, liver, or brain biotin, BSO, or BNB (Fig. 3-2). The distribution of biotin and metabolites in brain, liver, and serum was similar to results from our previous studies (124). Only brain biocytin was significantly elevated in rats consuming 3.4 g CBZ/kg diet compared to controls (3.6 ± 1.0 and 1.2 ± 0.26 pmol/g tissue, respectively, $p<0.05$).

Effect of CBZ Administration on PC Biotinylation, Activity, and Protein and mRNA Expression

The relative abundance of hepatic biotinylated PC was decreased 43% in the rats consuming 3.4 g CBZ/kg diet compared to controls ($p<0.05$) (Fig. 3-3a). Brain

biotinylated PC was decreased 29% ($p<0.05$) in rats consuming the 3.4 g CBZ/kg diet compared to controls (Fig. 3-3a). There was no difference in the relative abundance of

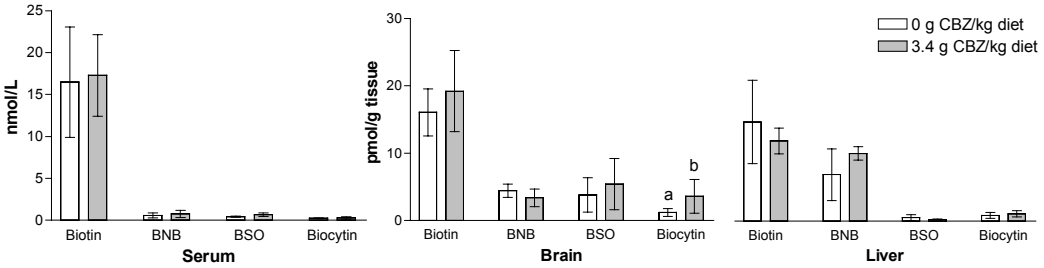


Figure 3-2. Effect of dietary carbamazepine (CBZ) on serum, brain, and liver biotin and biotin metabolites in rats. Results are means ± SD for control (0 g CBZ/kg diet, n=5) and CBZ-supplemented (3.4 g CBZ/kg diet, n=6). Means without a common letter differ, $p<0.01$.

biotinylated MCC, PCC, ACC1 or ACC2 in either brain or liver of rats consuming 3.4 g CBZ/kg diet compared to controls (data not shown). Hepatic PC specific enzymatic activity was decreased 30% in rats consuming the 3.4 g CBZ/kg diet compared to controls but brain PC activity was increased 175% in this group ($p<0.05$) compared to controls (Fig. 3-3b).

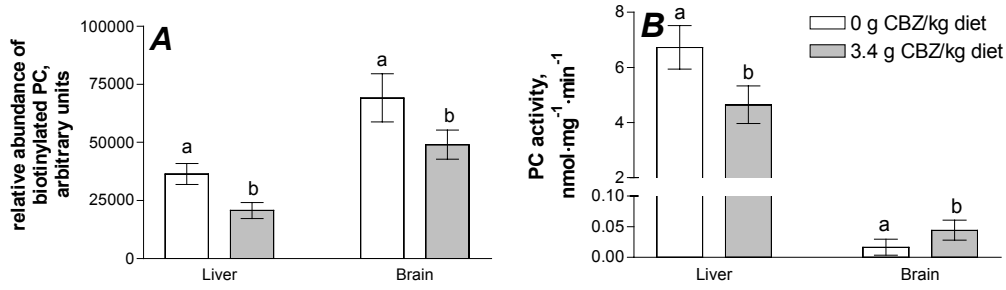


Figure 3-3. Effects of dietary carbamazepine (CBZ) on the relative abundance of biotinylated pyruvate carboxylase (PC) (A) and activity of PC (B) in rat liver and brain. Results are means ± SD for control (0 g CBZ/kg diet, n=5) and CBZ-supplemented (3.4 g CBZ/kg diet, n=6) rats. In (A), the relative abundance is the amount of fluorescence detected using a fluorescent-avidin blotting method. In (B), PC activity is expressed as nmol oxaloacetate formed per mg protein per min. Means without a common letter differ, $p<0.05$.

As assessed by avidin blotting following *in vitro* biotinylation of all available apocarboxylase, hepatic PC protein expression was significantly decreased by approximately 43% in rats consuming the 3.4 g CBZ/kg diet compared to controls (Fig. 3-4a).

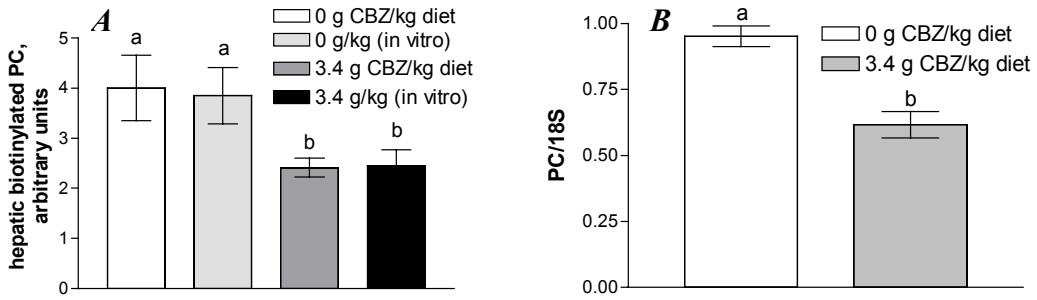


Figure 3-4. Effects of dietary carbamazepine (CBZ) on hepatic biotinylated pyruvate carboxylase (PC) before and after *in vitro* biotinylation (A) and hepatic PC mRNA determined by real-time PCR normalized to 18S mRNA (B). Results are means ± SD for control (0 g CBZ/kg diet, n=5) and CBZ-supplemented (3.4 g CBZ/kg diet, n=6) rats. Means without a common letter differ, $P<0.01$.

At the RNA level, after normalizing the hepatic PC mRNA with beta-actin, PC mRNA tended to be lower in the drug treated group compared to controls, but the difference was not significant when analyzed by northern blot (Fig. 3-5).

Real-time quantitative PCR was used to measure PC mRNA. The threshold cycle (C_t) was determined to be in the linear range of the curve by selecting an optimal R_n (emission of the fluorescent reporter over the starting background) value in the exponential phase of the amplification plot for each dilution. The standard curves were generated by plotting the C_t against the log of the mRNA concentration. A PC amplification plot for five dilutions of PC and 18S mRNA was used for the standard curves. The standard curve using the PC probe showed an R^2 of 0.999 and a slope of -3.42, compared to 18S values of 0.999 and –3.53 for R^2 and slope, respectively, indicating a high correlation between the linearity of both assays. Only concentrations of

less than 1 pg/μL mRNA deviated from the calculated standard curve, indicating that, at levels this low, quantification was not accurate. PC mRNA expression was significantly decreased 35% in rats consuming 3.4 g CBZ/kg diet (Fig. 3-4b).

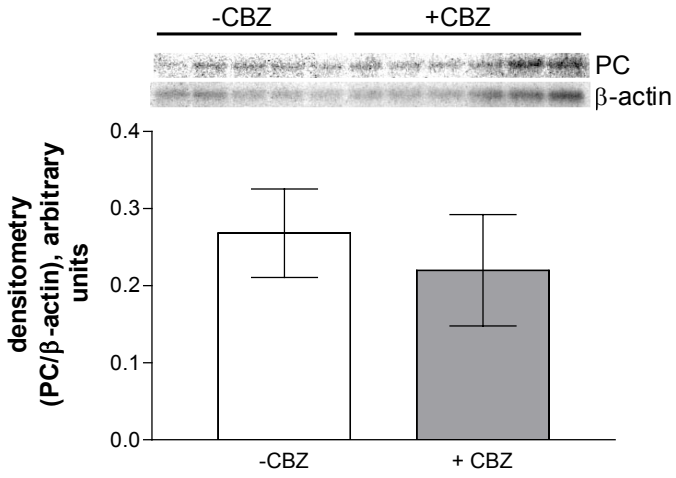


Figure 3-5. Hepatic PC mRNA determined by northern blotting. Results are means ± SD for control (0 g CBZ/kg diet, n=5) and CBZ-supplemented (3.4 g CBZ/kg diet, n=6) rats.

Discussion

It was shown in Chapter 2 that oral CBZ administration to rats alters biotin metabolism but not free biotin status. While we observed no changes in serum biotin concentrations in the present study, we observed alterations in biotin-dependent enzyme activity, mRNA, and abundance of biotinylated carboxylases. These biochemical changes in biotin metabolism may occur prior to observed decreases in serum biotin since it is known that serum biotin is not an early or sensitive indicator of biotin status (129). Since both the previous study and current study were relatively short term (21-28 d) compared to the long-term therapy associated with decreased serum biotin in epileptics (19), our protocols may not have been long enough to encounter biotin deficiency induced by CBZ administration. It is also noteworthy in light of the data generated in

this study that the dose of CBZ used may have been too low to elicit the full effects seen clinically. In our previous study, the dose of CBZ yielded a serum CBZ concentration of approximately 5 μmol/L (124), which is less than the therapeutic range for both rats and humans of 17 – 51 μmol/L (64,83). In the present experiment, we sought to increase the oral CBZ intake to result in a serum CBZ level more closely approximating the therapeutic range. Serum CBZ was approximately 12 μmol/L, which is still slightly below the therapeutic range for the treatment of epilepsy; thus, these results still could be interpreted as conservative in relation to what could occur at higher CBZ doses.

We previously found that oral CBZ administration to rats yields decreased hepatic abundance of biotinylated PC. Since the relative abundance of biotinylated PC depicts the holocarboxylase form of the enzyme and not the apocarboxylase, we wanted to determine whether the decrease was due to a reduction in the biotinylation (therefore the same concentration of total protein) or a decreased PC protein concentration. By biotinylating all of the available apocarboxylase form of the enzyme *in vitro* and then detecting by avidin-blotting (126), we determined that there was a change in total PC protein. The decrease in PC protein was confirmed by western blot using a polyclonal PC antibody (data not shown). To determine whether the decreased protein expression was due to protein degradation or altered mRNA expression, PC mRNA was quantified using both northern blotting and real-time PCR. After normalizing PC mRNA for beta-actin mRNA, there were no significant differences between the drug-treated group and controls as detected by northern blotting. Real-time PCR, however, showed that CBZ induced a statistically significant 35% decrease in hepatic PC mRNA in the drug-treated group after normalizing for 18 S rRNA. Two factors are likely involved that contribute

to the different results between methods, including the possible expression differences in beta-actin mRNA and 18 S rRNA between drug and control groups as well as the increased sensitivity and precision of real-time PCR. It is possible that CBZ alters beta-actin mRNA as well as PC mRNA. It is also true that normalizing for 18 S rRNA should be more accurate since this accounts for most of the RNA measured by an absorbance reading at 260 nm, and from that number, equal amounts were loaded in each reaction. The greater reproducibility of real-time PCR gives credence that there is in fact a significant decrease in hepatic PC mRNA in the drug-treated group compared to controls. The 35% decrease in PC mRNA was accompanied by approximately the same magnitude of decreases found for hepatic PC protein, biotinylation, and activity, which suggests the decreased mRNA expression was responsible for decreased PC protein and activity. Furthermore, the decreased PC protein provides an explanation for the decreased detection of biotinylated PC and PC specific activity observed in earlier studies (124). While the present study only utilized one dose of CBZ, we predict that CBZ decreases PC protein and mRNA expression in a dose-dependent manner based on results from our previous study where we found that CBZ decreases biotinylated PC in a dose-dependent manner (124).

The relative abundance of brain biotinylated PC was also reduced, but not to the extent that was seen in the liver (decreased 29% in brain compared to 43% in liver). This is consistent with a protective mechanism in the brain to preferentially maintain biotin status and biotin-dependent enzyme function compared to other tissues during periods of biotin deficiency (32,33,60). Biotinidase activity has also been found in previous studies to be lower in the brain compared to other tissues, which also suggests a slower turnover

of biotinylated proteins in the brain (59). This is supportive of our different results between brain and liver biocytin concentration and biotinylated PC.

The dietary administration of CBZ increased brain lactate but did not change serum lactate concentration. Elevated lactate in urine of patients undergoing long-term AED therapy is observed clinically and may be due to the same mechanism as the elevated brain lactate. Elevated lactate in the brain is associated with selective neuronal damage in cerebral ischemia and impaired neurological function (108-110,130) but more recently it has been argued that lactate may play an important role in nourishing oligodendrocytes and neurons (131-133). Whether elevated lactate concentration is detrimental to the brain is debatable, but it should be given some consideration since lactate does not easily cross the blood-brain barrier and it can act as a toxin in other tissues (49,52). One possible explanation of elevated lactate is a reduction in PC mRNA leading to decreased PC protein synthesis and decreased PC activity. PC catalyzes the carboxylation of pyruvate to form OAA and, as seen in PC deficiencies, decreased PC activity could lead to elevated lactate since pyruvate can build up and be shunted to alternate pathways, including lactate (107,134). Although we observed a decrease in hepatic mRNA, PC protein, and PC activity, there was a 175% increase in brain PC activity in the drug treated group compared to controls. This was not expected since there was a 30% decrease in the relative abundance of brain biotinylated PC, which is the active form of the enzyme. Although the mechanism underlying this discrepancy is not understood at this time, allosteric activators of PC may be responsible for the increased brain PC activity and warrants further study. Even though CBZ increased brain PC activity as measured *in vitro* and we would not expect lactate accumulation in compartments with

increased PC activity, lactate could be formed in other tissues and transported into the brain across the blood-brain barrier by the monocarboxylate transporter MCT1 that is present on vascular endothelial cells (135). In contrast, lactate accumulation in the brain that is observed in these studies may not be related to PC function *per se* and this will be investigated in the studies to follow.

