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CARNITINE DEFICIENCY IN THE PIVALATE-TREATED RAT

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# CARNITINE DEFICIENCY IN THE PIVALATE-TREATED RAT

By

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# ABSTRACT
# CARNITINE DEFICIENCY
# IN THE PIVALATE-TREATED RAT

By

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Sodium pivalate, a compound excreted in the urine bound to carnitine, was used to develop a rat model of a secondary carnitine deficiency. Rats received 20 mmol/L sodium pivalate or 20 mmol/L sodium bicarbonate in their drinking water for 4 days to 8 weeks and were fed a semi-purified AIN-76A diet low in carnitine. In some studies, rats were food-deprived for 24-48 hours, and to maximize fatty acid oxidation, were cold-stressed for 4 hours before tissue collection. Weight gain and food intake were unaffected by pivalate ingestion. Plasma carnitine concentrations were significantly lower ($p<0.05$) in the pivalate-treated groups than the control groups, and tissue carnitine concentrations were also significantly reduced in all studies using weanling rats. Acyl carnitine clearance by the kidneys was significantly greater in treated rats. Common indicators of fat metabolism were altered. Specifically, plasma and liver triglyceride levels and plasma ß-hydroxybutyrate and total ketone concentrations of the food-deprived rats receiving pivalate were significantly greater than those of controls. Concentrations of plasma and liver lipids, plasma free fatty acids, and plasma ß-hydroxybutyrate in the sodium bicarbonate control group and plain water control group were similar. Urinary excretion of ß-hydroxybutyrate was similar in control and treated animals. The higher lipid and ketone concentrations of pivalate-treated rats could not be attributed to excess substrate

supply, since measurement of hepatic arterio-venous differences demonstrated similar rates of free fatty acid uptake and ketone release in control and treated animals. Instead, the fasting ketosis of treated rats was attributed to decreased ketone utilization. Metabolism of $^{14}$Cβ-hydroxybutyrate to $^{14}CO_2$ was significantly lower in treated rats. Carnitine supplementation attenuated both the degree of lipid accumulation in the liver and plasma and the exaggerated fasting ketone response induced by pivalate. These results may have clinical significance, because the reduced plasma and muscle carnitine concentrations, fasting ketosis, and hepatic steatosis found in this animal model are findings also reported for human secondary carnitine deficiency due to organic acidurias.

## DEDICATION

This thesis is dedicated to my husband, Leonard Joseph Bianchi, Ph.D. His support through tribulation and separation made this work possible.

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# ABBREVIATIONS

| | |
|---|---|
| HCl | hydrochloric acid |
| Na | sodium |
| $NaHCO_3$ | sodium bicarbonate |
| SE | pooled standard error |
| CoA, CoASH | coenzyme A |
| ATP | adenosine triphosphate |
| AMP | adenine monophosphate |
| $P_i$ | inorganic phosphate |
| cAMP | cylic AMP |
| GRAMEC | Grand Rapids Area Medical Education Center |
| TCA | tricarboxylic acid |
| DNA | deoxyribonucleic acid |
| $KHCO_3$ | potassium bicarbonate |
| KOH | potasium hydroxide |
| EGTA | ethylene glycol-bis(ß-aminoethyl ether) N,N,N'-tetraacetic acid |
| MOPS | morpholinopropanesulfonic acid |
| $K_2HPO_4$ | dipotassium phosphate |

# INTRODUCTION AND BACKGROUND

## INTRODUCTION

Carnitine is a naturally occurring compound required for the mitochondrial oxidation of long chain fatty acids (1). As depicted in **Figure 1,** long chain fatty acids in the cell are "activated" by acyl CoA synthetase to form acyl CoA's in the cytosol. These are unable to cross the inner mitochondrial membrane for oxidation until transesterified to form acyl carnitines. After mitochondrial entry, free carnitine and acyl CoA's are reformed, and the latter undergo β-oxidation.

Although facilitating fat oxidation is the primary and best-studied function of carnitine, recent research suggests it plays a role in several other processes. Carnitine esterifies to certain xenobiotics (2) and organic acids (3), thereby enhancing their water solubility and facilitating elimination of these compounds by the kidneys. Because carnitine esterifies to acyl groups, it can modulate the acyl/free coenzyme A status by reducing the concentration of acyl CoA and increasing the free CoA level (4). The ratio of acyl CoA to free CoA regulates the rate of several enzymes, including pyruvate dehydrogenase. Hence, carnitine levels can indirectly influence carbohydrate metabolism. In rat heart mitochondria (5) and in human skeletal muscle (6), greater pyruvate metabolism is found in the presence of added carnitine. Other studies also implicate a role for carnitine in carbohydrate metabolism. Lactate release from patients with rapidly paced hearts was lower after carnitine infusion (7).

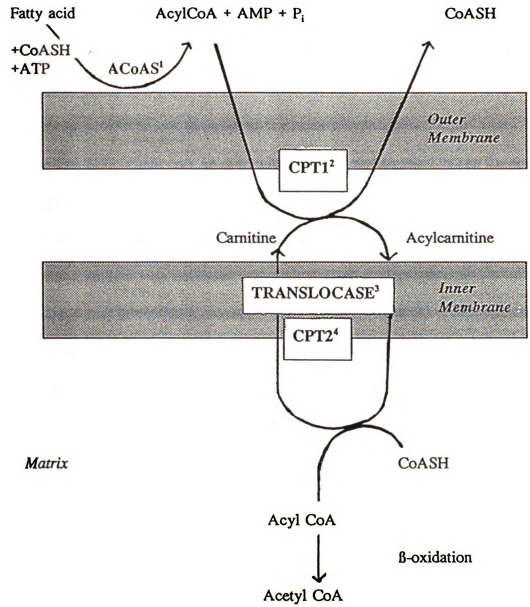


**FIGURE 1.** Carnitine facilitated transport of long chain fatty acids.

[1]Acyl CoA synthetase. [2]Carnitine palmitoyltransferase 1. [3]Carnitne-acylcarnitine translocase. [4]Carnitine palmitoyltransferase 2. Modified from McGarry et al (8).

The lower lactate levels suggest more pyruvate was metabolized. Finally, glucose utilization in diabetics was enhanced when they were infused with carnitine (9).

High concentrations of long chain acyl CoAs inhibit dicarboxylate and adenylate transport (10, 11), hence their reduction by carnitine may prove to be physiologically important. Higher myocardial ATP levels and lower long chain acyl carnitine concentrations were found in coronary bypass patients pre-treated with carnitine (12), which may be due to improved adenylate transport, as the authors suggested. However, heart biopsy ATP and lactate levels were inversely correlated, indicating enhanced pyruvate oxidation may also explain the higher ATP levels .

Carnitine also facilitates exit of short chain acyl groups from the mitochondria (13) and may act as an intercompartmental energy shuttle (14). The importance of these latter functions is less well established. After anaerobic exercise, a lower plasma lactate:pyruvate ratio and higher plasma short chain acyl carnitine concentration was found in subjects who had received a bolus dose of carnitine (15). The latter may represent a shuttle of excess metabolites from the mitochondria, thereby reducing the acyl CoA/CoASH ratio and facilitating pyruvate oxidation.

The results of a number of studies suggest L-carnitine or propionyl carnitine may enhance metabolism of ischemic, chronically hypertrophied, or dilated hearts (16, 17, 18). Cardiac carnitine levels are often found to be reduced with these conditions (19, 20). Improved exercise tolerance (19, 21) and less heart muscle damage (19) have been reported with carnitine supplementation. Acute carnitine administration to post myocardial infarction patients decreased the number of premature ventricular contractions (22). A number of mechanisms have been evoked to explain these

effects including augmented glucose oxidation (14), improved ATP generation (14), reduced induction of mitochondrial calcium efflux by palmitoyl carnitine (23), reduced lipid peroxidation of heart (24) and endothelial tissue (25), mitochondrial membrane preservation via reduced detergent action of long chain acyl CoAs and long chain acyl carnitines (19), and interaction with cardiolipin, a mitochondrial phospholipid (26), but firm evidence establishing which role(s) carnitine may play in improving heart function is lacking.

Altered carnitine metabolism (27) and, in those with advanced disease, reduced total carnitine concentrations (28) have been observed in the skeletal muscle of patients with peripheral vascular disease. Carnitine or propionyl carnitine supplementation has been able to increase exercise tolerance in many of these patients (29, 30, 31). In contrast, in normal volunteers carnitine is not rate-limiting for fatty acid oxidation during low to moderate intensity aerobic exercise (15).

Strenuous exercise decreases both plasma and muscle free carnitine concentrations and raises acyl carnitine levels (32, 33), whereas exercise at submaximal rates has little effect on carnitine metabolism (33). Concern that reduced free carnitine levels would limit fat oxidation or that high acyl CoA concentrations might limit TCA activity has prompted a number of studies examining the effect of carnitine supplementation on exercise metabolism and performance. The results have been mixed. With prolonged, moderate intensity cycling, in which 50% or more of the energy is derived from fat, carnitine supplementation does not increase leg fre fatty acid (FFA) uptake (34), reduce the respiratory quotient, or delay time to subjective fatigue (35). On the other hand, a double-blind cross-over trial of ten moderately

trained students given a single 2 g dose of L-carnitine and exercised to exhaustion showed greater work capacity, lower oxygen consumption, and lower blood lactate levels after carnitine than after placebo (36).

Carnitine may function in additional ways. A number of animal studies indicate carnitine administration, and particularly, acetyl-L-carnitine administration, may ameliorate the effects of ageing in the brain (37, 38). Chronic acetyl-L-carnitine (ALCAR) administration ameliorates the deposition of lipofuscin in old rats (39), opposes the loss of brain microsomal fluidity and glucocorticoid receptors associated with age (40), changes some brain phospholipid concentrations and levels of energy metabolites (41), reduces excess cAMP production induced by isopreterenol in fibroblasts (42), influences the pattern of hypothalamic diurnal β-endorphin concentrations (43), and enhances dopamine (44) and acetylcholine (45) release. Recently, using a model of apoptosis, acetyl carnitine delayed time to cell death (46). The researchers believed this finding may explain several of the effects described above. Another recent and related finding was that carnitine and acetyl carnitine enhanced the ability of cells to repair DNA strand breaks (47), a process reportedly defective in patients with Alzheimer's disease. The underlying mechanism(s) by which these effects are achieved is not clear. Whatever the mechanism(s) responsible, results in humans have shown some promise in attenuating brain function decline with aging. A double-blind, placebo-controlled study of acetyl-l-carnitine administration to 63 patients with Alzheimer's dementia showed a significantly slower rate of deterioration on 5 of the 14 tests administered, as well as a trend of less deterioration in 8 additional tests (48). A previous study of only 20 patients treated for 24 weeks

also showed a trend for delayed deterioration, but no significant differences in test results were found (49). Thus, although ALCAR is unlikely to prove a cure for Alzheimer's disease, early results suggest it may be a helpful treatment.

## BACKGROUND

### Carnitine deficiency

Investigation of carnitine deficiency states has provided some clues to carnitine functions. However, in nature, carnitine deficiency occurs rarely. Mammals synthesize carnitine from lysine and methionine (50) and also obtain preformed carnitine in the diet, especially from animal tissues. Carnitine deficiency may be found with defective carnitine transport (51), conditions which produce increased carnitine losses such as inborn errors of metabolism (3), valproic acid therapy for epilepsy (52), hemodialysis (53), and inadequate carnitine intake, which may occur with long-term, carnitine-free total parenteral nutrition (54). Impaired synthesis is another plausible etiology, but no cases have thus far been documented. Perhaps because of this wide variety of conditions which may produce it, carnitine deficiency is associated with a variety of signs and symptoms, which complicates the task of determining the spectrum of functions by carnitine.

#### Primary carnitine deficiencies

Carnitine deficiency syndromes have been broadly classified as myopathic or systemic, primary or secondary deficiencies. Myopathic carnitine deficiency (MCD), described by Engel and Angelini (55), is characterized by depressed levels of skeletal muscle carnitine, muscle weakness and lipid deposition, reduced myoblast uptake of carnitine, and normal to low normal levels of carnitine in plasma, liver, and kidney.

Muscle wasting may also occur, particularly in the proximal limb muscles and those of the jaw, neck and spine (56). The ratio of acyl carnitines to free carnitine is high, and serum ketone concentrations after fasting may be elevated. Treatment with oral L-carnitine, 50 $mg.kg^{-1}$ can ameliorate symptoms of muscle weakness even though muscle carnitine concentrations remain low (56).

Cases of systemic carnitine deficiency (SCD), characterized by depressed levels of carnitine in the plasma and liver, as well as muscle, were later documented (51). Primary systemic carnitine deficiency was diagnosed when no other illness causing the low carnitine levels could be ascribed. Children presented with signs of encephalopathy (57), myopathy, or cardiomyopathy (58) as their primary abnormality. Fasting hypoglycemia and hypoketonemia were sometimes found (59). Neutral lipid deposition could be found in several tissues: heart and skeletal muscle, liver, and kidneys (56). Response to carnitine treatment has been variable.

Recently, the distinctions between categories have blurred. In one family children who initially showed signs of cardiomyopathy and carnitine deficiency later developed skeletal muscle weakness (60). Some patients initially diagnosed with muscle carnitine deficiency subsequently developed systemic symptoms (61). In addition, several patients initially diagnosed with a primary systemic deficiency were later found to have deficiencies in enzymes of ß-oxidation, or glutaric aciduria type II (62), thereby making their carnitine deficiency a secondary phenomenon (51). This makes interpretation of the earlier literature difficult, because some conditions originally attributed to the carnitine deficiency may be due to the underlying disease. However, it may eventually clarify patients' seemingly anomalous responses to

carnitine treatment. Those with a defect in ß-oxidative enzymes would be less likely to improve, since low carnitine levels would not be the primary reason for the reduced fat oxidation in these patients.

A few recent cases of an autosomal recessive inherited primary carnitine deficiency have been diagnosed using the current sophisticated methodologies. These children suffered from a defect in intracellular uptake of carnitine (58). They had impaired carnitine uptake by fibroblasts, leukocytes, muscle and kidney. Patient presentation has been variable: fasting hypoglycemia and hypoketonemia, encephalopathy, or muscle weakness (58, 51) may occur, but most common is cardiomyopathy (58, 63). Plasma, muscle, and to a lesser extent, liver carnitine concentrations are consistently reduced. Levels are lower than are found with secondary carnitine deficiencies, and fasting urinary dicarboxylic acids concentrations are not increased, as with the ß-oxidation enzyme deficiencies. Carnitine administration corrects the myopathy and skeletal muscle weakness even though muscle carnitine concentrations remain severely depressed (58).

**Secondary carnitine deficiency**

The secondary carnitine deficiencies are those which result from increased losses of carnitine despite normal tissue carnitine uptake and transport. For example, carnitine losses incurred with hemodialysis reduce plasma and muscle carnitine concentrations, and may be responsible for intra-dialytic hypotension, muscle cramps, and post-dialysis asthenia (64). Often a secondary carnitine deficiency is due to increased carnitine losses in the urine such as with the Fanconi syndrome, genetic

disorders of metabolism such as the organic acidurias, and administration of valproic acid, an antiepileptic agent which metabolizes to a carnitine ester for excretion. An example of the organic acidurias is propionic carboxylase deficiency. The block in metabolism causes an abnormal accumulation of propionic acid, which conjugates to carnitine or glycine and is excreted in the urine as propionylcarnitine (3) and propionylglycine. Carnitine biosynthesis is unable to compensate for this loss, leading to a secondary carnitine deficiency in these individuals. The response to fasting is often hyperketonemia (65).

**Pivalate-induced carnitine deficiency-human**

Low plasma and tissue carnitine levels have been found in patients taking antibiotics conjugated to pivalic acid (66, 67). Melegh and coworkers (66) noted a fivefold increase in urinary carnitine excretion in patients treated with pivampicillin. The pivalic acid moiety of pivampicillin was excreted in large quantities bound to carnitine. Pivalic acid appears to act similarly to propionic acid. Both acyl acids are conjugated to carnitine and excreted in the urine. Consequently, pivalic acid administration is a potential mechanism for rapidly inducing a carnitine deficiency. Its effects on metabolism have not been fully investigated. Melegh (66) observed no effect on fasting glucose or triglyceride (TG) concentrations in children given pivampicillin for 7 days but saw a significant reduction in ß-hydroxybutyrate (ßOHB) and free fatty acid (FFA) concentrations. The former was attributed to reduced fatty acid oxidation with carnitine deficiency. Low tissue carnitine levels have been associated with impaired lipolysis (68), which could account for reduced plasma FFA levels with unchanged plasma TG concentrations. However, others have noted

reduced FFA levels after carnitine administration (12, 69). Treatment with pivaloyl-esterified antibiotics for 2-30 months has been shown to reduce total muscle carnitine concentrations to 10% of normal values (67). The effect of short-term pivampicillin administration on muscle carnitine concentrations has not been reported.

**Pivalate-induced carnitine deficiency-rat**

Pivalic acid is readily absorbed by the rat and quickly distributed throughout the body. Within 48 hours over 90% is eliminated from the body, primarily in the urine (70). In rat urine it is found in two conjugated forms, as pivaloylcarnitine (70) and pivaloyl-glucuronide (71, 72). Toxicity studies with rats over a 26 week period have indicated no adverse effects of pivalate administration (73). Consequently, pivalate administration is a potential mechanism for rapidly inducing carnitine deficiency in the rat.

Data from this laboratory, to be presented in chapter 1, indicate the pivalate-induced carnitine deficiency in rats shows several similar features to the carnitine deficiency induced by genetic lack of propionyl-CoA carboxylase or isovaleryl-CoA dehydrogenase. In each case there is an accumulation of a compound capable of binding to free CoA and forming a conjugated CoA: pivaloyl CoA, propionyl CoA, isovaleryl CoA. Some of the acyl groups are then conjugated to carnitine, which can restore the ratio of free CoA to acyl CoA in the tissues toward normal but also results in a loss of conjugated carnitine in the urine (74). A high acylcarnitine/free carnitine ratio in the urine and muscle (75, 76, 77), fasting ketosis (78, 79), and fat accumulation in the liver (80) and muscle (77) are findings common to the human and animal model deficiency states. A reduction in cardiac carnitine concentration of

concentration of 50% with pivalate administration for 24-26 weeks is associated with reduced rates of $^{14}$C-palmitate oxidation and diminished cardiac contractile function by isolated perfused working hearts (81). The pivalate-treated rat is a useful animal model of secondary carnitine deficiency due to organic acidurias. A similar model, the pivampicillin-treated rat, also produces a carnitine deficiency (82). Lower fasting plasma ßOHB concentrations were found after 3 days administration, but after 2-3 weeks of pivampicillin, plasma ßOHB levels were higher than those of the controls. The model was criticized by the researchers because it did not reproduce "pronounced alteration in liver metabolism as [indicated by] hypoglycemia" that has been reported in children receiving pivampicillin. However, low blood sugar is not a consistent finding in humans treated with pivalic acid containing drugs. After 7 days of treatment no significant change in plasma glucose concentrations were reported in one study (66), and in another only half of the children receiving longer-term drug treatment had fasting hypoglycemia (83). Hence, fasting normoglycemia is not a justifiable reason to abandon the model which does reproduce a number of other metabolic defects common to human secondary carnitine deficiency, including significant alterations in liver metabolism.

**B. Aims**

The goals of these studies were to characterize the pivalate-treated rat model of carnitine deficiency and to elucidate the cause of the exaggerated fasting ketosis observed in pivalate-treated rats. The experiments were designed to test the following hypotheses:

1. Carnitine depletion by pivalate will induce alterations in fat metabolism similar to those observed in humans.
2. Signs of altered fat metabolism induced by carnitine depletion with pivalate will be ameliorated by carnitine supplementation.
3. In the pivalate-treated rat with moderate carnitine depletion, diminished CoASH and/or succinyl CoA concentrations limit ketone utilization.
4. In the pivalate-treated rat with moderate carnitine depletion ketogenesis is enhanced due to increased free fatty acid (FFA) flux to the liver.

**C. Significance**

Hospitalization for episodes of ketoacidosis is a common problem encountered by patients with organic acidurias with secondary carnitine deficiency. Saudubray et al (84) stated "[with propionic and isovaleric acidemias] hyperketosis is thought to be mainly related to an excess of ketone body production, although this assumption is not always relevant and many mechanisms of hyperketosis still have to be elucidated." The mechanism of hyperketosis in an animal model of secondary carnitine deficiency induced by pivalate administration is the focus of this research. With an understanding of the mechanism, an appropriate preventive treatment may follow.

**D. Dissertation Format**

This dissertation is presented as a series of manuscripts based upon the above aims:

1. Sodium pivalate treatment reduces tissue carnitines and enhances ketosis in rats
   P.B. Bianchi and A.T. Davis
   J. Nutrition 121:2029-2036, 1991

2. Carnitine supplementation ameliorates the steatosis and ketosis induced by pivalate

   P.B. Bianchi and A.T. Davis

   FASEB J. 6:A3282, 1992

3. Reduced ketone utilization and unaltered hepatic arteriovenous differences in free fatty acids and ketones in the carnitine-depleted, pivalate-treated rat.

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# CHAPTER 1

## Sodium pivalate treatment reduces tissue carnitines and enhances ketosis in rats[1,2]

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## ABSTRACT

Sodium pivalate, a compound excreted in the urine conjugated to carnitine, was used to induce a secondary carnitine deficiency. In the first series of experiments, rats received in their drinking water either 20 mmol/L sodium pivalate (experimental) or 20 mmol/L sodium bicarbonate (control) for 4 days, 2 weeks, or 8 weeks. Tissues and urine were collected, and carnitine concentrations in liver, skeletal muscle, heart, plasma, and urine were determined. The total carnitine concentrations in tissues and plasma of pivalate-treated rats were significantly depressed ($p<0.05$) at all time points except at four days for skeletal muscle and at 4 days and 2 weeks for liver. The acylcarnitine/free carnitine ratios in urine and plasma of the pivalate-treated animals were significantly elevated at all time points relative to the controls. In the second experiment, rats received either the pivalate or the bicarbonate treatments for 15 days followed by a two day fast. After fasting, the plasma ß-hydroxybutyrate of pivalate treated rats was significantly elevated relative to controls, but there was no significant difference in plasma glucose concentrations. The reduced plasma and tissue carnitine concentrations, increased acylcarnitine/free carnitine ratio in plasma and urine, and fasting ketosis found in pivalate-treated rats are findings also reported for human secondary carnitine deficiency due to organic acidurias.



**INDEXING WORDS:**

carnitine, carnitine deficiency, pivalate, rats

A naturally occurring compound required for mammalian energy metabolism, carnitine is necessary for the mitochondrial oxidation of long chain fatty acids, and it is this role which has received the most attention (1). Other roles have been hypothesized for carnitine, including modulation of the acyl CoA/free CoA ratio, elimination of toxic metabolites, and acting as an intercompartmental energy shuttle (2, 3).