Although it is established clinically that chronic treatment with AEDs including CBZ is associated with biotin deficiency, the impact on tissue biotin and biotin-dependent enzymes involved in carbohydrate, lipid, and protein metabolism is less understood. Metabolic changes of biotin in the brain are of particular importance since alterations of brain biotin status and subsequent loss of function of biotin dependent enzymes can contribute to impaired neurological health. The potential protective role of biotin supplementation during CBZ administration is the next important question that will be addressed. This characterization will have important clinical significance as biotin may have future use in the medical management of epilepsy.

CHAPTER 4
PHARMACOLOGICAL BIOTIN SUPPLEMENTATION MAINTAINS BIOTIN STATUS AND FUNCTION IN RATS ADMINISTERED DIETARY CARBAMAZEPINE

The previous animal studies presented in Chapters 2 and 3 indicated that 1.5 – 3.4 g CBZ/kg diet fed to rats for 21 or 28 d decreased specific enzymatic activity and abundance of hepatic biotinylated pyruvate carboxylase (PC) (124). An elevation of brain lactate also accompanied these changes (124), a product that has been shown to accumulate when PC activity is decreased (107,134,136,137). Decreased PC activity can lead to an accumulation of pyruvate, which might increase the flux to lactate through lactate dehydrogenase. If the elevated serum and urine lactate observed in epileptic patients treated with AEDs (19) and in the brain of rats treated with CBZ (124) is due to decreased PC activity, then increasing PC activity should decrease lactate concentration. In previous studies, biotin supplementation to biotin-deficient primary culture hepatocytes or biotin-deficient chicks increased PC specific enzymatic activity (138,139). This suggests that biotin supplementation could increase flux from lactate to pyruvate by increasing PC activity and, therefore, decrease brain lactate in CBZ-treated rats. In this investigation, we supplemented CBZ-treated rats with a pharmacological dose of biotin to determine whether biotin supplementation could reduce brain lactate by maintaining biotin status and activity of biotin-dependent enzymes.

Materials and Methods

Materials

Male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Purified biotin-free rodent diet based on a modified AIN 76A formulation described previously was obtained from Research Diets (New Brunswick, NJ)(125). Carbamazepine (CBZ), 5-ethyl-5-p-tolylbarbituric acid, carbamazepine 10,11-epoxide (CBZ-e), d-biotin, protease inhibitor cocktail, [^{14}C]$NaHCO_3$, reduced β-nicotinamide adenine dinucleotide (NADH), and *o*-phenylenediamine dihydrochloride were purchased from Sigma (St. Louis, MO); avidin-horseradish peroxidase (Avidin-HRP) and avidin-alkaline phosphatase (Avidin-AP) was purchased from Pierce Chemical Company (Birmingham, AL); 96-well microtiter plates (Nunc Maxisorb) and bovine serum albumin (BSA) were purchased from Fisher Scientific (Pittsburgh, PA). Enhanced chemifluorescence (ECF) reagent was purchased from Amersham-Pharmacia (Picastaway, NJ). Biotinylated BSA and avidin-AlexaFluor 430 conjugate were synthesized as previously described (97).

Animals and Dietary Treatments

Male Sprague-Dawley rats (n=55), 50-75 g initial weight, were housed individually in hanging wire-bottom cages in an environmentally controlled room with constant temperature (22°C) and a 12 h light:dark cycle. The rats were acclimatized by feeding a powdered, egg-white based modification of the AIN 76A diet containing 0.06 mg free biotin/kg diet (97) described in Chapter 2 for 5 days to standardize their biotin status. After 5 days of acclimatization, 5 rats were killed and serum, liver, and brain were collected as described below. The remaining 50 rats were randomly assigned to 2 groups receiving either 0 g CBZ/kg diet (n=25) or 3.75 g CBZ/kg diet (n=25) for 21 days. Rats (n=5) from each treatment group were killed on day 26 of the study, and serum, liver, and

brain were collected. Those from the remaining groups were then divided into two further treatment groups (n=10) receiving either 0.06 mg biotin/kg diet to represent a physiologic dose of biotin or a pharmacological dose of 6.0 mg biotin/kg diet. After 21 d (day 47 of the study) of biotin supplementation, 5 rats from each of the 4 groups were killed and serum, liver, and brain were collected. The remaining 5 rats from each group were killed 21 d later and serum, liver, and brain were also collected (Fig. 4-1). The entire study was 68 d. Food intake and body weights were recorded every other day. All procedures were approved by the University of Florida Animal Care and Use Committee.

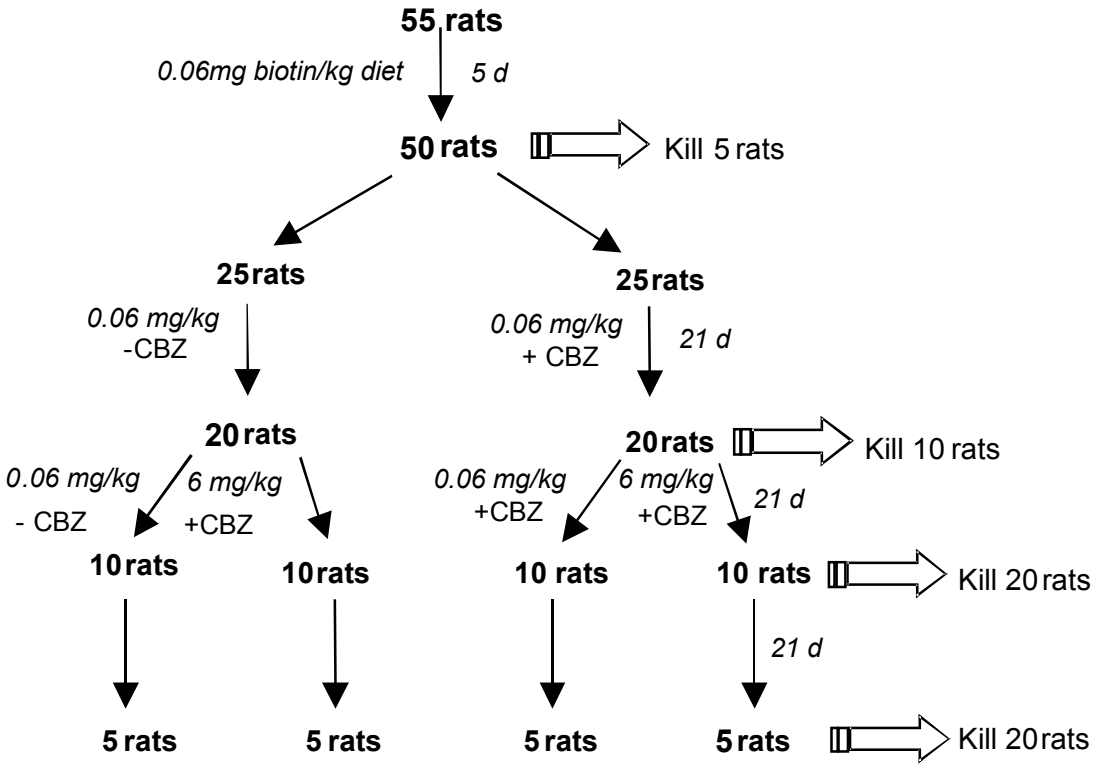


Figure 4-1. Experimental protocol.

Sample Preparation

On days 5, 26, 47, and 68, rats were anesthetized with halothane and killed by cardiac puncture and exsanguination. Whole blood was allowed to coagulate for 30 min,

and then centrifuged at 10,000 x g for 5 min to separate serum. Whole brain was removed and homogenized in 10 volumes of ice-cold HEM homogenization buffer (30 mmol/L HEPES, pH 7.2, 1 mmol/L EDTA, 300 mmol/L mannitol, and protease inhibitor cocktail). The left lobe of the liver was divided into 5 sections and all samples were immediately frozen in liquid nitrogen and stored at –80°C until needed. For protein assays and metabolite analysis, a frozen liver portion was thawed and homogenized in 10 volumes of ice-cold HEM. A portion of each homogenate was saved for protein analysis and the remainder was centrifuged at 200,000 x g for 30 min at 4°C to collect the soluble fraction for measurement of biotin, biotin metabolites, and CBZ. All samples were immediately frozen in liquid nitrogen and stored at –80°C until assayed.

Pyruvate Carboxylase and Acetyl CoA Carboxylase Activity Assays

PC activity in brain and liver was determined using a method previously described (102) with slight modifications (124). For the experiment to determine whether NADH is an activator of PC activity, the reaction mixture was similar to the modified method (124), except 0 – 2.5 mmol/L NADH was added. Background activity was determined by omitting pyruvate from the reaction mixture. Specific enzymatic activity for PC was expressed as nmol OAA formed·mg protein^{-1}·min^{-1}.

ACC activity was determined using a method described by Thampy et al. (140) with minor modifications. Briefly, frozen liver was homogenized in 10 volumes of homogenization buffer (50 mmol/L potassium phosphate, pH 7.4, 10 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L dithiothreitol, protease inhibitors) using a Polytron homogenizer. To measure activity, each tube contained 0.1 mg of either liver or brain homogenate, along with a mixture consisting of 50 mmol/L potassium phosphate,

pH 7.4, 2.5 mmol/L $MgCl_2$, 2 mmol/L dithiothreitol, 1.17 mmol/L acetyl CoA, 4 mmol/L ATP, 12.5 mmol/L [^{14}C] $NaHCO_3$ (specific activity 40.7 MBq/mmol), 0.75 mg/ml bovine serum albumin, and 10 mmol/L citrate in a final volume of 0.15 mL. After 15 min at 37°C, the reaction was terminated by adding 0.05 mL 200 g/L trichloroacetic acid (TCA), and the precipitated protein was sedimented by centrifugation at 13,000 x g for 5 min. The supernatant was transferred to a scintillation vial and 0.1 mL of 100 g/L TCA was added to wash the protein pellet. After centrifugation (13,000 x g, 5 min), the supernatant was combined with the initial extract. Pooled supernatants were dried under a stream of nitrogen at 65°C for 30 min and dissolved in 0.5 mL distilled water. Scintillation cocktail (5 mL) was added to each vial and the samples were counted by liquid scintillation counting. Specific enzymatic activity for ACC was expressed as pmol malonyl CoA formed·mg protein^{-1}·min^{-1}.

Detection of Biotinylated Biotin-Dependent Carboxylases

The five biotin-dependent carboxylases, ACC1, ACC2, PC, PCC, and MCC were detected using the avidin-blotting technique described previously in Chapter 2 (97).

Measurement of Metabolites

The measurement of biotin and its metabolites in serum, liver, and brain was performed using a coupled HPLC/competitive binding assay (97,99). Serum, liver, brain, and dietary CBZ were measured using a method previously described (100) with slight modifications (124).

NADH was measured using a method described by Uppal and Gupta (141). Briefly, 100 μL brain homogenate (as prepared above) was added to tubes containing 2 mL alcoholic 0.5 mol/L KOH [prepared by dissolving KOH in a mixture of

ethanol/doubly distilled water (1:1, v/v)] preheated in a heating block at 70°C. The tubes were immediately capped, heated for 60 s, and then quickly cooled on ice for 10 min. Then the extract, which contained both mitochondrial and cytosolic NADH, was neutralized with triethanolamine-HCl phosphate (0.5 mol/L triethanolamine-HCl, 0.4 mol/L KH_2PO_4, 0.1 mol/L K_2HPO_4) to pH 7.8 and kept at 37°C for 20 min. After incubation, the extract was centrifuged at 30,000 x g for 10 min and the supernatant was collected. Spectrophotometric measurements at 340 nm were recorded upon mixing 2 mL extract with 5 μL 1 mol/L pyruvate. Then 5 μL 0.5 mg/mL lactate dehydrogenase (D-lactate dehydrogenase from *Lactobacillus leichmannii*, 270 units activity/mg, Sigma Chemical, St. Louis, MO, prepared by diluting stock with 2.1 mol/L ammonium sulfate) was added to the cuvette, mixed, and incubated for 3 min prior to reading the final absorbance at 340 nm. NADH concentration from brain samples was determined by extrapolation from a standard curve with known NADH concentrations.