Carnitine deficiency syndromes have been broadly classified as primary or secondary deficiencies. Recently, because several patients initially diagnosed with a primary deficiency were later found to have secondary carnitine deficiencies (4), the distinctions between categories have blurred. There are varying manifestations of carnitine deficiency as well. Myopathic carnitine deficiency, described by Engel and Angelini (5), is characterized by muscle weakness and lipid deposition, depressed levels of skeletal muscle carnitine, and normal to low normal levels of carnitine in plasma, liver, and kidney. Systemic carnitine deficiency was described by Treem et al (4), who found depressed levels of carnitine in the plasma and liver, as well as muscle. Common features of these syndromes are muscle weakness and lipid deposition.

The secondary carnitine deficiencies are those which result from increased losses of carnitine. Though this may occur in persons undergoing dialysis therapy, a secondary carnitine deficiency is usually due to the increased carnitine losses in the urine that occur with the Fanconi syndrome and genetic disorders of metabolism such as the organic acidurias. For example, with propionic carboxylase deficiency, the block in metabolism causes an abnormal accumulation of propionic acid, which conjugates with carnitine and is excreted in the urine as propionylcarnitine (6). The

implication is that carnitine biosynthesis is insufficient to compensate for this loss, leading to a secondary carnitine deficiency in these individuals.

Many animal models have been developed to study carnitine deficiency. Methods include the prolonged feeding of choline deficient (7), lysine deficient (8), or D-carnitine substituted diets to weanling rats (9); giving multiple intraperitoneal injections of D-carnitine, the non-metabolizable isomer of the amino acid (10); or dialysis (11). Each of these methods was developed for a different purpose and therefore will be better suited for some studies than others. Dialysis, though quick and effective in depleting plasma carnitine, is a relatively expensive process. The lysine and choline deficient diets are time-consuming and also produce growth failure and other effects not totally reversible by carnitine supplementation (8, 12), thus complicating interpretation of studies using these models. D-carnitine inhibits carnitine transferases as well as reduces L-carnitine tissue concentrations (10).

Melegh and coworkers (13) noted an increased urinary carnitine excretion in patients treated with pivampicillin for 7 days. Total carnitine excretion was increased five-fold, whereas the amount of free carnitine in the urine declined. Plasma total acid soluble carnitine concentrations were reduced 38.6%, and plasma carnitine decreased 75%. The pivalic acid moiety of pivampicillin was found excreted in large quantities as pivaloylcarnitine. Pivalic acid (trimethyl acetic acid) appears to be acting similarly to propionic acid in persons with propionic acidurias. Both acyl acids are conjugated to carnitine and excreted in the urine in large quantities. Consequently, pivalic acid administration is a potential mechanism for rapidly producing a carnitine deficiency. The purpose of these studies was to determine if a carnitine deficiency can be induced

in rats by providing sodium pivalate in the drinking water.

## MATERIALS AND METHODS

***Animals and Experimental Design.*** Male Sprague-Dawley rats (100-125 g) were purchased from Charles River (Portage, MI) and maintained in metabolic cages at the animal facilities of the Laboratory Animal Care Service of Michigan State University (MSU). This project was approved by the All-University Committee for Animal Use and Care of MSU. For all experiments, the rats were fed *ad libitum* a nutritionally complete purified diet, AIN-76A (TEKLAD, Madison, WI). Analysis in this laboratory determined the carnitine content of the diet to be 1.2 nmol/g (personal communication, A.T. Davis, Dept. of Surgery, MSU). The experimental rats received 20 mmol/L sodium pivalate in their drinking water (which was freely available), and control rats received equimolar sodium bicarbonate. Previous results suggested that this pivalate dosage would induce carnitine excretion without modifying normal food and water intake (personal communication, A.T. Davis, Dept. of Surgery, MSU, see appendix, Table B-2). Solutions were adjusted to pH 7.0. The first series of experiments tested whether pivalate administration would induce a carnitine deficiency. Rats received the treatments for 4 days (n=6 rats per treatment group), 2 weeks (n=5), or 8 weeks (n=5). For the four day study, food and water intake were monitored daily. For the 8 week study food intake was monitored on the last day of the study. Urine was collected on the last day of the experiments, using 0.5 mL of 6 mol/L hydrochloric acid as a preservative, and stored at -20 °C prior to analysis. In an additional experiment to determine whether fat metabolism would be affected by the pivalate treatment, male, Sprague-Dawley rats (100-125 g) received the above

treatments for fifteen days, and then were starved for two days while continuing with the same treatments in the water.

***Blood and tissue samples.*** At the end of each study, rats were killed by decapitation. Blood was collected from the neck into heparinized tubes. A section of the median lobe of the liver was excised and quickly freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen. The heart and a sample of hindlimb muscle were obtained and frozen in like manner.

Carnitine in urine, tissues and plasma was determined as described previously (14). Briefly, protein was precipitated from plasma by the addition of 6% perchloric acid and centrifugation. Frozen tissues were homogenized on ice in 3% perchloric acid and centrifuged. The pellets were saved for assay of long chain carnitines. For free carnitine analysis, $KHCO_3$, 2 mol/l, was added to remove the acid, and samples were held on ice for 30 min while the reaction came to completion. Samples were neutralized with MOPS, 2 mol/l, and centrifuged to eliminate the potassium perchlorate. An aliquot of the supernatant was assayed in a neutral MOPS buffer containing EGTA, sodium tetrathiontate and $^{14}$C-acetyl CoA by a radioistope exchange reaction in which addition of carnitine acetyl transferase prompted the transfer of $^{14}$C acetyl groups to carnitine. Excess $^{14}$C-acetyl CoA was removed by applying the reaction mixture to Dowex 1-x chloride form columns. Unreacted $^{14}$C-acetyl CoA binds to the columns, whereas, the acetyl carnitine, with no net charge, was eluted with water. Aliquots of the elutions were added to Picofluor, and the amount of carnitine present was determined using a Packard Minaxi Tri-carb 4000 series scintillation counter. Total acid soluble carnitine is measured similarly, except after

the protein precipitation step, short-chain acyl carnitines were hydrolyzed with 1 N KOH for 30 minutes before continuing with the procdures. Short chain carnitines were determined by taking the difference between free carnitine and total acid-soluble carnitine concentrations. Pellets containing acid insoluble carnitine were hydrolyzed with 0.5 N KOH for 2 hours in a 65° C water bath, neutralized with MOPS, and centrifuged prior to running the radioisotope exhange reaction. Urine, which lacks protein, was assayed for free and acyl carnitines without the protein precipitation step. Total carnitine is reported as the sum of free, short-chain, and long-chain acylcarnitines for plasma and tissues, and free and acylcarnitines for urine.

For the starvation study, liver was analyzed for triglycerides, and blood obtained from tail veins before and after the fast was analyzed for ß-hydroxybutyrate (ßOHB) and glucose. Sigma Chemical Co. (St. Louis, MO) Kit No. 336 was used for measuring serum triglycerides. Triglyerides were hydrolyzed to glycerol and free fatty acids. The quantity of glycerol present was determined by spectrophotometric measurement of the induced formation of formazan, which has an absorbance maximum at 500 λ. Sigma Kit No. 405 was used to measure triglycerides in liver homogenates, 0.2 g liver/ml. Triglycerides were extracted in propanol and hydrolyzed to glycerol and free fatty acids. The amount of glycerol present was measured spectrophotometrically by the amount of diacetyl dihydrolutidine formed, which absorbs light at 410 λ. ßOHB was measured by the change in absorbance of light at 340 λ when NAD is reduced and ßOHB is oxidized to acetoacetate using Sigma Kit No. 310-UV.

Analyses for urine acylcarnitines were graciously provided by Dr. Loran

Bieber of Michigan State University, Department of Biochemistry, using a modification of the method described previously (15). Briefly, acyl carnitines were twice extracted from the urine in 19 volumes of chloroform/methanol (3:2), and the supernatants were combined and evaporated to dryness under $N_2$. Aliqouts were incubated with L-[$^{14}$C]carnitine in a buffer of 250 mmol/l $K_2HPO_4$, ph 7.4, 50 μmol/l CoASH in 10 mmol/l dithiothretol, and carnitine acetyl transferase, isolated and purified from rat liver. After a one hour incubation at 30 °C N-ethylmaleimide was added to sequester free CoA and convert acyl CoA derivatives to acylcarnitines. Protein was precipitated wtih 6% perchloric acid and removed after centrifugation. The pH of the supernatant was adjusted to 6.5-7.0 with 1 N KOH, and the samples were filtered through 0.2-μm pore centrifugal filter tubes. Twenty microliter aliquots were applied onto a $C_{18}$ reverse phase Partisil 10 ODS-3 column (250 x 4.6 mm) and eluted with a gradient of two solvents, 5 mmol/L butanesulfonic acid brought to pH 3.4 with acetic acid, and 100% methanol.

***Materials.*** [$^{14}$C]-acetyl coenzyme A was obtained from NEN (Boston, MA), and carnitine acetyl transferase was obtained from Boehringer Mannheim (Indianapolis, IN). The pivalic acid, sodium salt was obtained from Aldrich (Milwaukee, WI). All other chemicals used were of reagent grade.

***Statistical Analysis.*** Data for the 4-day, 2-wk, and 8-wk groups were analyzed by two-way ANOVA (16) using the Number Cruncher Statistical System (Kaysville, UT). Comparisons between individual means were made with the Student-Newman-Keuls test (16). Because of non-normality of distribution, urine carnitine, urine and liver acylcarnitine/free carnitine data and urine carnitine clearance data were analyzed

after log transformation. The data from the starvation study were analyzed with Student's t-test. A significance level of $P<0.05$ was used for all tests.

## RESULTS

In the initial 4-d study, no significant differences in weight gain, percentage of weight gain, food intake or water intake were noted between groups. No differences in the general appearance or activity of the rats were observed. Carnitine intake for the 4 d was $100\pm5$ nmol for the treated rats and $105\pm12$ nmol for the controls. Food intakes on the last day of the 8-wk study were not significantly different between the two groups. Body weights at the end of the 2 and 8 week studies, as well as at the end of the starvation study, showed no significant differences between the two groups.

Total carnitine concentrations for tissues and plasma are shown in **Figure 1.** Carnitine levels in plasma and heart were significantly depressed after only 4 d of pivalate administration, and showed no significant further decline after 2 wk or 8 wk of treatment. By 2 wk of treatment the muscle total carnitine concentration was also significantly diminished compared to control values and showed a further decrease by 8 weeks. In the liver total carnitine levels for the pivalate-treated group were reduced only at 8 wk.

**Table 1** describes the acylcarnitine:free carnitine ratios in the tissues. When free carnitine concentrations are reduced and/or excess long-chain or short-chain acylcarnitines accumulate, the ratio is increased. In all tissues studied, factorial ANOVA testing indicated the main effects of comparing pivalate and bicarbonate treatments were significantly greater acylcarnitine/free ratios for the pivalate groups.

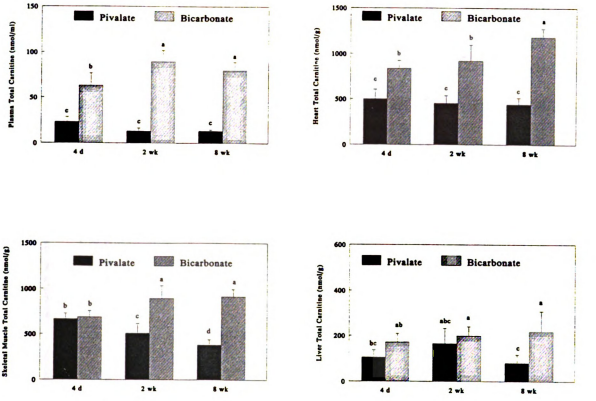


**FIGURE 1** Plasma, heart, hindlimb muscle, and liver total carnitine concentrations of rats given 20 mmol/L sodium pivalate or sodium bicarbonate in their drinking water for 4 d, 2 wk, or 8 wk. Values are means ± SD for 6 rats per group for the 4 d data, with 5 rats each for the 2 and 8 wk data. Bars with common superscripts are not significantly different ($P > 0.05$) by the Student-Newman-Keuls test.