Serum and tissue lactate concentrations were measured using a kit based on spectrophotometric endpoint method of measuring NADH (Sigma Diagnostics, St. Louis, MO).

Statistical Analysis

Results are expressed as means ± SD. Differences in concentrations of biotin and biotin metabolites among treatments were tested using a Student's t-test on day 26 (2 treatment groups), and a 2-way ANOVA for data from days 47 and 68 (with biotin intake and CBZ exposure as main factors). Data for biotinylated carboxylases and lactate from day 68 of the study were tested using a 2-way ANOVA. Comparisons of CBZ concentrations between control and biotin-supplemented groups were conducted using a

Student's t-test. In all statistical procedures, a difference was considered significant at $p<0.05$. Homogeneity of variance was routinely verified and no data transformation was necessary for these experiments.

Results

Concentration of CBZ in the Diet, Serum, Liver and Brain

Analysis of the diet containing CBZ revealed 3.75 g CBZ/kg diet, which confirmed the accuracy of diet formulation. Brain, serum, and liver CBZ concentrations were determined on day 68 of the experiment (42 d after starting biotin supplementation in 2 groups). Biotin supplementation did not alter brain CBZ (46 ± 2.4 and 44 ± 5.3 nmol/g for rats consuming 0.06 or 6.0 mg biotin/kg diet, respectively), liver CBZ (53 ± 0.7 and 54 ± 0.7 nmol/g for rats consuming 0.06 or 6.0 mg biotin/kg diet, respectively), or serum CBZ (1.5 ± 0.4 and 1.2 ± 0.3 μmol/L for rats consuming 0.06 or 6.0 mg biotin/kg diet, respectively).

Effect of CBZ on Metabolites

Brain, serum, and liver lactate concentrations were determined on day 68 of the study. CBZ administration was associated with greater lactate concentrations in both serum and brain in rats consuming 0.06 mg biotin/kg diet ($p<0.05$). Biotin supplementation (6.0 mg biotin/kg diet) in CBZ-treated rats effectively prevented the increase of lactate in serum and brain as seen in Fig. 4-2 ($p<0.05$).

CBZ reduced serum biotin by 60% compared to controls ($p<0.05$) after day 47, but biotin supplementation maintained serum biotin concentration in these rats (Fig. 4-3a). Biotin-supplementation increased serum biotin concentration in both control and CBZ-

treated group, but serum biotin in the CBZ-treated group that was biotin-supplemented was only increased to 17% of the biotin supplemented control rats (61 ± 20 versus

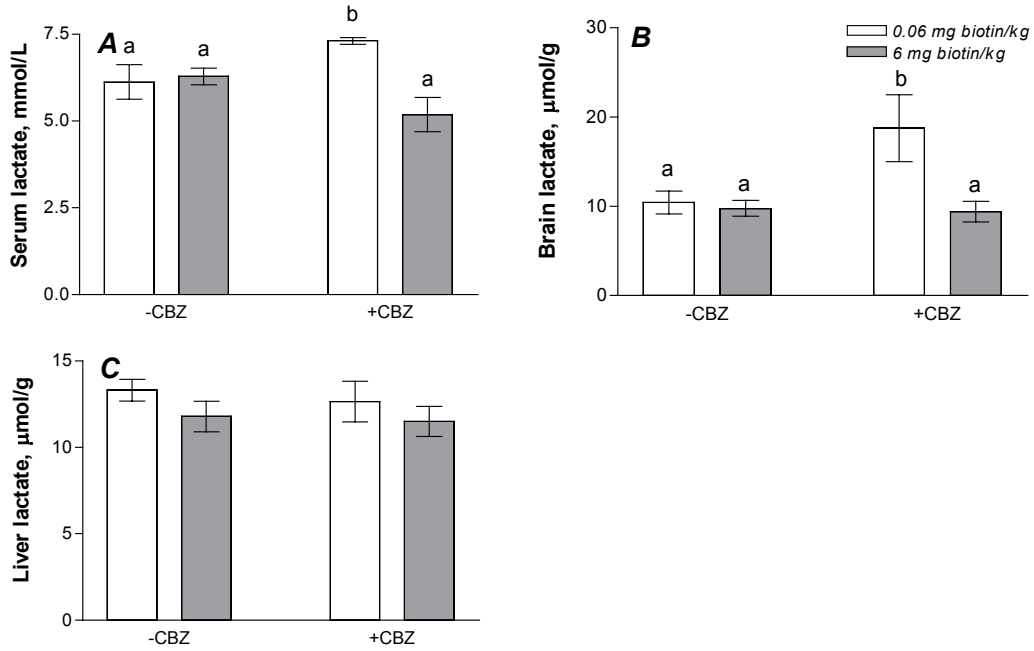


Figure 4-2. Effect of dietary carbamazepine (CBZ) on (A) serum, (B) brain, and (C) liver lactate in rats after 68 d. Results are means ± SD for controls and CBZ-treated rats (n=5/group). Means without a common letter differ, $p<0.05$.

350 ± 143 nmol/L, respectively, $p<0.05$). CBZ treatment did not alter brain biotin concentration (Fig. 4-3b). Biotin supplementation increased brain biotin concentration in both groups; however, the elevation was 12% less in the CBZ-treated group compared to the control group on day 68 (Fig. 4-3c). Hepatic biotin was reduced in CBZ-treated rats by approximately 50% compared to controls ($p<0.05$) (Fig. 4-3c), while biotin supplementation prevented the decrease of free hepatic biotin concentration in these rats. Effects of both CBZ and biotin intake were observed on concentrations of the biotin catabolites in serum and tissues. Biotin supplementation increased serum BSO concentration similarly in both controls and CBZ-treated groups, but CBZ alone had no

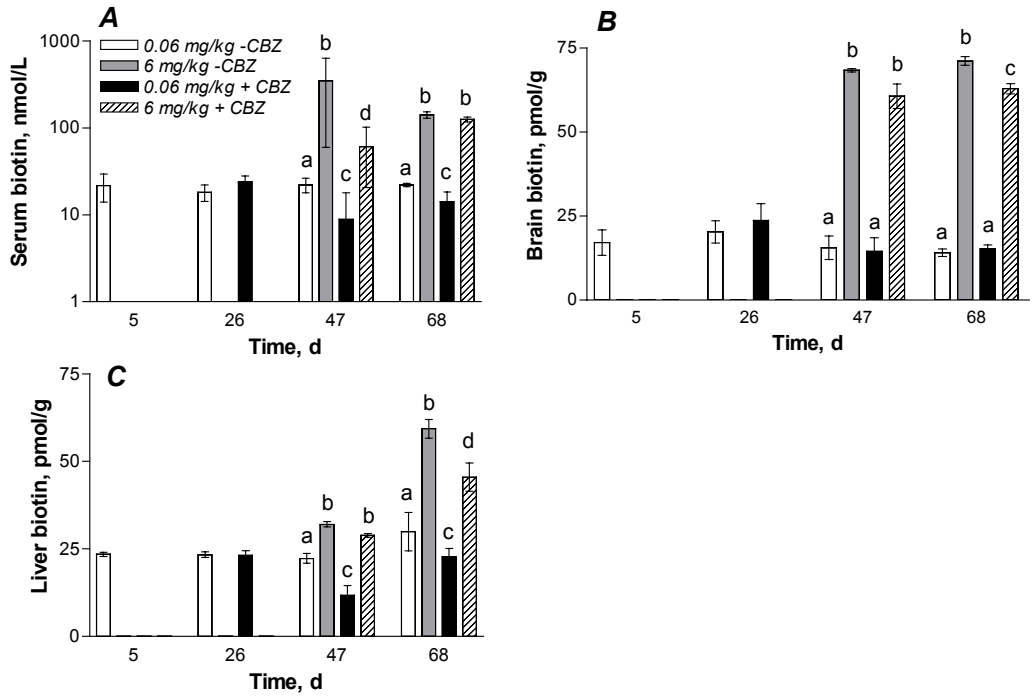


Figure 4-3. Effect of dietary carbamazepine (CBZ) on biotin concentrations in (A) serum, (B) brain, and (C) liver in rats on day 68 of the study. Results are means ± SD, n=5. Means without a common letter at each time point differ, $p<0.05$.

effect (Fig. 4-4a). CBZ increased hepatic BSO approximately 123% compared to controls on day 47 but there was no difference observed on day 68. Biotin supplementation also increased hepatic BSO in both groups (Fig. 4-4b). Brain BSO was reduced 40% in CBZ-treated rats on day 26 ($p<0.05$) but was not different from control values on days 47 and 68 (Fig. 4-4c).

CBZ increased serum BNB 27% on day 26, 163% on day 47, and 410% on day 68 (Fig. 4-5a). Biotin supplementation also elevated serum BNB in both groups where, on day 68, CBZ-treated rats had serum BNB concentration equal to biotin supplemented control and CBZ-treated rats. Hepatic BNB was elevated 89% in CBZ-treated rats on day 68 and biotin supplementation similarly elevated hepatic BNB in control and CBZ-treated rats (Fig. 4-5b). CBZ administration significantly increased brain BNB by 75%

and 45% on days 47 and 68, respectively, to approximately the same brain BNB concentration as both biotin-supplemented groups (Fig. 4-5c).

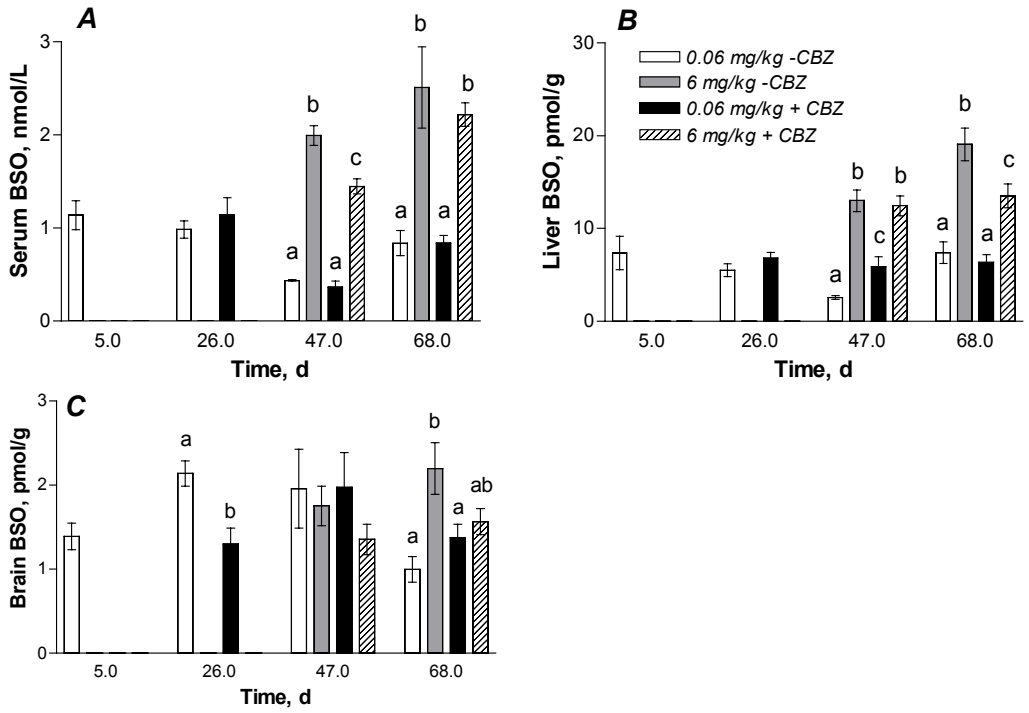


Figure 4-4. Effect of dietary carbamazepine (CBZ) on biotin sulfoxide (BSO) concentration in (A) serum, (B) liver, and (C) brain in rats on day 68 of the study. Results are means ± SD, n=5. Means without a common letter at each time point differ, $p<0.05$.

Biotin supplementation increased serum biocytin 146% in control rats but only 116% in CBZ-treated rats (Fig. 4-6a). CBZ had no effect on hepatic or brain biocytin (Fig. 4-6b, 4-6c).

CBZ had no effect on the abundance of hepatic biotinylated MCC or PCC; however, the drug significantly decreased hepatic biotinylated ACC1, ACC2, and PC by approximately 33%, 60%, and 43%, respectively (Fig. 4-7). Biotin supplementation prevented the decrease of hepatic biotinylated ACC1 and ACC2 (Fig. 4-7). All biotin-dependent carboxylases in brain were significantly decreased by CBZ (31%, 42%, 43%,

and 43% for PC, MCC, PCC, and ACC1, respectively, $p<0.05$) but only decreased biotinylated ACC1 was prevented by biotin supplementation (Fig. 4-7).

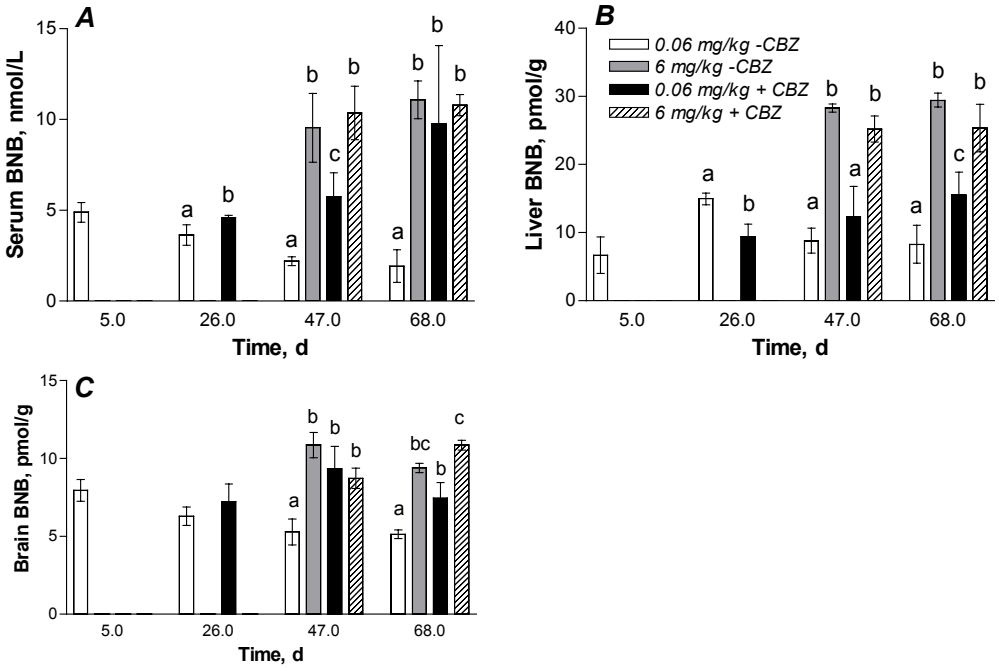


Figure 4-5. Effect of dietary carbamazepine (CBZ) on bisnorbiotin (BNB) concentration in (A) serum, (B) liver, and (C) brain in rats on day 68 of the study. Results are means ± SD, n=5. Means without a common letter at each time point differ, $p<0.05$.

CBZ significantly reduced hepatic ACC (both ACC isoforms) specific enzymatic activity 30% in rats consuming 0.06 mg biotin/kg diet but not in rats consuming 6.0 mg biotin/kg diet ($p<0.05$, Fig. 4-8a). Brain ACC specific enzymatic activity was significantly decreased 48% in CBZ-treated rats consuming 0.06 mg biotin/kg diet compared to controls (Fig. 4-8b, 73 ± 15 versus 39 ± 13 pmol malonyl CoA formed·mg protein^{-1}·min^{-1}, respectively, $p<0.05$). The biotin supplemented group (6.0 mg biotin/kg diet) exhibited higher brain ACC activity in CBZ-treated rats compared to CBZ-treated rats consuming 0.06 mg biotin/kg diet (Fig. 4-8b, $p<0.05$). Hepatic specific enzymatic activity of PC was reduced by CBZ and but the decrease was prevented by biotin supplementation (Fig. 4-9a). The increase in PC activity due to biotin supplementation is

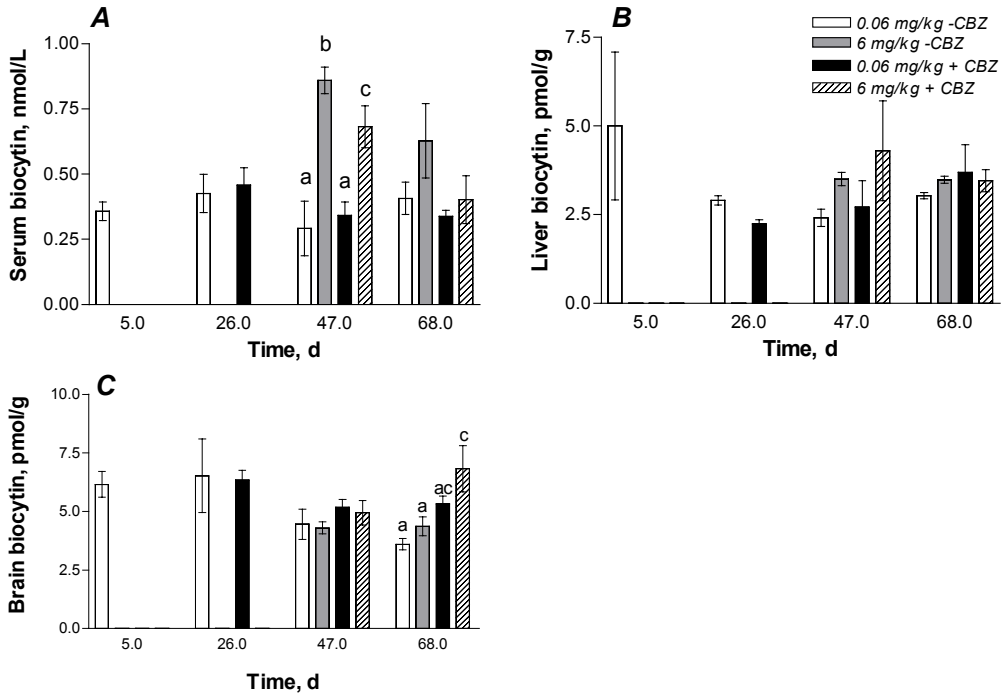


Figure 4-6. Effect of dietary carbamazepine (CBZ) on biocytin concentrations in (A) serum, (B) liver, and (C) brain in rats on day 68 of the study. Results are means ± SD, n=5. Means without a common letter at each time point differ, $p<0.05$.

not limited to rats consuming CBZ, since it was also found that biotin supplementation increased PC activity in control rats consuming 0.06 mg biotin/kg diet ($p<0.05$). Relative to non-supplemented controls, biotin supplementation only increased PC activity in CBZ-treated rats by 16% compared to a 30% increase in activity of rats not consuming CBZ (Fig. 9, $p<0.05$). A similar relationship was observed for brain (Fig. 4-9b). The 48% decrease in brain PC activity induced by CBZ was prevented by biotin supplementation (Fig. 4-9b, $p<0.05$).

Effect of NADH on Hepatic PC Activity

In order to determine the mechanism by which PC activity increased in brain and liver following biotin supplementation in the CBZ-treated rats, it was hypothesized that there are activators of PC besides the Mg^{2+} and acetyl CoA already accounted for in the activity assay. Elevated brain NADH would likely accompany decreased lactate. Since

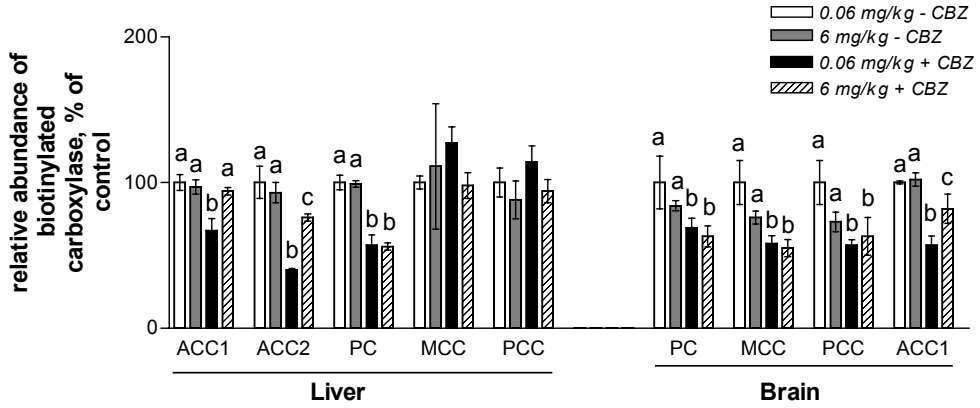


Figure 4-7. Effect of dietary carbamazepine (CBZ) and biotin supplementation on the relative abundance of biotinylated biotin-dependent enzymes in rat liver and brain. Results (means ± SD, n=5) are expressed as a percentage of the control group (0.06 mg biotin/kg diet – CBZ). Means for each carboxylase without a common letter differ, $p<0.05$.

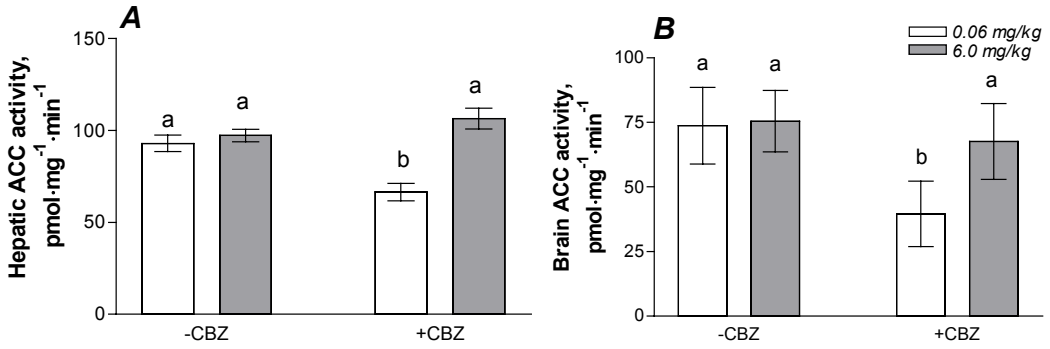


Figure 4-8. Acetyl CoA carboxylase (ACC) specific enzymatic activity in (A) liver and (B) brain on day 68 of the study. Results (means ± SD, n=5) are expressed as pmol malonyl CoA formed·mg-1·min^{-1}. Means for each activity without a common letter differ, $p<0.05$.

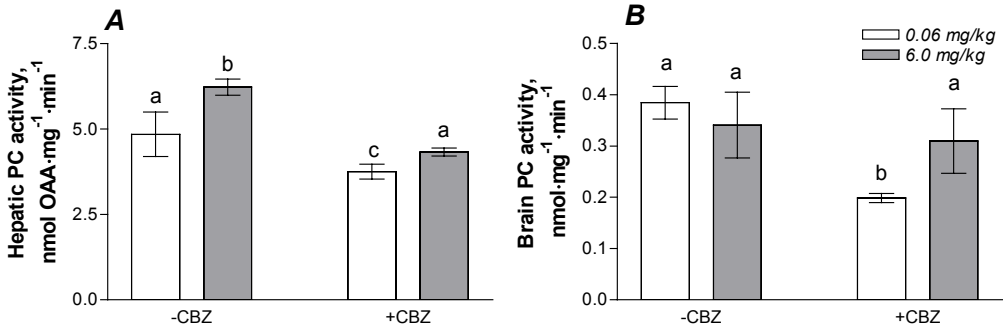


Figure 4-9. Pyruvate carboxylase (PC) specific enzymatic activity in (A) liver and (B) brain on day 68 of the study. Results (means ± SD, n=5) are expressed as nmol oxaloacetate formed·mg protein^{-1}·min^{-1}. Means for each activity without a common letter differ, $p<0.05$.

biotin supplementation prevents the increase in brain lactate and therefore maintains NADH concentration, the possibility of NADH being an activator of PC was studied. Using crude liver homogenates, we observed that NADH activates hepatic PC in both control and CBZ-treated rats. The addition of NADH to the reaction mixture brought hepatic PC concentration from CBZ-treated rats to equal hepatic PC activity from control rats having no added NADH (Fig. 4-10).

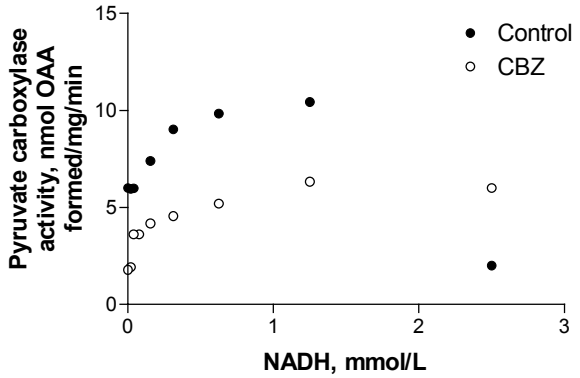


Figure 4-10. Activation of pyruvate carboxylase (PC) specific enzymatic activity by NADH in vitro using crude homogenate. PC specific enzymatic activity was determined in rat liver homogenate with 0-2.5 mmol/L NADH added to the reaction mixture for control and carbamazepine (CBZ)-treated rats.

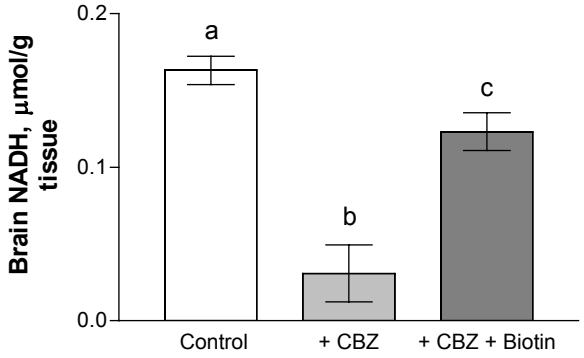


Figure 4-11. Effects of carbamazepine (CBZ) and biotin supplementation on brain NADH concentrations of rats on day 68 of the study. Results (means ± SD, n=5) are expressed as µmol NADH/g wet brain weight. Means for each bar without a common letter differ, $p<0.05$.