**TABLE 1 Ratio of acylcarnitine to free carnitine in liver, heart, skeletal muscle, plasma and urine of pivalate-treated or control (bicarbonate-treated) rats[1]**

| Tissue/fluid | Group | Time point | | | SE[2] | P[3] |
|---|---|---|---|---|---|---|
| | | 4d | 2 wk | 8 wk | | |
| | | *Acylcarnitine:free carnitine ratio* | | | | |
| Liver[4] | Pivalate-treated | $0.37^{b}$ | $0.40^{b}$ | $1.36^{a}$ | —[5] | P, T |
| | Control | $0.29^{b}$ | $0.13^{c}$ | $0.96^{a}$ | | |
| Heart | Pivalate-treated | $0.71^{cd}$ | $1.70^{b}$ | $2.81^{a}$ | 0.09 | P, T, P× T |
| | Control | $0.33^{d}$ | $1.02^{bcd}$ | $1.26^{bc}$ | | |
| Skeletal muscle | Pivalate-treated | $0.88^{b}$ | $0.42^{b}$ | $1.87^{a}$ | 0.08 | P, T, P× T |
| | Control | $0.43^{b}$ | $0.14^{b}$ | $0.38^{b}$ | | |
| Plasma | Pivalate-treated | $3.39^{a}$ | $1.69^{b}$ | $1.69^{b}$ | 0.1 | P, T, P × T |
| | Control | $1.11^{bc}$ | $0.54^{c}$ | $0.94^{c}$ | | |
| Urine[4] | Pivalate-treated | $25.79^{ab}$ | $11.77^{b}$ | $53.51^{a}$ | —[5] | P, T, P × T |
| | Control | $0.89^{c}$ | $0.76^{c}$ | $0.14^{d}$ | | |

[1]Values are means, except where indicated; n=6 for 4-d time point data; n=5 for the 2 and 8 week data. Values for a particular tissue with common superscript letters are not significantly different (P > 0.05), Student-Newman-Keuls test.
[2]Pooled standard error from the two-way ANOVA.
[3]Significant main and interaction effects from the two-way ANOVA; P, significant effect of pivalate: T, significant effect of time; P × T, significant interaction.
[4]Because of the variability of the data, values are presented as medians. To analyze the data via a two-way ANOVA, data were transformed prior to analysis, using the natural log of the values.
[5]Because the data are expressed as medians, and the pooled SE term is based on log-transformed data, this value has been omitted.

In addition, for the liver, heart and skeletal muscle the ratios in the pivalate-treated group at 8 wk were significantly higher than at 4 d. The opposite temporal relationship was true for plasma.

Urine total carnitine data are shown in **Figure 2.** The data are expressed as micromoles of total carnitine excreted per 100 g body weight per day, in order to compensate for the differences in body weights of rats at 4 d, 2 wk, and 8 wk. Despite a large but not statistically significant carnitine excretion by the pivalate group at 4 d, ANOVA testing showed the overall total carnitine excretion of pivalate-treated rats was significantly lower than the carnitine excretion of control rats, ($p<.05$). This was due to a significant depression of total carnitine excretion by the pivalate treated group at 8 wk. Carnitine excretion of the control rats remained constant over time. However, pivalate-treated rats excreted significantly more acylcarnitines than control rats at each time point. Acylcarnitine excretion of treated rats per 100 g body weight, expressed as the median, was 1.161 μmol/day at 4 d, 0.295 μmol/day at 2 wk, and 0.043 μmol/day at 8 wk. Excretion by control rats at the same time points was 0.124 μmol/day, 0.131 μmol/day, and 0.026 μmol/day. Urine acylcarnitine:free carnitine ratios were significantly elevated in the pivalate group at the three time points (Table 1).

Clearance of acylcarnitines was significantly greater in the pivalate treated rats relative to controls at all time points. Acylcarnitine clearances of the pivalate-treated rats per 100 g of body weight, expressed as the median (x $10^{-3}$ mL/min), were 62.2, 34.8 and 5.8 at 4 d, 2 wk and 8 wk, respectively. Acylcarnitine clearances of control

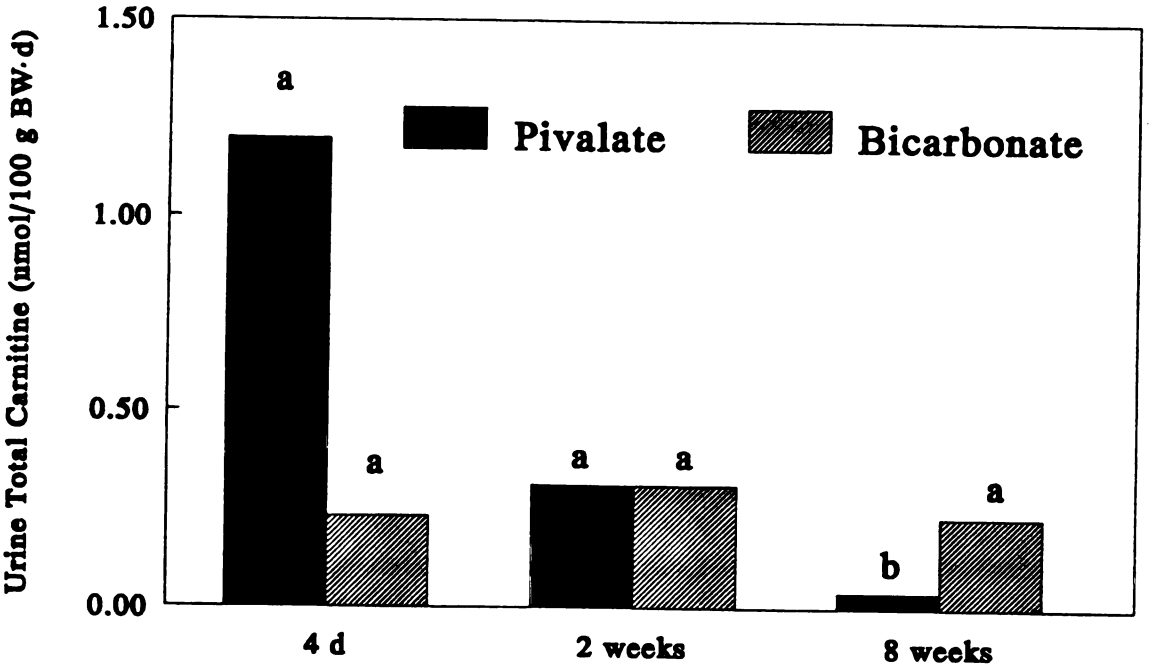


**FIGURE 2** Urinary total carnitine excretion of rats given 20 mmol/L sodium pivalateor sodium bicarbonate in their drinking water for 4 d, 2 wk, or 8 wk. Values are medians for 6 rats per group for the 4 d data, with 5 rats each for the other treatment groups. Due to the non-normality of the data, values were transformed using the natural log of the original number, prior to analysis via a two-way analysis of variance. Bars with common superscripts are not significantly different (P>0.05) by the Student-Newman-Keuls test.

rats at the same time points were 8.1, 8.3 and 1.3 x $10^{-3}$ mL. min, respectively. Main treatment effects showed that control rats had a significantly higher free carnitine clearance than the pivalate-treated rats (data not shown, see Appendix, Table A-2).

**Figure 3** shows chromatograms of rat urine acylcarnitines from the 4-d study. The upper panel is from a bicarbonate-treated rat; the lower panel is from a pivalate-treated rat. Peak 1 in both chromatograms represents radiolabelled free carnitine, which is a by-product of the assay used for these analyses. Peak 2 in the lower panel was identified as pivaloylcarnitine, which constituted the major acylcarnitine fraction in the urine of the pivalate treated rat. As expected, Peak 2 was not present in the chromatogram of the control rat.

Data from the rats deprived of food for 2 d are shown in **Table 2.** Plasma and tissue total carnitine concentrations in the rats given pivalate for 15 d and starved for two d were significantly lower than control values. The extent of carnitine depletion was similar to that of rats given pivalate for two wk without the food deprivation. The additional metabolites measured in the starved rats are also shown in Table 2. No significant differences in plasma glucose concentrations were noted between the two treatment groups in either the pre-fasting or the post-fasting samples (pre-fasting data not shown). After fasting, plasma ß-hydroxybutyrate and liver triglycerides were significantly elevated in the pivalate-treated rats, to almost twice the level seen in the controls.

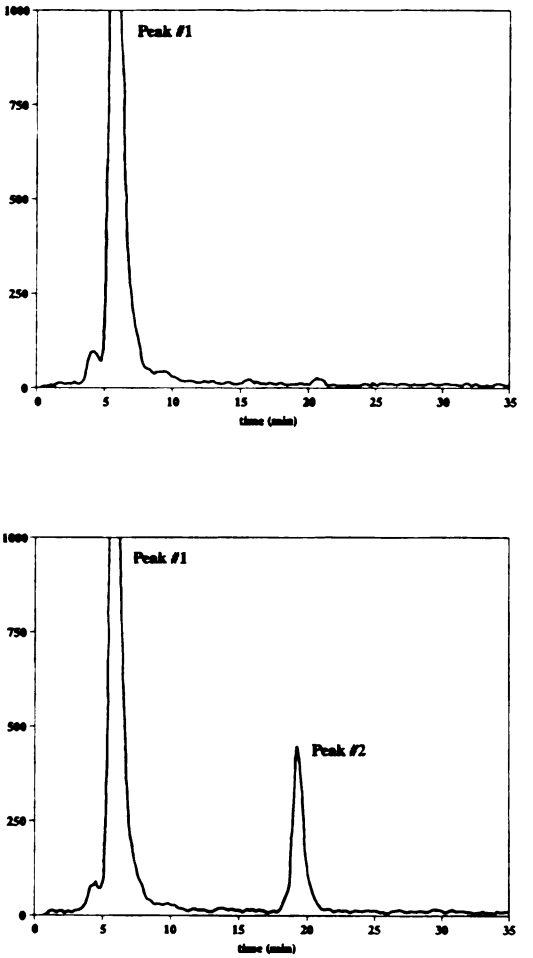


**FIGURE 3** HPLC chromatography of urinary acylcarnitines from rats on treatment for four days. The Y-axis represents counts per minute. Figure 3a - Urine from rat receiving oral bicarbonate. Figure 3b - Urine from rat receiving oral pivalate. Peak #1 is free carnitine generated by the assay, while peak #2 represents urinary pivaloylcarnitine.

**TABLE 2 Effect of oral pivalate (pivalate-treated) or bicarbonate (control) administration for 15 d on tissue total carnitine, plasma glucose, plasma ß-hydroxybutyrate and liver triglyceride concentrations in rats fasted for 2 d[1].**

| | Group | | |
|---|---|---|---|
| | Pivalate-treated | Control | SE[2] |
| Plasma total carnitine, *nmol/mL* | 21.7[a] | 60.7 | 4.2 |
| Heart total carnitine, *nmol/g* | 503[a] | 927 | 68 |
| Skeletal muscle total carnitine, *nmol/g* | 514[a] | 924 | 35 |
| Liver total carnitine, *nmol/g* | 303[a] | 410 | 47 |
| Plasma glucose, *mmol/L* | 3.2 | 2.8 | 0.5 |
| Plasma ß-hydroxybutyrate, *mmol/L* | 5.4[a] | 3.0 | 1.0 |
| Liver triglycerides, µ*mol/g* | 27.2[a] | 14.0 | 46.6 |

[1]Values are means; n = 6 for pivalate-treated and n = 5 for control rats. Differences between treatments were determined using the *t*-test and are indicated: [a]$P < 0.05$.
[2]Pooled standard error.