NADH concentration was significantly lower in the brain of rats consuming 3.75 g CBZ/kg diet (Fig. 4-11). Biotin supplementation (6 mg/kg diet) partially prevented the

decrease of brain NADH concentration in CBZ-treated rats to a range similar to that of controls.

Discussion

We investigated whether biotin supplementation could alleviate the antagonistic effects of CBZ on biotin status and function observed clinically. Clinical studies show that over 80% of epileptics undergoing long-term AED therapy exhibit some degree of biotin deficiency (90) but there are no animal studies that have been conducted regarding this phenomenon. Previous studies presented in Chapters 2 and 3 examined this effect, but showed no changes in serum, liver, or brain free biotin in rats after consuming CBZ for 21 or 28 d (124). In the present report, there was also no change in free biotin in these tissues after 26 d; however, CBZ significantly decreased serum and liver free biotin after day 47 and continued through day 68 d of the study. The observation that changes in biotin status were seen only after > 21 d of CBZ administration in this study supports the human clinical data where changes in biotin status were observed in epileptics undergoing long-term therapy (19).

In the present study, biotin and CBZ were incorporated into the diet, and therefore, they were consumed simultaneously. This is a clinically relevant design since taking CBZ with meals is recommended to patients. Since biotin and CBZ were consumed together, this provided the greatest chance for the pharmacological dose of biotin to affect CBZ uptake in the intestine. In view of other studies finding that CBZ competitively inhibits biotin uptake in the intestine (66), there was a possibility that excess biotin may likewise inhibit CBZ uptake. Our results do not support this possibility since CBZ concentrations in serum, brain, and liver were unaltered by biotin supplementation.

To determine whether elevated brain lactate during CBZ administration in rats (124) was due to decreased biotin status and PC activity, lactate was measured after biotin supplementation in rats administered 3.75 g CBZ/kg diet for 68 d. Serum and brain lactate were elevated approximately 15 and 100%, respectively, in rats consuming CBZ compared to controls, and supplementation with a pharmacological concentration of biotin (6 mg/kg diet) prevented this elevation. The elevation of brain lactate was ~10 fold higher than the increase found in our previous study where rats consumed 2.9 g CBZ/kg diet for 21 d. Not only did biotin supplementation in this present study prevent elevated brain and serum lactate in the drug-treated group, it did this without altering CBZ concentrations. This is striking evidence that decreased biotin status may, in fact, be related to elevated lactate in clinical (19) and animal studies (124) and that biotin supplementation can reduce lactate concentration.

In CBZ-treated rats, the abundance of biotinylated ACC and PC were decreased in brain and liver. As expected, specific enzymatic activities of these carboxylases also were decreased in CBZ-treated rats since the biotinylated form is the active form of the enzyme. Biotin supplementation maintained the abundance of biotinylated ACC in these tissues; however, the abundance of biotinylated PC in brain and liver remained decreased. Biotin supplementation prevented the CBZ-induced decrease of both ACC and PC specific enzymatic activity. The likely mechanism for decreased ACC specific activity involves decreased biotin nutritional status, which led to under-biotinylation of all available ACC. Biotin supplementation increased the pool of free biotin available to biotinylate any remaining apo-ACC and, therefore, increased the amount of the active form of the enzyme. The mechanism is not as easily explained for the elevated brain and

hepatic PC specific enzymatic activity following biotin supplementation since the abundance of biotinylated PC remained decreased. Whereas the precise mechanism of increased PC activity without changes in biotinylated PC is not known, changes in the concentration of an activator of PC could explain this phenomenon.

Since biotin supplementation decreased lactate concentration in CBZ-treated rats to concentrations similar to control rats, it plausible that flux from lactate to pyruvate was increased or it was excreted from the body. Since lactate does not easily cross the blood-brain barrier, it is likely that lactate in the brain was at least partially shunted back to form pyruvate, a pathway that also involves the formation of NADH. In *in vitro* analysis, NADH maximally increased PC specific activities 350 and 170% in drug-treated and control groups, respectively, compared to PC activity when measured with no NADH in the reaction mixture (Fig. 4-9). We have purified rat liver PC and the kinetics of NADH activation are characterized in Chapter 5. Along with this activation data, the observation that CBZ decreased brain NADH and biotin supplementation prevented the decrease (Fig. 4-10) supports the hypothesis that higher NADH levels in CBZ-treated rats supplemented with biotin compared to CBZ-treated rats could account for the increased PC activity.

The precise mechanism of action of anti-convulsant properties of CBZ is not completely known, but there is evidence showing it may enhance sodium channel inactivation (71). This stabilizes neuronal membranes pre- and postsynaptically by reducing high-frequency repetitive firing of action potentials or by slowing synaptic transmission (71). Another proposed mechanism is the blocking of N-methyl-D-aspartate (NMDA) receptor mediated events (73). NMDA is a glutamate receptor with involvement in seizure generation. It must be considered whether decreased biotin status

contributes to the mechanism of action of the drug. Decreased function of biotin-dependent carboxylases, particularly PC, could result in higher cerebral concentrations of carbon dioxide, which has been shown to raise seizure threshold (142,143). Likewise, excessive carbon dioxide concentrations can induce seizures (144) and, therefore, could account for seizures unresponsive to CBZ. Decreased biotin status and PC activity in the brain could also lead to decreased concentration of OAA, which is an essential precursor for *de novo* synthesis of aspartate, an excitatory neurotransmitter. One study found a modest decrease in brain aspartate concentration following phenytoin treatment in rats (145). Further research is needed to determine precisely whether biotin deficiency during CBZ therapy is involved in the mechanism of action of the drug. If it is not involved in the mechanism of action, then it seems possible that maintaining biotin status during CBZ therapy could prevent some of the side effects that some 50% of patients on CBZ experience (71).

The protective role of biotin supplementation during CBZ administration on biotin status and biotin-dependent enzyme function was demonstrated in this investigation. This characterization could serve as groundwork for future studies to determine whether biotin supplementation should be included as a concurrent strategy in the medical management of epilepsy.

CHAPTER 5
CHARACTERIZATION OF PYRUVATE CARBOXYLASE ACTIVATION BY NADH

Pyruvate carboxylase (PC) [EC 6.4.1.1] is a mitochondrial enzyme that catalyzes the formation of OAA from pyruvate and HCO_3^-, with concomitant ATP cleavage (146). The active site of PC consists of two separate subsites with biotin acting as a mobile 1-carbon carrier that is carboxylated at one subsite upon cleavage of ATP and then transfers the carboxyl group to the second subsite where pyruvate is carboxylated to form OAA (147,148). The synthesis of OAA provides a substrate for gluconeogenesis and it also replenishes tricarboxylic acid cycle intermediates used for amino acid and fatty acid synthesis.

Both PC activity and the association state of the enzyme are regulated by several factors, including various activators and the dependence of association state on enzyme concentration. Studies have shown that upon decreasing PC enzyme concentration *in vitro*, there is dissociation of the catalytically active tetrameric enzyme into inactive dimers and, hence, loss of PC activity (149). It was also found that ATP, Mg^{2+}, and acetyl CoA can shift the equilibrium back to the tetrameric form to reactivate the enzyme (149). PC shows a dependence on acetyl CoA in certain species and also requires Mg^{2+} for activity (150-153). PC mRNA expression is regulated by insulin, glucagon, and glucocorticoid concentrations during gluconeogenesis and lipogenesis (154-156). Siess et al. first suggested that mitochondrial NADH supply might be a factor in the regulation of PC activity (157). They found that NADH concentrations were elevated after PC

activation by 3-hydroxybutyrate in *in vitro* studies (157), which suggests that the activation could be due to the increase in NADH concentration. Whether NADH exerts a direct regulatory effect on PC activity has not been previously investigated.

We have demonstrated in Chapter 4 that NADH activates hepatic PC in a crude rat liver homogenate. The mechanism of activation is currently not known. In addition, it is not known whether other components of the homogenate contributed to the activation, including the possibility of oxidation of NADH to NAD^+. In this chapter, we present data showing that NADH is an activator of purified PC at high concentrations *in vitro*. We also report data regarding the influence of NADH on the kinetic constants for the different substrates from which we formulate a mechanistic hypothesis.

Experimental Procedures

Materials

Male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN) and fed a standard rodent diet. D-biotin, protease inhibitor cocktail, [^{14}C]$NaHCO_3$, reduced β-nicotinamide adenine dinucleotide (NADH), β-nicotinamide adenine dinucleotide (NAD^+), β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), and Sephacryl S200 were purchased from Sigma (St. Louis, MO); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher Scientific (Pittsburgh, PA); an ImmunoPure monomeric avidin column was purchased from Pierce (Rockford, IL). Avidin-AlexaFluor 430 conjugate was synthesized as previously described (97). Polyvinyldifluoride (Immobilon-P) was obtained from

Millipore Corporation (Bedford, MA). All other chemicals used were analytical grade and of the highest purity available.

Purification of Pyruvate Carboxylase

PC was purified using a method adapted from Oei and Robinson (158). Rat liver obtained immediately after killing of the animals by exsanguination was minced into pieces and frozen in liquid nitrogen until use. Approximately 16 g was thawed and homogenized in 20 mL HEM buffer on ice (30 mmol/L HEPES, pH 7.2, 1 mmol/L EDTA, 300 mmol/L mannitol, and protease inhibitor cocktail) for 1 min using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). The homogenate was centrifuged at 200,000 x g at 4°C for 30 min, and the soluble fraction recovered was immediately applied to an ImmunoPure monomeric avidin column (Pierce, Rockford, IL) at 4°C. Prior to application of the protein, the column was first washed with 6 mol/L guanidine-HCl, 0.2 mol/L KCl (pH 1.5) and then equilibrated with running buffer comprised of 0.1 mol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 0.1 mmol/L dithiothreitol, 5% (v/v) glycerol (Buffer A). The column was washed with 2 mmol/L biotin in the running buffer to saturate biotin-binding sites and washed with 10 column volumes of 0.1 mol/L glycine-HCl (pH 2.8) to remove exchangeable biotin. After reequilibration with running buffer, the soluble protein fraction was applied to the column and non-biotinylated proteins were eluted with 10 volumes of Buffer A. After A_{280} was < 0.05, biotinylated proteins were eluted with 2 mmol/L biotin in Buffer A. Fractions containing PC activity were pooled and directly applied to a Sephacryl S200 column equilibrated with Buffer A. PC activity was measured in collected fractions, and those containing the majority of the activity were pooled. PC activity was also assayed at each step during the purification procedure.

Purity was determined by SDS-PAGE analysis using 8% w/v total acrylamide followed by an avidin-western blot (124).

Rat liver PC was also purified using the above method for the experiment testing whether physiological NADH concentrations activate PC *in vitro*.

Pyruvate Carboxylase Activity Assay

PC activity was determined as described previously (124). Activity assays were conducted under conditions that allowed measurement of initial rate, and activity was a linear function of enzyme concentration. Protein concentration was determined by the method of Markwell et al. (101).

All of the kinetic data presented in this paper were obtained using the purified PC. Various concentrations of NADH were added to the activity assay reaction mixture (0, 1.25, and 2.5, mmol/L) to evaluate the effects of NADH on PC activity with no preincubation. In order to determine whether other pyridine nucleotides affect PC activity, a similar experiment was performed with the addition of 5 mmol/L NAD^+, NADPH, or $NADP^+$ added to the reaction mixture. K_m and V_{max} values were calculated from saturation curves of velocity versus concentration of each substrate (pyruvate, ATP, or HCO_3^-) by nonlinear regression using GraphPad Prism Enzyme Kinetics Software (GraphPad Software, Inc. San Diego, CA). For these experiments, the non-variable substrates were saturating concentrations (3.14 mmol/L ATP, 4 mmol/L HCO_3^-, and 7.5 mmol/L pyruvate).

Results

Pyruvate Carboxylase Purification

Avidin-affinity chromatography successfully isolated biotin-dependent enzymes, which were then applied to a size-exclusion column to purify PC. PC activity was eluted

approximately with the void volume of the Sephacryl S200 column. Approximately 14% of initial PC activity was recovered after the last purification step, and this yielded a 270-fold purification (Table 5-1).

Table 5-1. Purification table for rat liver pyruvate carboxylase (PC).

	Protein (mg)	vol (ml)	Activity (nmol/min)	Specific activity (nmol/min/mg)	Recovery of activity (%)	Purification (-fold)
Homogenate	1900	24	17000	9.2	100	1.0
Cytosol	490	7	7100	15	42.0	1.6
Post-Avidin column	6.0	8	2500	420	14.0	45
Post-Sephacryl S200	1.0	4	2400	2500	14.0	270

The amido stained gel and avidin-western blot (Fig. 5-1a & b) revealed that other biotin-dependent carboxylases were present in the avidin column eluant but they were not present in the Sephacryl S200 elution fraction containing PC activity.

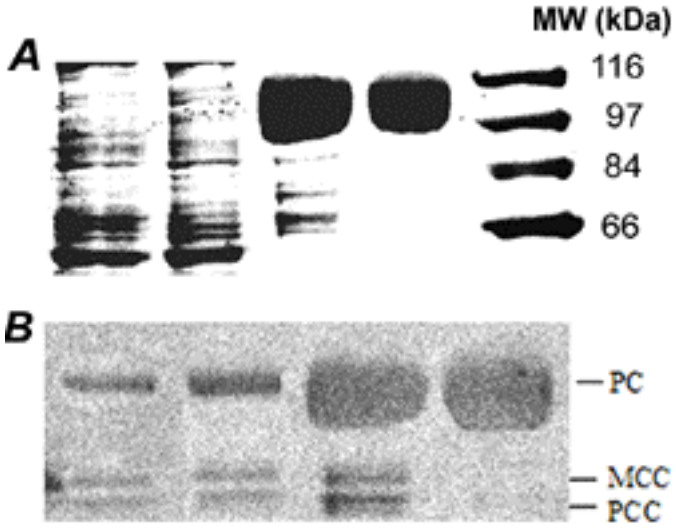


Figure 5-1. Amido-stained proteins following SDS-PAGE (8% w/v total acrylamide) (A) and avidin-western blot following PC purification (B). For both blots: lane 1, liver homogenate; lane 2, 200,000 x g supernatant; lane 3, post-avidin column; lane 4, post Sephacryl S200 column; lane 5 (A only), protein MW standards. Equal volumes of sample were applied to each lane. Pyruvate carboxylase, PC; methylcrotonyl CoA carboxylase, MCC; propionyl CoA carboxylase, PCC.

Kinetic Constants

Regardless of the concentration of NADH (0, 2.5 and 5 mmol/L) in the reaction mixture, PC obeyed Michaelis-Menten kinetics (Fig. 5-2). The Hill coefficient was determined to be 0.89 ± 0.1, which suggests that NADH does not bind PC cooperatively.

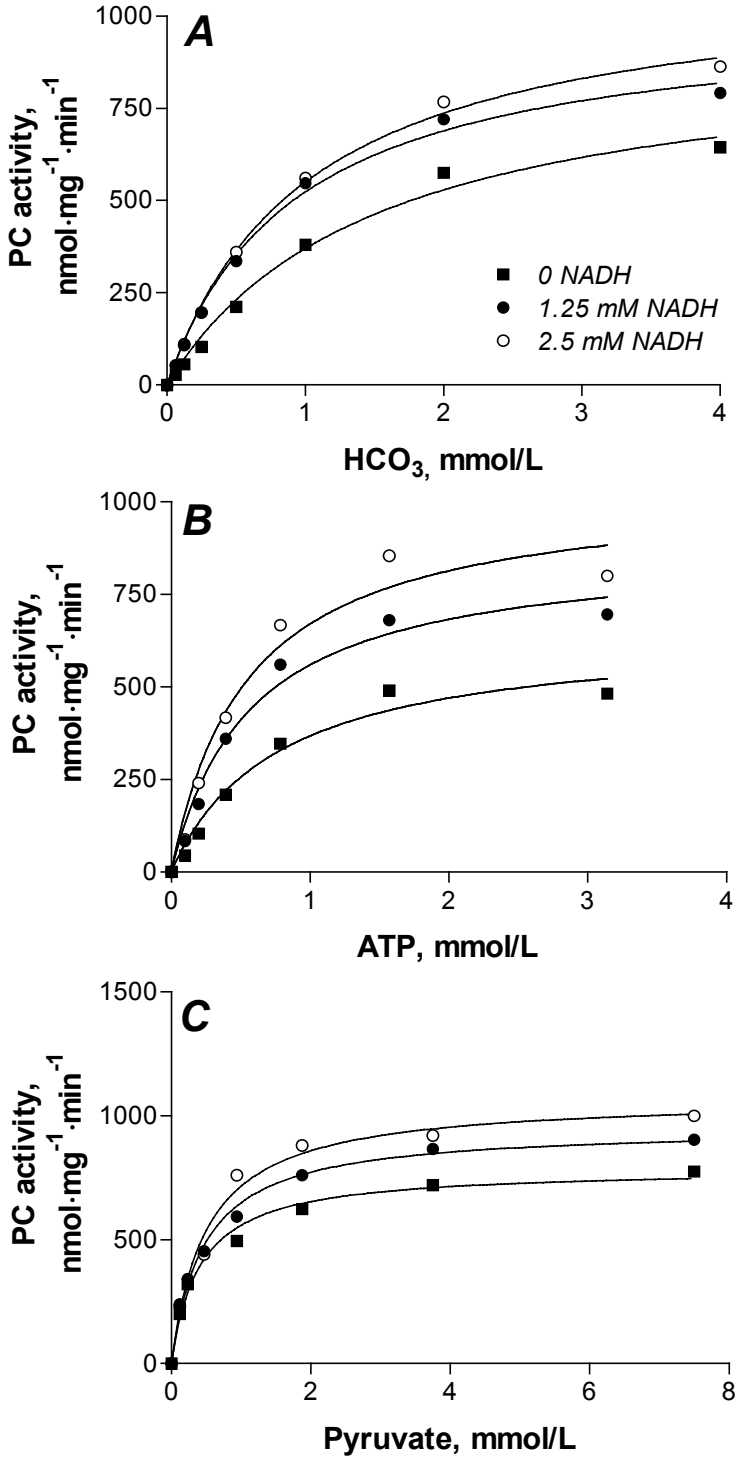


Figure 5-2. Effect of NADH concentration on the kinetics of pyruvate carboxylation by pyruvate carboxylase (PC) using variable HCO_3^- with 3.14 mmol/L ATP and 7.5 mmol/L pyruvate(A), variable ATP with 4 mmol/L HCO_3^- and 7.5 mmol/L pyruvate (B), and variable pyruvate with 3.14 mmol/L ATP and 4 mmol/L HCO_3^- (C). Each data point represents means from two independent assays with duplicate samples for each assay.

NADH altered Michaelis-Menten kinetic constants for the three substrates for the enzyme. Increasing concentrations of NADH increased V_{max} of PC for pyruvate (Table 5-2). NADH increased V_{max} 62 and 21% for PC with both ATP and HCO_3^-, respectively. The K_m for these substrates was reduced 29 and 31 % for ATP and HCO_3^-, respectively, upon the addition of 2.5 mmol/L NADH to the reaction mixture.

Table 5-2. Effects of increasing NADH concentrations on Michaelis-Menten kinetics for various substrates for pyruvate carboxylase (PC).

	0 NADH	1.25 mM NADH	2.5 mM NADH
HCO_3^-			
Km (mmol/L)	1.5 ± 0.26	0.93 ± 0.09	1.0 ± 0.09
ATP			
Km (mmol/L)	0.77 ± 0.20	0.55 ± 0.12	0.55 ± 0.15
Pyruvate			
Km (mmol/L)	0.40 ± 0.07	0.50 ± 0.05	0.50 ± 0.08

Since the conditions of this activity assay do not constitute an oxidizing environment (124), it is not likely that NADH is being converted to NAD^+ in the reaction mixture. However, to determine unambiguously whether PC activation is specific for NADH, other pyridine nucleotides were also tested (NAD^+, $NADP^+$, and NADPH). These compounds did not activate PC, but instead decreased activity with increasing concentrations (Fig. 5-3).

Unlike the effects observed with PC from crude liver homogenate seen in Chapter 4, physiological concentrations of NADH (0.05-0.30 mmol/L) did not activate purified PC (Fig. 5-4).

Discussion

There is reason to believe that physiological and pharmacological concentrations of NADH activate PC activity based on our preliminary studies using unpurified PC in Chapter 4. In order to determine explicitly whether NADH is an activator of PC specific

enzymatic activity, PC from rat liver was purified. We found that pharmacological concentrations of NADH alter Michaelis-Menten kinetic constants for pyruvate, ATP, and HCO_3^-, which indicates that pharmacological concentrations of NADH are capable of regulating PC activity. Because $NADP^+$, NAD^+, and NADPH did not activate PC and actually suppressed PC activity, we conclude that NADH is a specific activator for PC.

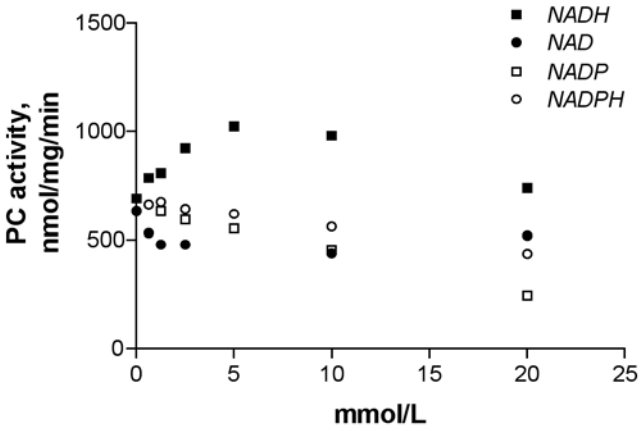


Figure 5-3. Purified rat liver pyruvate carboxylase (PC) specific enzymatic activity determined with and without 5 mmol/L NADH, NAD^+, $NADP^+$, or NADPH added to the reaction mixture. Each data point represents means from two independent assays with duplicate samples for each assay.

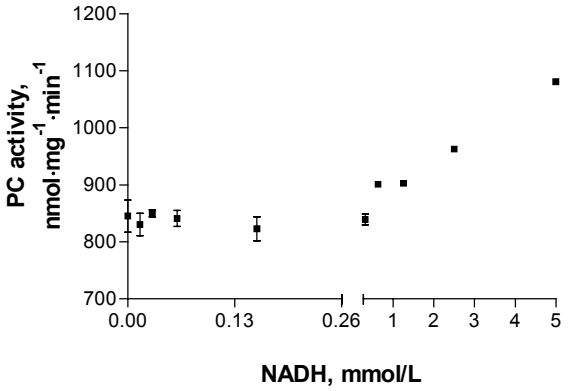


Figure 5-4. Physiological and pharmacological NADH effects on specific enzymatic activity of purified pyruvate carboxylase (PC).

Data from Chapter 4 indicate that physiological concentrations of NADH also activate PC, which suggests that cellular NADH concentration may regulate PC activity. We did

not find this to be the case, however, with purified PC *in vitro*. At physiological concentrations of NADH (0.05-0.30), there was no change in PC specific enzymatic activity. The fact that PC from the crude homogenate and the purified PC do not behave similarly suggests that there is an unknown factor in the crude homogenate that is responsible for the activation. It is possible that the endogenous NADH in the crude homogenate may also have an effect on PC.

The reaction catalyzed by PC is a sum of two partial reactions that occur at different sites on the enzyme (147,148). The valeric acid side chain of biotin is covalently bound to a specific lysine residue 35 amino acids from the C-terminus of the enzyme (159) and the long chain can act as a hinge to allow for the carboxylated biotin molecule to move from one reaction site to the next. In the first reaction site, biotin is carboxylated and ATP is concurrently hydrolyzed. Carboxylated biotin is then shifted to the second reaction site where the 1-carbon unit is transferred to pyruvate to form OAA. Since the decrease in K_m was specific for ATP and HCO_3^- but not pyruvate, we can speculate that NADH is acting at the site where biotin is carboxylated in the first partial reaction. While the 3-dimensional structure of PC has not been fully determined, there may be a region present that resembles a Rossmann fold that is a common binding site for enzymes requiring nucleotide binding for activity (160). The ATP/HCO_3^- binding domain (site of first partial reaction) of yeast PC is between amino acids 157 and 468, while the pyruvate binding domain (site of second partial reaction) is between amino acids 559 and 913. A conserved motif among proteins with a Rossmann fold is KMSKS, and the closest match in the PC sequence is between amino acids 442-446 with KMSRA.

This could possibly allow similar folding since the charges would be similar at physiological pH.

Regulation of PC activity plays a crucial role in the control of gluconeogenesis. The design of metabolic reactions is such that anabolic and catabolic pathways are regulated in order to minimize futile cycling. Glycolysis and gluconeogenesis are examples of such metabolic pathways that are carefully regulated. It recently has been found that NAD^+ is an allosteric activator of pyruvate kinase in rat liver cells (161). Increased cytosolic NAD^+ concentration was associated with decreased gluconeogenesis and enhanced glycolysis (161). Our data, which indicate that NADH activates purified rat liver PC at high concentrations and at both high and low concentrations for PC from crude liver homogenate (Chapter 4), suggest that increased NADH may stimulate gluconeogenesis *in vivo*. In the present studies, we may not be accounting for an unknown factor or effect of NADH on PC from crude liver homogenate and we cannot rule out this same effect *in vivo*. Thus, these data provide evidence for another mechanism by which gluconeogenesis might be regulated *in vivo* but requires further investigation.