# DISCUSSION

Increased excretion of acylcarnitines has been noted in subjects given drugs containing an ester moiety of pivalic acid. Melegh et al (13) reported that the oral ingestion of pivampicillin (pivaloyloxymethyl-ampicillin) resulted in a fivefold increase in total carnitine excretion. After 1 wk of study, the plasma free carnitine concentration in children receiving pivampicillin was significantly depressed, and the short-chain acylcarnitine level was significantly increased. Vickers et al (17) showed the appearance of pivaloylcarnitine in the urine after a single dose of an ester of methyldopa containing pivalate. Similar observations have been reported by Holme et al (18) and Melegh et al (19). Based upon these results, pivalate administration seems to be a unique way of generating an animal model of carnitine deficiency. Toxicity studies with rats over a 26 wk period have indicated no adverse effects of pivalate administration (20). Within 48 hours, over 90% is eliminated from the body, primarily in the urine (21). However, in an earlier study, Dziewiatkowski and Lewis (22) showed that pivalic acid was metabolized to pivaloyl-glucuronide in the rat. Similarly, Vickers et al (17) have shown that the cynomolgus monkey excreted 70% of a dose of labeled pivalate as a glucuronide, with only 10% of the dose administered present in the urine as pivaloylcarnitine, whereas in humans, close to 100% of the excreted radioactivity was in the form of pivaloylcarnitine. Thus, the different metabolism of pivalate in humans compared to other species could obviate the use of pivalate in the development of an animal model of carnitine deficiency. The present studies were designed to test this hypothesis.

The results of our experiments were similar to those reported for patients with secondary carnitine deficiency due to organic acidurias. Acylcarnitine excretion was greater and plasma and tissue total carnitine concentrations were lower in pivalate-treated rats. On d 4 of pivalate treatment, the acylcarnitine excretion of the treated rats, expressed as the median, was 2.56 μmol/d, compared to 0.27 μmol/d for the controls. As indicated in Figure 3, the predominant acylcarnitine fraction in the treated group was pivaloylcarnitine. Pivalate intake for the same period was 1.82 mmol. Although <0.5% of the pivalate intake was excreted as pivaloylcarnitine, data shown in Figure 1 indicate that by 4 d a rapid depletion in carnitine was produced in the plasma and heart. Total carnitine concentrations in these tissues were reduced by 64% and 40% respectively. After 2 weeks of pivalate administration, the carnitine concentration of skeletal muscle was reduced 43% relative to controls. We did not measure muscle function or tissue lipids in these studies. However, lipid vacuoles in both muscle (23, 24) and liver (25) and hypotonia have been found in children with a similar degree of carnitine depletion.

Greater depressions in plasma and muscle carnitine concentrations have been reported for patients with a carnitine deficiency (26), including two patients receiving pivaloyloxymethyl-esterified antibiotics (18). In the latter study muscle carnitine levels were reduced to approximately 10% of normal after 22-30 months. However, after only 2 wk of pivalate administration we found the rat plasma and tissue total carnitine concentrations were in the range reported for secondary carnitine deficiency due to organic acidurias (6, 25-27). We cannot say whether extending the duration of pivalate treatment to rats would cause the profound carnitine depletion reported for

humans. Since the total carnitine concentration in skeletal muscle at 8 wk was significantly lower than at 2 wk, the depletion process had not stabilized within the period of our experiments.

In children with organic acidurias, the increased metabolic demand for elimination of acyl groups leads to increased acylcarnitine to free carnitine ratios in plasma and urine (27). An elevated acylcarnitine:free carnitine ratio has also been reported in muscle (6, 25) and liver (25) of patients with organic acidurias or myopathic carnitine deficiency (28). High ratios are associated with disturbed CoA homeostasis (29). We found ratios were significantly greater in the plasma, urine and tissues of pivalate-treated fed animals, and increased in the plasma and tissues over time. At 8 wk, the ratio in urine for the pivalate group was 54, which is comparable to the ratio of 46 reported for a series of patients with propionic acidemia (30).

As we expected, pivalate ingestion led to a significantly greater acylcarnitine excretion. Subsequent analyses demonstrated that most of it was conjugated to pivalate. Acylcarnitine clearance was also greater in pivalate-treated rats. This is consistent with carnitine excretion in patients with organic aciduria. An increased total carnitine excretion is frequently (30) but not always (25, 27, 30-32) observed in these children as well. The median carnitine excretion of the pivalate-treated rats at 4 d was 2.65 μmol/day compared to 0.64 μmol/day for the controls. Due to the variability in total carnitine excretion, these values were not significantly different. Unlike the control rats, in which urinary carnitine excretion relative to body weight was constant, total carnitine excretion in the pivalate treated rats diminished over time. This decrease in excretion may be a compensatory response. Between 4 d and 8 wk,

acylcarnitine clearance in the pivalate group decreased 44%, and free carnitine clearance decreased 92%. The reduction in excretion may have been due to increased pivaloyl glucuronide excretion. The reduction in free carnitine clearance implies considerable renal conservation of free carnitine, and is consistent with urine excretion patterns of children with organic acidurias. Children with secondary carnitine deficiency due to isovaleryl aciduria may also have a low total carnitine excretion and markedly reduced free carnitine excretion (27).

The second experiment was conducted to determine whether the carnitine depletion produced by pivalate ingestion would have any effects on fat metabolism. Hypoketotic hypoglycemia is seen in children with a primary carnitine deficiency (4). This syndrome has been attributed to insufficient free carnitine to facilitate fatty acid entry into the mitochondria for oxidation (4). Conversely, a normal rise in plasma ketone concentrations after fasting or hyperketosis has been reported in children with secondary carnitine deficiencies due to organic acidurias or skeletal muscle carnitine deficiency (28, 32, 33).

In the pivalate treated rats, there was a perturbation in fat metabolism consistent with that reported in children with carnitine deficiency secondary to organic aciduria or skeletal muscle carnitine deficiency. In the starvation experiment, after 15 d of pivalate treatment, plasma glucose concentrations were not different between the two groups either before or after the fast, but fasting plasma ß-hydroxybutyrate levels were significantly higher in the pivalate-treated animals. This pattern is similar to that reported by DiDonato et al (28), in which after fasting the plasma ß-hydroxybutyrate concentration of a patient with muscle carnitine deficiency was six times that found in

normal controls, whereas the plasma glucose remained unchanged. Wolff et al (32) reported elevated fasting blood ketones in four patients with propionic aciduria. Carnitine supplementation was able to attenuate the rise in blood ketones. Chalmers et al (30) also reported a case of fasting ketosis in a child with methylmalonic aciduria which resolved after carnitine supplementation.

In the current study, liver carnitine concentrations of the pivalate-treated animals showed less depletion at the early time points studied, possibly because liver is the site of carnitine biosynthesis in the rat, and is not dependent upon an external supply of carnitine. In vitro, half-maximal rates of oleate oxidation by rat liver have been reported with only 10-15 μmol/L carnitine (34), which is far lower than the concentrations found in these studies at any time. If the liver carnitine concentration, though reduced, were not rate-limiting for fatty acid transport into the mitochondria, it would explain why animals with low tissue carnitine concentrations retained the capacity to increase their plasma ß-hydroxybutyrate concentrations after fasting. Why blood ß-hydroxybutyrate concentrations were higher in the carnitine depleted rats remains to be established. The ability of carnitine to modify the ratio of acyl CoA to free CoA may be involved. Wolff (32) hypothesized a number of mechanisms whereby through this function carnitine might diminish ketosis, including enhancing acetyl-CoA disposal via the tricarboxylic acid cycle and increasing peripheral utilization.

Liver triglyceride concentrations in the pivalate treated rats were higher than in controls. This was an unexpected finding, since the plasma ketone levels in these rats were also greater. Increased plasma free fatty acid concentrations (35) and lipid

vacuoles in the liver (27) have been reported in children with carnitine deficiency. Van Harken et al (36) showed that in perfused rat livers, triglyceride accumulation and ketogenesis were dependent upon the free fatty acid concentration of the perfusate. Consequently, elevated plasma free fatty acid concentrations may have produced the effects seen in our study.

We infer that the effect of 2 wk of pivalate administration in the rat has effects similar to those seen in human secondary carnitine deficiency due to organic acidurias. Diminished muscle carnitine concentrations, fasting ketosis, greater acyl to free carnitine ratios in the plasma and urine, and greater acylcarnitine clearances are all common features. Pivalate treatment may provide a useful animal model of carnitine deficiency under conditions of acyl-CoA accumulation. Thompson et al (37) have recently stated that the mechanisms by which carnitine supplementation could benefit children with impaired propionate metabolism require further study. Further experimentation to determine the role of carnitine in modulating ketogenesis and ketone body utilization is also needed (38). This model may provide an opportunity for exploring these questions.

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## TEXT FOOTNOTES

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# CHAPTER 2

## Carnitine supplementation ameliorates the steatosis and ketosis induced by pivalate.

Peri B. Bianchi and Alan T. Davis

## ABSTRACT

Sodium pivalate, a compound excreted in the urine bound to carnitine, was used to induce a carnitine deficiency. Weanling rats received 20 mmol/L sodium pivalate or 20 mmol/L sodium bicarbonate in their drinking water for 2 weeks. They fasted for 24 hours, and to maximize fatty acid oxidation, were cold-stressed for 4 hours. Plasma heart, muscle, and liver carnitine concentrations were significantly lower in the pivalate treated group ($p<0.01$) than the control group. Several common indicators of fat metabolism were altered. Plasma and liver triglyceride (TG) levels and plasma free fatty acid and ß-hydroxybutyrate (ßOHB) concentrations were significantly greater than those of controls ($p<0.01$). The reduced plasma and muscle carnitine concentrations, fasting ketosis, and lipid accumulation in liver and muscle are findings also reported for human secondary carnitine deficiency due to organic acidurias. Supplementing the diet with L-carnitine significantly raised plasma and tissue carnitine concentrations ($p<0.05$), and reduced the plasma ßOHB and liver TG concentrations to levels not significantly different from control values. Carnitine supplementation ameliorates the degree of liver lipid accumulation (steatosis) and exaggerated fasting ketone response (ketosis) induced by pivalate.



Key words: carnitine deficiency, carnitine supplementation, pivalate, ketosis, steatosis

Carnitine is a compound necessary for mitochondrial oxidation of long-chain fatty acids (1). Long chain fatty acyl CoAs cannot cross the mitochondrial membrane. Carnitine palmitate transferase (CPT) esterifies fatty acyl groups from CoA to carnitine, thus enabling their mitochondrial entry as acyl carnitines via carnitine translocase activity. Carnitine also modulates the acyl CoA:free CoA ratio in the cell (2), helps eliminate toxic metabolites (3), serves as an intercompartmental energy shuttle (4), and may be important in brain (5) and heart function (6).

Because the body can synthesize carnitine, documented cases of primary carnitine deficiency are few. Profound carnitine deficiency has been found in patients with a recessively inherited defect in muscle and kidney carnitine transport (7). More common are the secondary deficiencies, which result from increased losses of carnitine due to some other condition. Examples of secondary deficiencies include patients receiving dialysis treatments (8), patients with the Fanconi syndrome, who have a generalized renal loss of many small molecules including carnitine (9), and patients with genetic disorders of metabolism such as the organic acidurias and deficiencies of the enzymes of ß-oxidation (10). With each of the inborn errors of metabolism, the metabolic block leads to accumulation of metabolites which conjugate to carnitine and are excreted in the urine in quantities exceeding that of carnitine synthesis. Drug therapy with valproate and pivalate-containing compounds can also lead to increased losses of carnitine esters and carnitine deficiency (11).