CHAPTER 6
DISCUSSION

This project describes the use of a rat model to address various aspects of altered biotin metabolism during oral CBZ administration. CBZ alters both biotin status and function after long-term therapy in rats. Most investigations of biotin metabolism during CBZ administration done previously were from clinical trials and have included plasma or urine biotin, BNB, and BSO measurements. Only one animal study examined the effects of three days of i.p. administration of CBZ on biotin, BNB, and BSO concentrations in the plasma (103). There were no differences found among these metabolites following CBZ administration, and it was suggested that a rat might not be a good model for human response of biotin after CBZ. There were several potential problems with this study, however, including the i.p. mode of administration. As stated earlier, one of the potential mechanisms of decreased biotin status is decreased biotin uptake across the intestinal brush border (66), and i.p. administration of CBZ would bypass this competitive uptake. Also, CBZ was only administered for three days in the study. Clinical data showing that AEDs decrease plasma biotin 45-50% are from patients undergoing long-term therapy for many years (19,20) and, therefore, three days may not have been long enough to elicit a response.

Our rat model of oral CBZ administration is also clinically relevant in that the CBZ concentrations used in all of our studies were not toxic based on observations that all rats continued to gain weight for the entire duration of the studies. Other rat studies of oral CBZ administration report evidence that dosages were toxic based on decreased weight

gain during the study and food intakes were not reported (83). The dietary model used in the experiments outlined for this project was useful because CBZ was administered orally, which closely models normal administration of the drug in humans, and biotin intake was physiologically relevant. Although serum CBZ concentrations in all three experiments were lower than the 4-12 mg/L therapeutic range for humans, we still observed alterations of biotin status and function. Therefore, our animal model could be considered as conservative compared to studies in humans with plasma CBZ between 4-12 mg/L.

These studies demonstrated that biotin status and function of biotin-dependent enzymes at the tissue level are decreased during CBZ administration and that this decrease is prevented by biotin supplementation. Previously, changes in biotin status and function in tissues were poorly understood since most data of decreased plasma biotin status during AED therapy were from clinical trials. We, however, speculated that there would be a decrease in biotin-dependent enzyme function in tissues based on clinical observations that patients undergoing long-term AED therapy excreted high concentrations of organic acids including lactic acid, propionic acid, and 3-HIA. Although not definitive, these organic acids imply a loss of function of PC and ACC, PCC, and MCC, respectively.

It is not well understood how pharmacological biotin supplementation prevented decreases in hepatic and brain PC specific enzymatic activity without altering the abundance of the biotinylated form of PC, which is the active form of the enzyme. One possibility is that there is an activator of PC not accounted for in the enzyme activity reaction mixture. Since biotin supplementation also prevented the decrease of ACC

activity, the flux through this pathway could be increased along with an increased flux of lactate back to pyruvate (Fig. 6-1). This reaction, catalyzed by lactate dehydrogenase, is also accompanied by the formation of NADH (Fig. 6-1). We found that physiological concentrations of NADH activate PC from crude homogenate *in vitro* but not from purified PC. Since we also found that CBZ significantly reduced brain NADH concentration and that biotin supplementation partially prevented this metabolic effect, it is possible that the NADH generated after biotin supplementation and increased ACC activity was enough to increase PC activity *in vivo* without changing the abundance of the active form of the enzyme.

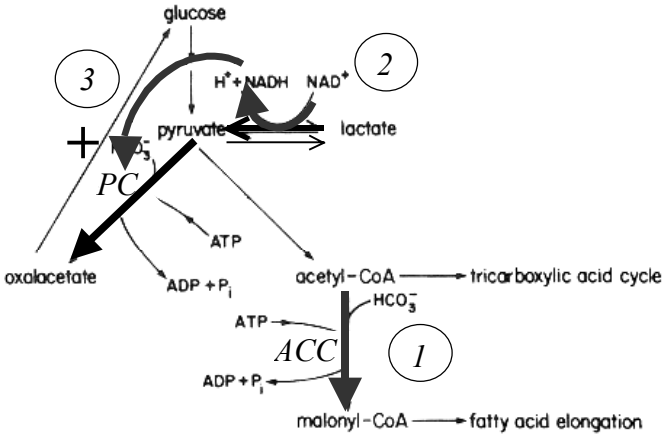


Figure 6-1. NADH as an activator of pyruvate carboxylase (PC). It is proposed that, upon biotin supplementation, ACC activity is increased and flux through the pathway catalyzed by this enzyme is increased (1). As a result of increased flux through this pathway, there is an increased flux of lactate to pyruvate and a resulting increase in concentration of NADH (2). NADH then activates PC without changing the abundance of biotinylated PC (3).

The studies presented in this project show that oral CBZ administration to rats increases brain and serum lactate concentrations, and that biotin supplementation can completely prevent these increases. It is evident that biotin supplementation during CBZ therapy may be effective in preventing the lactic aciduria experienced by epileptics during long-term AED therapy (19,20). Before we can suggest that biotin

supplementation should be considered for future medical management for the treatment of epilepsy to prevent this side effect, we must demonstrate that lactic acid buildup in the brain is actually an adverse effect. It is assumed that the elevated brain and serum lactate during CBZ administration to rats is an adverse side effect based on adverse effects of lactate accumulation in other tissues. Intravenous infusion with racemic D-, L-lactate into rats was accompanied by bradycardia when serum lactate was 1-2 mmol/L and fibrillation and ventricular standstill at 11 mmol/L (51). In other studies, patients with short bowel syndrome had elevated D-lactate (0.5 mmol/L) that was produced from colonic bacteria and was correlated with confusion, loss of memory, slurred speech, unsteady gait, and visual blurring (162). There is a possibility, however, that increased lactate could be involved with the mechanism of action of the drug.

One of the most important questions that must be asked as a result of this research is whether decreased biotin status is a part of the mechanism of action of CBZ. If this were the case, then biotin supplementation during CBZ therapy would be expected to decrease the efficacy of the drug in the prevention of seizures. Biotin deficiency is accompanied by decreased PC activity, an enzyme that catalyzes the conversion of pyruvate to OAA. The overall effect of decreased PC activity is a relative diminution of the OAA pool and a consequent shift of the aspartate aminotransferase reaction away from aspartate, an excitatory neurotransmitter (Fig. 6-2). Increased glutamate concentrations would allow more substrate to be available for the glutamate decarboxylase reaction favoring γ-aminobutyric acid (GABA) production, which is an inhibitory neurotransmitter. Increased inhibitory neurotransmitter and decreased excitatory neurotransmitter concentrations that accompany a biotin deficiency could

potentially decrease seizure activity and, therefore, could be a mechanism of action for CBZ. Ketogenic diets are used to manage epileptic seizures, and the mechanism of action may be similar to metabolic perturbations that are observed during a biotin deficiency (163). One proposed mechanism of action of the ketogenic diet is a decreased pool of OAA since β-hydroxybutyrate utilizes available coenzyme A to produce acetyl CoA and, therefore, there is a decreased supply of TCA cycle intermediates. This is proposed to lead to decreased OAA, which would then yield increased inhibitory and decreased excitatory neurotransmitters.

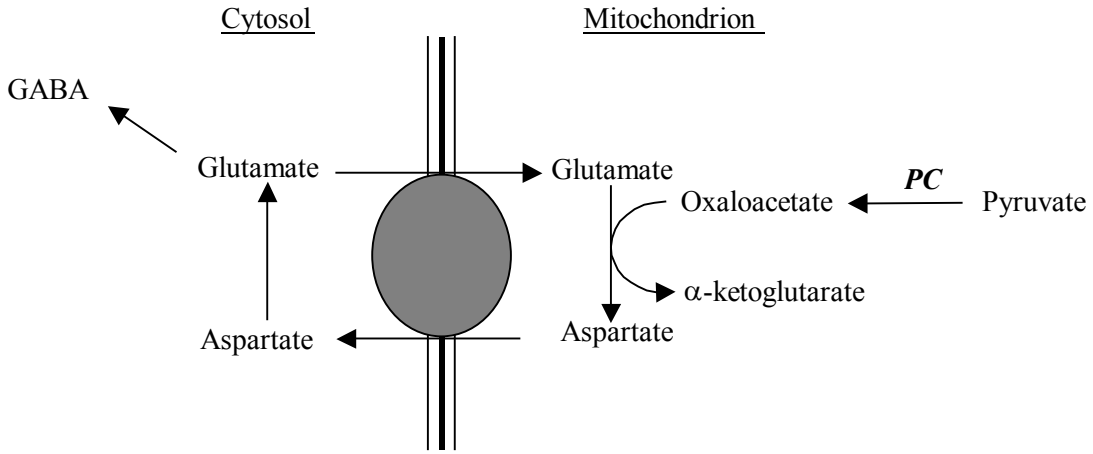


Figure 6-2. Mitochondrial glutamate-aspartate translocator. PC, pyruvate carboxylase; GABA, γ-aminobutyric acid.

If it were the case that decreased biotin status is part of the mechanism of action of CBZ, it would obviously not be recommended that epileptic patients receive concomitant biotin supplementation to increase status. Then, however, the consequences of long-term biotin deficiency must be addressed for patients who may require CBZ therapy for an entire lifetime. Seizure control among epileptic patients will be top priority for clinicians, but implications of biotin deficiency may need to be considered since it is apparent that CBZ therapy decreases biotin status and function of biotin-dependent enzymes. While studies have not been done directly addressing the effects of a long-term

marginal biotin deficiency, it is likely that the metabolic pathways of carbohydrate, amino acid, and fatty acid metabolism that involve biotin-dependent enzymes would be altered. Since it has been shown in animal studies that biotin deficiency during pregnancy is teratogenic (164,165), this could also be a potential public health concern for women taking CBZ. CBZ administration during pregnancy increases chances for birth defects and developmental delay among the fetus (166-170).

The future direction of this research may involve an investigation of the efficacy of CBZ in seizure control with concurrent biotin supplementation in an animal model of epilepsy. There are several animal models of epilepsy, including induction of seizures in an animal model with electrical stimulation, chemoconvulsants, and genetic models including a genetically epilepsy-prone rat strain. Only after it has been tested in an animal model and it has been found that CBZ is still effective in preventing seizures with concurrent biotin supplementation will this be likely to be tested in humans.

The current project has demonstrated that biotin supplementation can completely prevent decreases in biotin status, biotin-dependent enzyme function, and increases in lactate concentration induced by long-term oral CBZ administration. The public health significance of the decreases in biotin status and function during CBZ therapy is important, particularly for epileptics, and may explain some of the adverse side effects experienced by nearly 50% of patients undergoing long-term AED therapy. Biotin supplementation during pregnancies of women taking CBZ should especially be considered to possibly reduce teratogenic risks commonly associated with CBZ therapy during pregnancy. This project has expanded the area of nutrient-drug interactions and the data presented here should provide a basis for the direction of future studies in this

field to determine whether biotin supplementation should be included in the medical management of epilepsy.

APPENDIX
GENERAL METHODS

Protein Concentration Measurement

According to a method by Markwell et al. (101), the protein concentration in tissue samples was determined as follows. Samples or a standard curve using various concentrations of known amounts of BSA (0 – 100 mg/mL) diluted in dH_2O were made in duplicate. An aliquot (1 mL) of solution C [100:1 solution A (2% Na_2CO_3, 0.4% NaOH, 0.16% Na^+, K^+ Tartrate, 1.0% SDS (w/v) to solution B (4% $CuSO_4$)] was added to each sample or standard, vortexed, and incubated at room temperature for 10 min. Folin phenol (100 μL, 1 mol/L) was then added, immediately vortexed, and incubated at room temperature for 45 min and the absorbance was read at 650 nm. A nonlinear regression (second order polynomial) was performed and unknown sample protein concentrations were extrapolated from this curve.

Preparation of HPLC Standards

Synthesis of L and D-Biotin Sulfoxides

Acid peroxide (0.1 mol/L HCl, 1% v/v H_2O_2) was mixed with an equal volume of either 100 μmol/L [^{3}H] biotin (32 Ci/mmol) or unlabeled biotin and incubated for 24 h at room temperature. The reaction was stopped by adding 1.5 volumes of 0.1 mol/L NaOH and the samples were dried under N_2 at 37°C. Finally, biotin sulfoxides (BSO) were resuspended in 10 mmol/L KP_i, pH 7.0.