The pivalate-induced animal model of carnitine deficiency reproduces several features of the carnitine deficiency observed in patients with organic acidurias (12). Total and free carnitine concentrations were reduced in plasma, heart, and skeletal

muscle after only 2 weeks of treatment. Excess liver triglycerides were present in the rats, analogous to the presence of lipid vacuoles in the liver of children with carnitine deficiency (13). In addition, an elevated acylcarnitine:free carnitine ratio, which suggests disturbed CoA homeostasis (14), is found in the plasma, urine, and tissues of both rats and patients. Furthermore, the response to fasting of both patients with propionic acidura and pivalate-treated rats is increased ketone production (12), unlike the hypoketonemia most often associated with what was formerly termed "primary systemic carnitine deficiency" (10).

Why the blood ßOHB concentrations are higher with this form of carnitine deficiency remains to be established. Despite the high plasma ketone levels in the pivalate-treated group, suggesting accelerated hepatic fatty acid oxidation, their liver triglyceride concentrations are elevated. Van Harken et al (15) showed that in perfused rat livers, both triglyceride accumulation and ketogenesis were dependent upon the free fatty acid concentration of the perfusate. High free fatty acid (FFA) levels are sometimes found in patients with carnitine deficiencies (16). Consequently, elevated plasma FFA concentrations may have produced the effects initially reported.

The goal of the following studies was to extend and confirm the work described in chapter 1. The following questions were raised: *1)* Would differences between control and treated rats be further accentuated by using younger animals and by attempting to maximize their rates of fatty acid oxidation?, *2)* Were there differences in plasma FFA concentrations between treated and control rats? *3)* Were the differences in fat metabolism related to the changes in carnitine concentration or to an unrelated pivalate toxicity? Specifically, would carnitine supplementation attenuate

the effect of pivalate treatment?

## MATERIALS AND METHODS

***Experiment 1.***

**Animals and experimental design.** To answer the first two questions weanling rats were used, because their weight triples during the two-week study period. Their need for carnitine to maintain tissue concentrations during a rapid growth period should make them highly susceptible to depletion strategies. Twelve (12) male, Sprague-Dawley, weanling rats were randomized into two groups of equal number and allowed to adjust to the AIN-76A diet, which was used for this and all subsequent experiments, for three days. The rats continued the diet and received either 20 mmol/l sodium bicarbonate (CON) or 20 mmol/L sodium pivalate as their drinking water (PIV) for 2 weeks. The last day food was withheld for 24 hours, and the rats were kept at 6° C for an additional 4 hours to further increase their rate of fatty acid oxidation (17). Urine was collected during the last 24 hours of the study, using 0.5 mL of 6 mol/L hydrochloric acid as a preservative, and stored at -20° C prior to analysis.

***Experiment 2.***

**Animals and experimental design.** For the carnitine supplementation study, three groups of male, weanling rats were given a 20 mmol/L solution of sodium pivalate as their drinking water for two weeks. One group of 6 rats (PIV) received a low carnitine AIN-76A diet without carnitine supplementation. A second group (+CN),

n=7, received the same diet supplemented with carnitine to provide 0.3 mmol/day additional carnitine, a dose approximately equimolar to the pivalate dose. Because Melegh (11) found children supplemented with carnitine equimolar to pivampicillin were not able to maintain stable plasma total carnitine concentrations, a third group (++CN), n=7, received a diet intended to provide 0.8 mmol/day (++CN). The 6 rats making up the fourth group, the controls (CON), received unsupplemented diet and a 20 mmol/L sodium bicarbonate solution as their water. Solutions were adjusted to pH 7.0. Food and water intake were monitored 3 times per week. After two weeks, food was withheld for 24 hours. During the last four hours they were housed at 6° C. Urine was collected as described previously. After the cold exposure rats were killed, and blood and tissues samples were collected. Because rats in some metabolic cages had significantly reduced food intake and weight gain, they were omitted from data analysis. One treatment group was, therefore, reduced to only three animals. Consequently, a second supplementation study was done, experiment 3.

***Experiment 3.***

**Animals and experimental design.**

Our primary goal for this experiment was to confirm the effects of carnitine supplementation on the fasting ketosis. For the second supplementation study 2 groups of male, weanling rats were given rats were given a 20 mmol/L solution of sodium pivalate as their drinking water for two weeks. One group of rats received a low carnitine AIN-76A diet without carnitine (PIV). Because the initial supplementation study showed providing rats with 0.3 mmol/day additional carnitine had only a modest

effect, only the higher carnitine supplementation level. was fed to the second group of rats (**PIV/CN+**). A control group received 20 mmol/L sodium bicarbonate in their water and the unsupplemented diet (**CON**). In this experiment, the fourth group received the standard AIN-76 diet and plain water (**$H_20$**). In previous experiments sodium bicarbonate was used for the control solution to maintain electrolyte balance with the sodium pivalate group(s). However, because bicarbonate infusion has been shown to increase hepatic ketone release in rats (18) and to promote an increase in total plasma ketones in humans (19), it was prudent to test for an effect of oral ingestion of bicarbonate in this model.

***Blood and tissue samples.*** At the end of each study, rats were killed by decapitation and blood was collected from the neck into heparinized tubes. Tissue samples for Experiments 1 and 2 were obtained in the following manner. A section of the median lobe of the liver was excised and quickly freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen. The heart and a sample of hindlimb muscle were then obtained and frozen in like manner. In experiment 3, the gastrocnemius muscle, which has a mixture of red and white fibers, was obtained.

***Chemical analyses.*** Carnitine in urine, tissues, and plasma was determined by the radiochemical technique of Cedarblad and Lindstedt (20) as modified by Brass and Hoppel (21). Plasma and urine ßOHB were measured using kit No 310-UV, Sigma Chemical Co. (St. Louis, MO). Sigma Kit No. 405 was used to measure triglycerides in liver homogenates, 0.2 g liver/mL. Sigma Kit No. 336 was used for measuring serum triglycerides. Acetoacetate was measured using the method of Mellanby and

Williamson (22) as modified for unacidified plasma (23). Blood samples were promptly spun and the plasma kept on ice until the assay was run. Plasma was assayed in a phosphate buffer pH 6.8, made by combining equal volumes of 0.1 mol/L potassium dihydrogen phosphate and dipotassium hydrogen phosphate. Reduced nicotinamide-adentine dinucleotide, 6 mmol/L, was included in the reaction mixture. The change in absorbance at 340 λ after the addition ß-hydroxybutyrate dehydrogenase was measured to determine the amount of acetoacetate present. Plasma free fatty acids were measured with an enzymatice colorimetric assay using Kit No. 1383 175 from Boehringer Mannheim Biochemicals. The free fatty acids are esterified to CoA, and the acyl CoA oxidized to form enoyl CoA and hydrogen peroxide. The latter induces the formation of a red dye which is measured at 546 λ.

Urinary dicarboxylic acids were determined in Dr. Denis C. Lahotay's laboratory by a modification of the method of Goodman and Markey (24). Aliquots of acidified, salt saturated urine specimens containing 0.25 μmoles of creatinine were diluted to 2 ml with water, extracted once with two volumes of ethyl acetate, and once with ether. The extracts were combined, dried under nitrogen, and derivitized with 100 μL of N,O-Bis(trimethyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) to form trimethylsilyl derivatives. A one μL aliquot of the derivatized mixture was injected in splitless mode into a Hewlett Packard 5890 Series II Gas Chromatograph equipped with a 0.25μ, 30 m, DB-1 glass capillary chromatographic column (J&W Scientific, Folsom, California) with an on-column injector. The injector and transfer line were kept at 250°C. The column temperature

was initially set to 60° C for 1 minute, increased by 20° $min^{-1}$ to 90° C, then raised at 8° C $min^{-1}$ to 310° C. Samples eluting from the column were analyzed and quantitated by mass spectrometry in electron ionization mode with an HP-5971 quadrupole Mass Spectrometer. The identity of the individual organic acids was confirmed by comparing the mass spectrum of each with the mass spectra of crystalline standards stored in a mass spectral library. Prior to extraction, 0.5 $\mu$moles of this standard was added to a volume of urine containing 2.5 $\mu$moles of creatinine. Organic acid concentrations were calculated based on the ratio of the individual organic acid to the internal standard, using a Windows-based target compound analysis software program provided with the detector from Hewlett-Packard. A unique, prominent ion was selected for each organic acid or dicarboxylic acid, and the ratio of the ion abundance of this ion to that of the internal standard, pentadecanoic acid, was used for constructing standard curves. Some of the unsaturated dicarboxylic acids are not available commercially. Standard curves for these were constructed assuming that the abundance of the total ion current of such a compound is the same as that of a structurally closely related, commercially available saturated analog of the same concentration. This means of calculating the concentration of commercially unavailable organic acids has been validated in several laboratories involved in the diagnosis of inborn errors of organic acid metabolism (25). Recovery of standards is approximately 100%. Results are expressed per unit amount of creatinine to correct for differences in urine concentration.

***Histology.*** Slides of hindlimb muscle or gastrocnemius were stained with oil red O.

Lipid droplets, which stain orange with oil red O, were ranked on a 0-3 scale of "no lipid" to "much lipid" in ten, high power fields per coded slide. Readings from the first study were confirmed by a histology technician unaware of the treatment groups and in the second study by a pathologist also uninformed of the treatment group codes.

***Materials.*** [$^{14}$C]-Acetyl CoA was obtained from NEN Products, E.I. DuPont (Boston, MA), and carnitine acetyl transferase was obtained from Boehringer Mannheim (Indianapolis, IN). The pivalic acid, sodium salt, was obtained from Aldrich Chemical (Milwaukee, WI). All other chemicals used were of reagent grade.

***Statistical analysis.*** Differences between the control and pivalate-treated groups were analyzed using Student's T-test. When there were more than two treatments, differences between groups were analyzed by one-way analysis of variance. Where significant differences were found, differences between treatment means were tested with the Student-Newman-Keuls procedure. The urine data was not normally distributed and, therefore, was analyzed with the Mann-Whitney test when there were two treatment groups, and the Kruskall Wallis procedure followed by Dunn's test for multiple treatments. Correlations between groups were determined using Pearsons' r. A probability level of Type I error of $p<0.05$ was selected to signify statistical significance.

# RESULTS

## *Experiment 1.*

***Body weight.*** Body weights of the rats at the end of the 2-week period were similar for both groups, 118.4 ± 6.8 g for the treated group and 121.8 ± 4.2 g for the control group.

***Carnitine levels.*** Total carnitine levels of treated rats, shown in **Table 1** , were reduced by 60% or more in plasma, skeletal muscle, and heart, and by 33% in the liver ($p<0.01$). Excretion of free carnitine in urine was significantly decreased, and an increase in the acylcarnitine/free carnitine ratio was found ($p<0.01$), **Figure 1**.

***Lipid metabolism and histology.*** Plasma ßOHB concentrations and FFA concentrations were significantly greater ($p<0.01$) in the pivalate-treated animals. Plasma and liver triglyceride concentrations of treated rats were also significantly increased. These and other indices of fat metabolism in plasma and liver are shown in **Table 2**. Plasma free fatty acid and ßOHB concentrations were significantly correlated, $R=0.75$, $p<0.007$. There were also significant correlations between plasma free fatty acid levels and the plasma triglyceride concentrations, $r=0.74$, $P<0.004$, and the liver and plasma triglyceride concentrations, $r=0.64$, $P<.02$. Lipid droplets were more prevalent in the muscle of the pivalate-treated rats. Representative tissue samples are depicted in **Figure 2**.

**TABLE 1 Plasma and tissue carnitine levels with 2 weeks of pivalate treatment[1,2]**

| Group | Total Carnitine | | | |
|---|---|---|---|---|
| | Plasma | Heart | Muscle | Liver |
| | *nmol/ml* | *nmol/g* | *nmol/g* | *nmol/g* |
| Control | 20.4 | 558 | 508 | 314 |
| Pivalate | 7.0 | 216 | 184 | 213 |
| SE | 2.1 | 68 | 62 | 46 |

[1]Values are means, n=7 for each group.

[2]For all tests, differences between groups were significantly different, $p < 0.01$, Student's *t*-test.

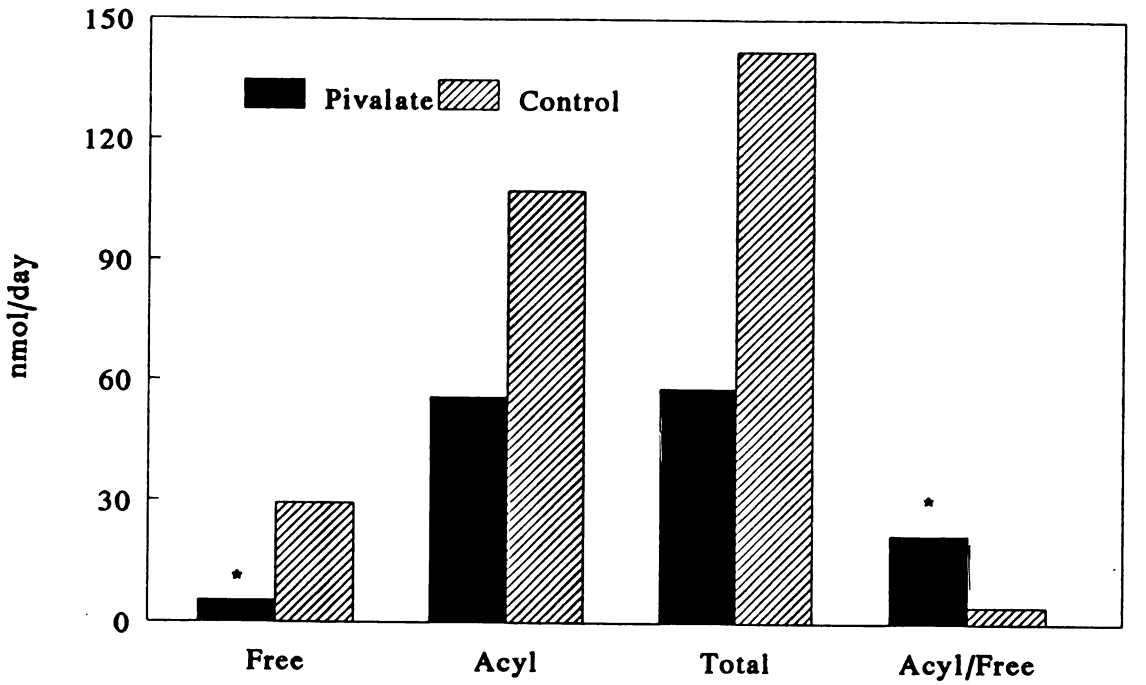


**FIGURE 1** Urine carnitine concentrations of rats given 20 mmol/L sodium pivalate or sodium carbonate in their water for 2 weeks. Values are medians for 6 rats per group. Bars with asterisks represent data that are significantly different from control values ($p < 0.01$), Mann-Whitney U test.

**TABLE 2 Altered fat metabolism in a pivalate-induced rat model of carnitine deficiency[1,2]**

| Group | Triglycerides | | Free Fatty Acids | ß(OH)butyrate |
|---|---|---|---|---|
| | Plasma | Liver | Plasma | Plasma |
| | *mM* | *mg/g* | *mM* | *mM* |
| Control | 0.84 | 8.8 | 1.5 | 2.4 |
| Pivalate | 1.62 | 90.2 | 2.3 | 5.0 |
| SE | .25 | 18.1 | 0.38 | 0.6 |

[1]For all tests, differences between groups were significantly different, $p < 0.01$, Student's *t*-test.

[2]Values are means, n=7 for each group.

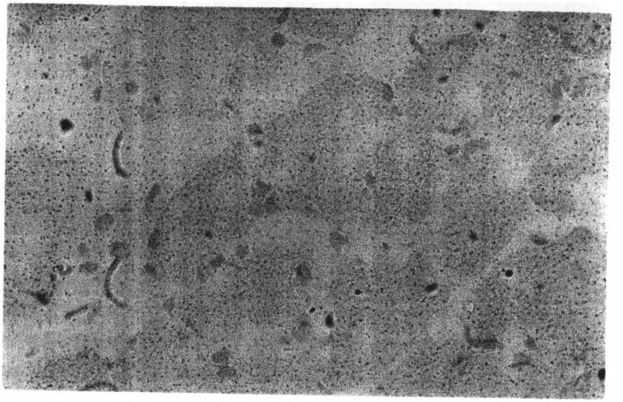

**FIGURE 2A** Representative slide of hindlimb muscle rat stained with oil red O, magnification 400 x, **Control** rat.

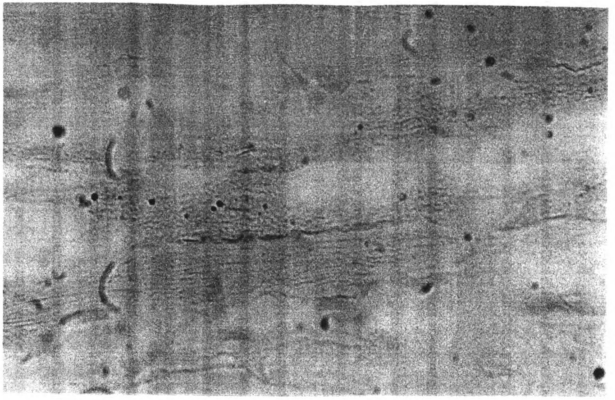

**FIGURE 2B** Representative slide of hindlimb muscle stained with oil red O, magnification 400, **Pivalate-treated** rat.

*Experiment 2.*

***Food and pivalate intake and weight gain.*** Carnitine supplemented rats consumed 0.33 mmol carnitine/d (Cn+) or 0.65 mmol carnitine/d (Cn++), which corresponded to 38 mg/kg or 74 mg/kg, respectively. Average daily pivalate consumption by pivalate-treated rats was 0.36-0.40 mmol. Neither weight gain, final weights after food deprivation, nor weight loss was significantly different between treatment groups.

***Carnitine levels.*** Pivalate did not prevent tissue uptake of carnitine in the supplemented rats. Their tissue carnitine concentrations, **Table 3**, were 200% of control levels or more.

***Lipid metabolism.*** Urinary excretion of ßOHB was similar for all groups, **Figure 3.** As before, plasma ßOHB concentrations of the pivalate-treated rats were significantly greater than those of the controls, **Figure 4.** There was a dose dependent decrease in the plasma ketone concentration with carnitine supplementation. Levels in the group receiving the greater supplementation were not significantly elevated. The same relationship was found for the liver and plasma triglyceride concentrations, **Figures 5 and 6.** Concentrations were significantly greater in the pivalate group than the control group, but for rats supplemented with 0.65 mmol carnitine/d (Cn++), levels were not significantly greater than control levels. Unlike Experiment 1, the plasma free fatty acid concentrations of the unsupplemented pivalate-treated rats, **Figure 7,** were not significantly greater than control concentrations, though carnitine supplementation reduced the FFA concentration. Oil red O stains of gastrocnemius muscle (not shown)

**TABLE 3 Rat plasma and tissue carnitine levels after 2 weeks of pivalate treatment[1,2]**

| Group | | Total Carnitine | | | |
|---|---|---|---|---|---|
| | | Plasma | Heart | Muscle | Liver |
| | | *nmol/ml* | *nmol/g* | *nmol/g* | *nmol/g* |
| Control[3], | n=5[2] | 21.9[a] | 545[a] | 457[a] | 277[a] |
| Pivalate[4], | n=3 | 7.1[a] | 234[b] | 170[b] | 217[a] |
| Piv + Cn[5], | n=5 | 74.1[b] | 1104[c] | 1214[c] | 793[b] |
| Piv++Cn[6], | n=6 | 101.1[b] | 1044[c] | 1420[c] | 566[a,b] |
| SE, pooled | | 9.6 | 57 | 75 | 115 |

[1]Groups with common subscripts are not significantly different ($P > 0.05$), Student-Keuls-test.

[2]Male, Sprague-Dawley weanling rats.

[3]Rats received water providing 20 mmol/L sodium bicarbonate and a semipurified AIN-76A diet.

[4]Rats received water providing 20 mmol/L sodium pivalate and a semipurified AIN-76A diet.

[5]Rats received water providing 20 mmol/L sodium pivalate and a semipurified AIN-76A diet providing 0.334 mmol supplemental L-carnitine/ day.

[6]Rats received water providing 20 mmol/L sodium pivalate and a semipurified AIN-76A diet providing 0.646 .mmol supplemental L-carnitine/ day.

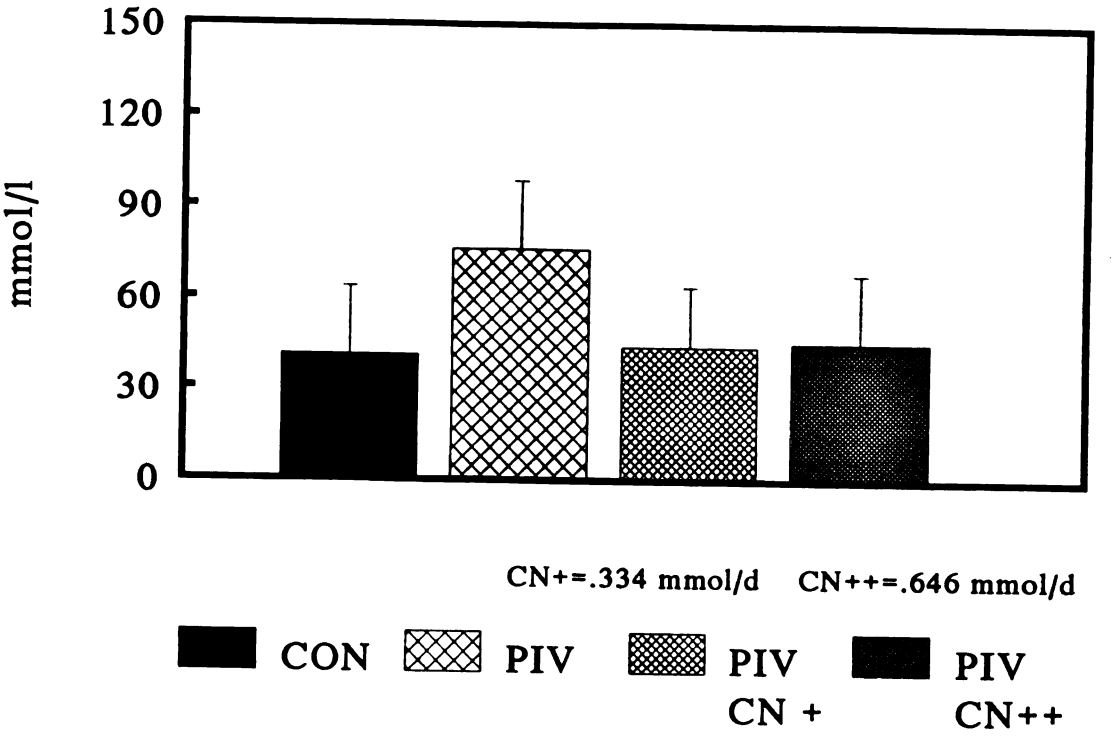


**FIGURE 3** ßOHB concentrations in the urine of weanling rats given the following solutions in their water for two weeks: **Con** 20 mmol/L sodium bicarbonate, n=5; **Piv** 20 mmol/L sodium pivalate, n=3. Both supplemented groups also received the sodium pivalate solution. The **Cn+** group consumed 0.334 mmol supplemental L-carnitine/day mixed in their diet, n=5. The **Cn++** group consumed 0.646 mmol/day L-carnitine, n=6. Values, shown as medians, are not significantly different ($P > 0.05$), Kruskall Wallis test.