Synthesis of Bisnorbiotin

Bisnorbiotin (BNB) was made using a previously described method with minor modifications (50). *Rhodotorula rubra* (ATCC) was grown in YPD broth (50 g/L, Becton Dickinson, Sparks, MD) and then washed 4 times in 0.9% saline (w/v) by centrifugation (10 min at 1,500 x g) to remove growth medium. The cells were resuspended in 10 mL of 0.25 mol/L phosphate buffered saline, pH 7.2. [^{14}C] biotin was diluted to 50 μCi/mL in 2 mL of 0.25 mol/L PBS. 1.75 mL (87.5 μCi) of the [^{14}C]biotin was added to 1 mL of cells. The yeast was cultured for 48 h with constant gentle shaking at 190-200 rpm at 25°C. Then the cells were removed by centrifugation (20 min, 1,500 x g, 4°C), and the supernatant that contained the [^{14}C]BNB was retained. The supernatant was centrifuged at 1,500 x g for 10 min at 4°C to remove any remaining cells. BNB was separated from any remaining biotin by reversed phase HPLC described below. It was determined that the conversion of biotin to BNB was 92% efficient and the theoretical concentration of the newly synthesized BNB was adjusted accordingly (Fig. A-1).

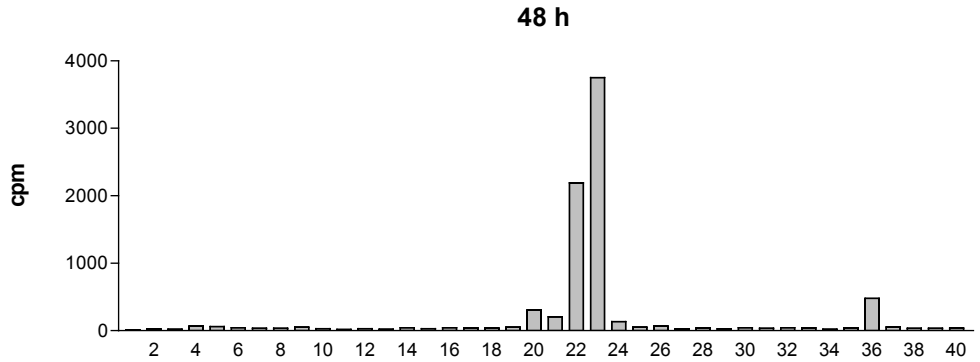


Figure A-1. Bisnorbiotin (BNB) synthesis. 48 h after incubation of *Rhodotorula rubra* with [^{14}C]biotin, 92% is converted to [^{14}C]BNB.

HPLC Conditions for Biotin Measurements

All samples were ultrafiltered using a 5000 molecular weight cutoff filter prior to injection to remove protein bound biotin. Therefore, this method is solely for the measurement of free biotin. Biotin and its metabolites were separated using a mobile phase gradient with two solutions. Solution 1 consisted of 0.05% (v/v) trifluroracetic acid (Sigma, St. Louis) adjusted to pH 2.5 with ammonium acetate, and Solution 2 was a 1:1 (v/v) dilution of Solution 1 mixed with HPLC grade acetonitrile (Fisher). At time zero, the gradient was 0% Solution 2, 100% Solution 1 and by 35 min, the gradient reached 60% Solution 1 and 40% Solution 2. Nonpolar molecules in the sample were then removed from the column after the analytical gradient by increasing Solution 2 to 100% for 5 min. The column was then re-equilibrated in 100% Solution 1 for the next 20 min for a total run time per sample of 60 min. Biotin eluted at fractions 35-36, BSO eluted at fractions 19-20, biocytin eluted at 30-33, and BNB eluted at fractions 22-23.

Method Validation for the Measurement of Carbamazepine in Serum Using HPLC

A Waters Model 501 HPLC (Millipore, Bedford, MA) with a Waters U6K injector (Millipore), a Waters 486 Model tunable absorbance detector (Millipore, Bedford, MA), and a 5 μm, Luna C-18 reversed-phase (Phenomenex) 250 X 2 mm column was used to measure CBZ concentration in the serum (100). A 1 mg/mL stock standard of CBZ was made in HPLC-grade methanol. Various concentrations of CBZ working stock standards (2.5 – 20 mg/L) were prepared freshly in 10% distilled water and 90% phosphate buffer (0.4 mM potassium phosphate, adjusted to pH 6.0 with 0.9 mol/L phosphoric acid). The mobile phase consisted of 17:28:55 acetonitrile/methanol/phosphate buffer. 5-Ethyl-5-p-tolylbarbituric acid (tolylbarb) was used as an internal standard to compare the peak

height ratio with that of CBZ. Samples were prepared by precipitating proteins with acetone containing 10 mg/L tolylbarb added to an equal volume of serum as described in Chapter 2. Sample or standard (5 μL) was injected and eluted with mobile phase at ambient temperature and 0.2 mL/min, monitoring the effluent at 195 nm. Tolylbarb consistently eluted around 15.5 min and CBZ eluted at 19.5 min as seen in spiked serum samples in Fig. A-2.

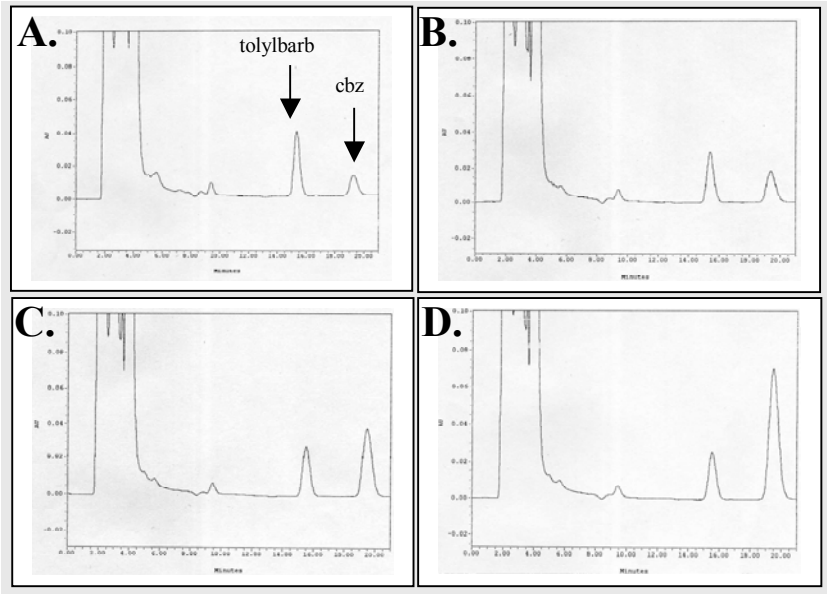
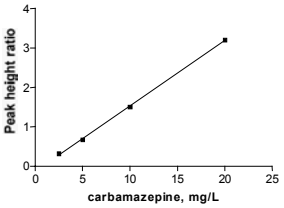


Figure A-2. The 4 chromatograms represent spiked serum samples separated by HPLC with known amounts of carbamazepine (CBZ): 2.5mg/L (A), 5 mg/L (B), 10 mg/L (C), and 20 mg/L (D). 10 mg/L of the internal standard tolylbarb was used with each run.

The peak areas were determined by Millennium 2010 Chromatography Manager software and the peak area ratio between CBZ and tolylbarb was determined for each standard and sample. The concentration of CBZ in the sample was then determined by extrapolation from the linear regression of the standard curve. In order to determine % recovery with spiked samples, various concentrations of CBZ were added to a serum

sample and the peak area ratio was determined (Fig. A-3). The interassay error between days (same sample was measured 10 different days) was determined to be 1.66%.



Spiked samples	Mean Ratio ± SEM	% recovery
2.5 mg/L	0.34 ± 0.012	110 ± 2.4
5 mg/L	0.69 ± 0.014	98 ± 1.4
10 mg/L	1.5 ±0.008	99 ± 0.27
20 mg/L	3.0 ± 0.013	95 ± 0.58

Figure A-3. Standard curve for carbamazepine (CBZ) with table of values where serum was spiked with known concentrations of CBZ.

Separation of Propionyl CoA Carboxylase and Methylcrotonyl CoA Carboxylase

In order to determine the proper conditions to separate MCC and PCC, the following experiment was done (Fig. A-4). We found that loading 80 μg of liver homogenate onto an 8% (w/v total acrylamide) SDS-PAGE gel with a separating gel buffer pH 8.0 maximally separated these two carboxylases that only differ in molecular weight by 0.5 kDa.

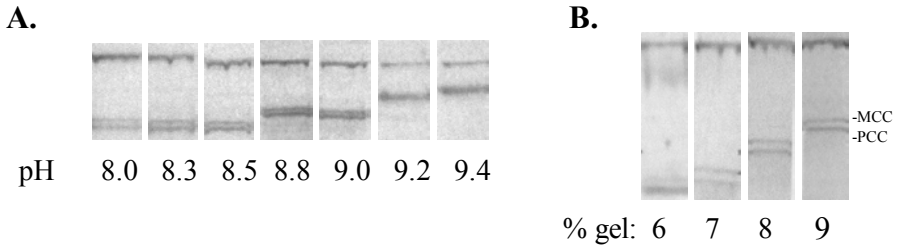


Figure A-4. Separation of propionyl CoA carboxylase (PCC) and methylcrotonyl CoA carboxylase (MCC) using SDS-PAGE. In (A), separating gel buffers with different pH were used to optimally separate PCC and MCC (lower 2 bands in gels). In (B), a separating gel buffer with pH 8.0 was used with different % (w/v) total acrylamide.

Linearity of Avidin-AlexaFluor 430 Detection Using STORM Fluorescent Scanner

Loading 80 μg liver homogenate using the conditions defined above to separate PCC and MCC has been determined to be within the linear range of the detection limits

of avidin AlexaFluor-430 using a STORM fluorescent scanner (Fig. A-5). To determine the linear range of detection, a range of homogenate masses were loaded on a 10% (w/v total acrylamide) SDS-PAGE gel and detected with avidin AlexaFluor-430. The specificity of this detection was confirmed through competition with excess avidin. Liver homogenate (80 μg) was resolved by SDS-PAGE and transferred to PVDF as described in Chapter 2.

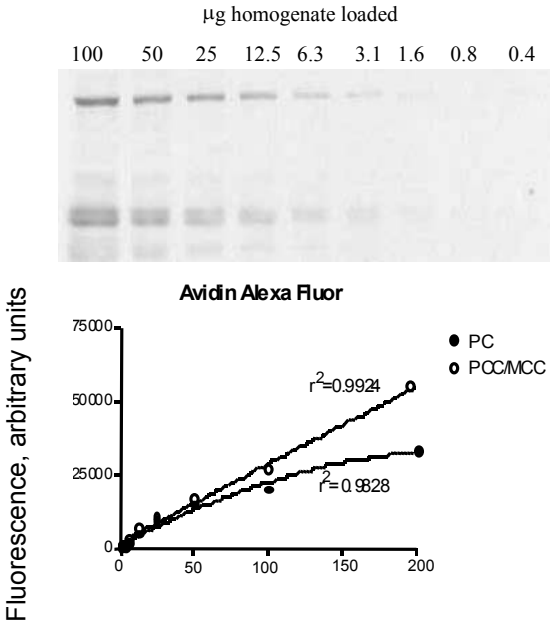


Figure A-5. Linearity of liver homogenates using avidin AlexaFluor-430 for detection.

Biotinylated Protein Competition with Biotin Using Avidin-AlexaFluor 430 Detection

In order to determine specificity of the detection of biotinylated proteins using avidin-AlexaFluor-430, an avidin-western blot was performed with excess biotin to compete for binding to the avidin. For the competition, 0.1 μmol/L biotin was competed with 1:750 avidin AlexaFluor-430 (Fig. A-6).

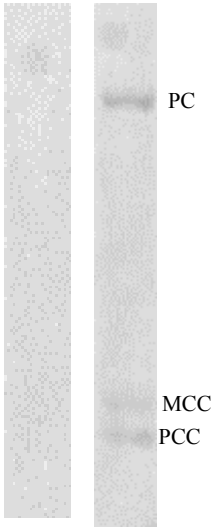


Figure A-6. Avidin competition. Biotin-dependent enzymes, pyruvate carboxylase (PC), methylcrotonyl CoA carboxylase (MCC), and propionyl CoA carboxylase (PCC) are competed in the blot on the left with excess biotin.

Diet Preparation

A diet commonly used for the analysis of dietary components formulated by the American Institute of Nutrition (AIN 76A) was used in the following experiments (Research Diets, Inc., New Brunswick, NJ). Although similar to diets used in other studies on biotin nutriture, this diet differs significantly from those used earlier in terms of carbohydrate source and amount, fatty acid composition, and some vitamins and minerals (95). The composition of the diet is spray dried egg white (20%), corn starch (15%), sucrose (50%), cellulose (5%), corn oil (5%), AIN 76A mineral and vitamin mix (with 0.06 mg/kg biotin), and choline bitartrate (0.2%). This diet has been modified to include spray-dried egg white as its sole protein source. The avidin protein of the egg white in the purified diet binds approximately 1.44 mg biotin/kg of purified diet, inhibiting biotin absorption (98). A 0.06 mg biotin/kg diet represents the biotin in excess of the binding capacity of the dietary egg white avidin. This daily dietary intake of biotin closely resembles the average daily biotin intake of a 70 kg person. CBZ was added to

this diet in the appropriate amounts selected for the groups in the studies presented in Chapters 2-4.

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BIOGRAPHICAL SKETCH

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