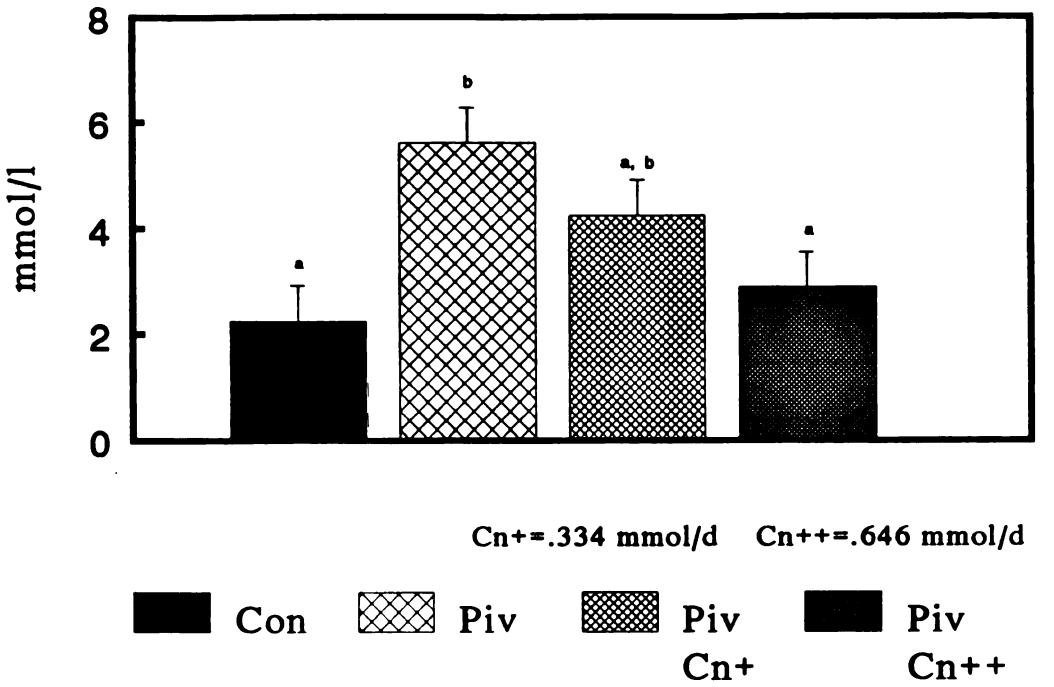


**FIGURE 4** ßOHB concentrations in the plasma of weanling rats given the following solutions in their water for 2 weeks: **Con** 20 mmol/L sodium bicarbonate, n=5; **Piv** 20 mmol/L sodium pivalate, n=3. Both supplemented groups also received the sodium pivalate solution. The **Cn+** group consumed 0.334 mmol supplemental L-carnitine/day mixed in their diet, n=5. The **Cn++** group consumed 0.646 mmol/day L-carnitine, n=6. Bars with common superscripts are not significantly different ($P > 0.05$), Student-Newman-Keuls test.

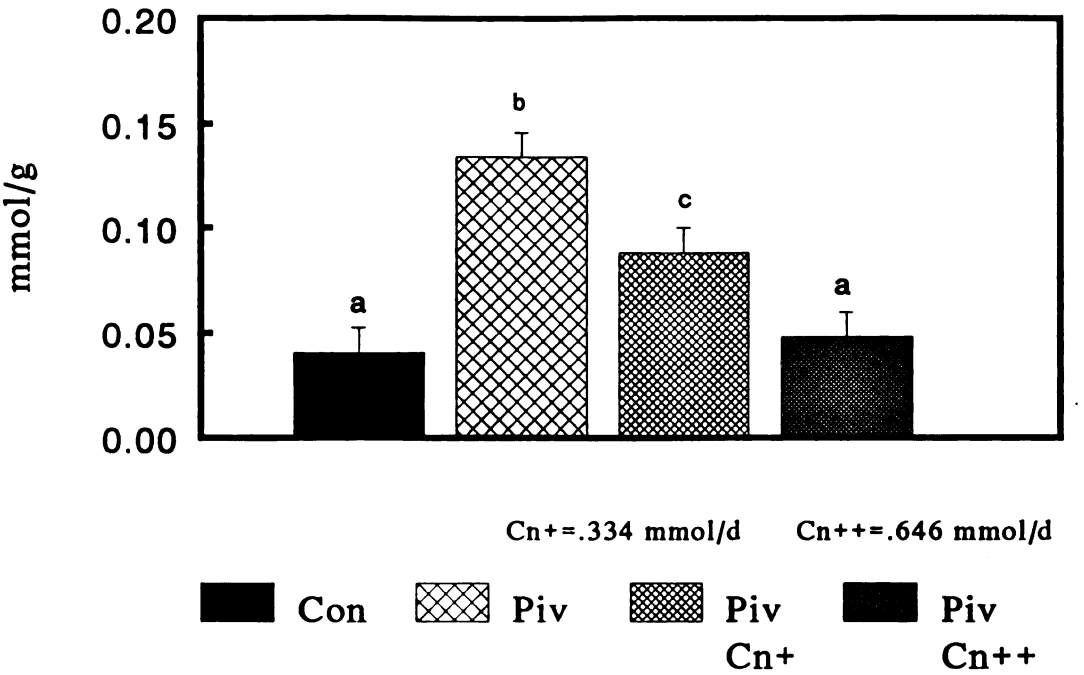


**FIGURE 5** Liver triglyceride (mmol/g wet tissue weight) concentrations of weanling rats given the following solutions in their water for two weeks: **Con** 20 mmol/L sodium bicarbonate, n=5; **Piv** 20 mmol/L sodium pivalate, n=3. Both supplemented groups also received the sodium pivalate. The **Cn+** group consumed 0.334 mmol supplemental L-carnitine/day mixed in their diet, n=5. The **Cn++** group consumed 0.646 mmol/day L-carnitine, n=6. Bars with common superscripts are not significantly different ($P > 0.05$), Student-Newman-Keuls test.

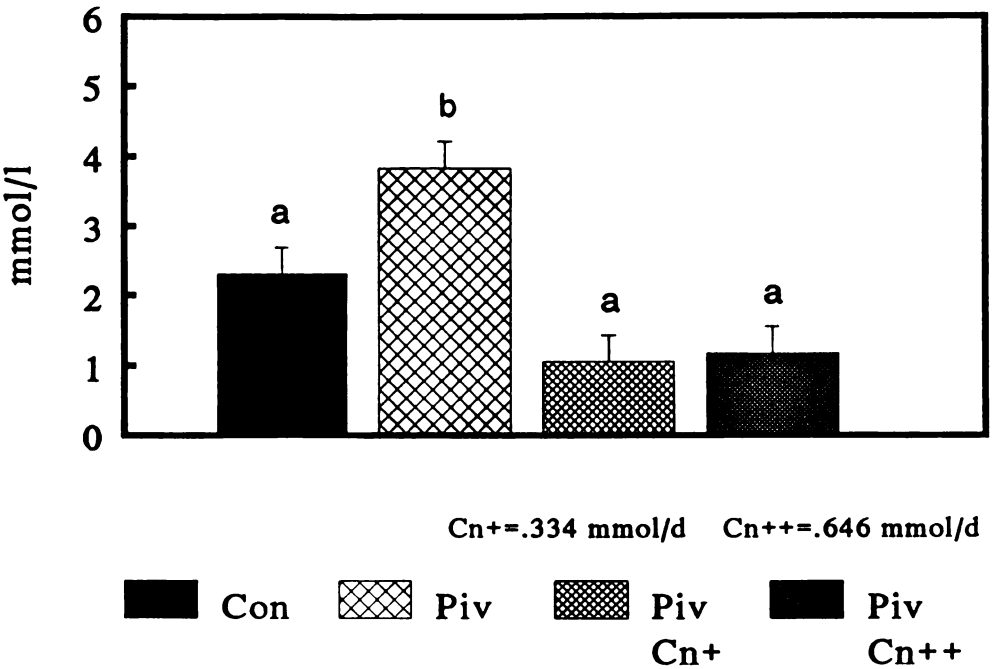


**FIGURE 6** Plasma triglyceride concentrations of weanling rats given the following solutions in their water for two weeks: **Con** 20 mmol/L sodium bicarbonate, n=5; **Piv** 20 mmol/L sodium pivalate, n=3. Both supplemented groups also received the sodium pivalate. The **Cn+** group consumed 0.334 mmol supplemental L-carnitine/day mixed in their diet, n=5. The **Cn++** group consumed 0.646 mmol/day L-carnitine, n=6. Bars with common superscripts are not significantly different ($P > 0.05$), Student-Newman-Keuls test.

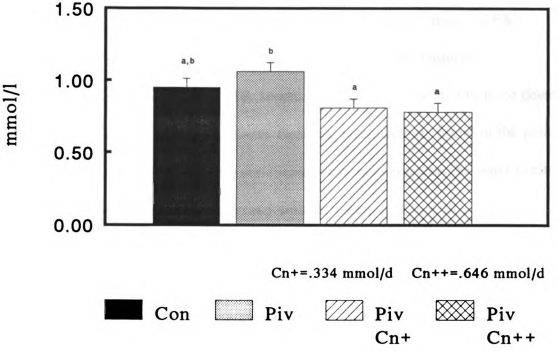


**FIGURE 7** Experiment 2. Plasma FFA concentrations of weanling rats given the following solutions in their water for two weeks: **Con** 20 mmol/L sodium bicarbonate, n=5; **Piv** 20 mmol/L sodium pivalate, n=3. Both supplemented groups also received the sodium pivalate. The **Cn+** group consumed 0.334 mmol supplemental L-carnitine/day mixed in their diet, n=5. The **Cn++** group consumed 0.646 mmol/day L-carnitine, n=6. Bars with common superscripts are not significantly different (P > 0.05), Student-Newman-Keuls test.

had no pattern of lipid deposition among the treatment groups.

***Experiment 3.***

**Fat Metabolism.** The second supplementation study confirmed the ability of carnitine supplementation to ameliorate the effects of pivalate administration upon plasma ketones. Again, there were no significant differences in plasma FFA concentrations between treated and control rats, but, as before, carnitine supplementation reduced plasma FFA levels. The data are presented in more detail in **Figures 8-9**. Significantly more urinary dicarboxylic acids were found in the pivalate group, **Figure 10**. There were no significant differences between the water control group and the bicarbonate control group with any assay.

## DISCUSSION

The pivalate-treated, weanling rat was a good model of secondary carnitine deficiency like that seen in patients with organic acidurias. As found previously, plasma and tissue carnitine concentrations were lower, and the urine acylcarnitine:free carnitine ratio was higher in pivalate-treated rats than controls. Plasma ßOHB levels of pivalate-treated rats were, as in the original study, approximately twice those of the controls. However, the degree of liver triglyceride accumulation was greater in the younger treated rats. In the previously reported study triglyceride concentrations were approximately twice that of control levels, while these studies with weanling rats gave values 3-10 times the control numbers.

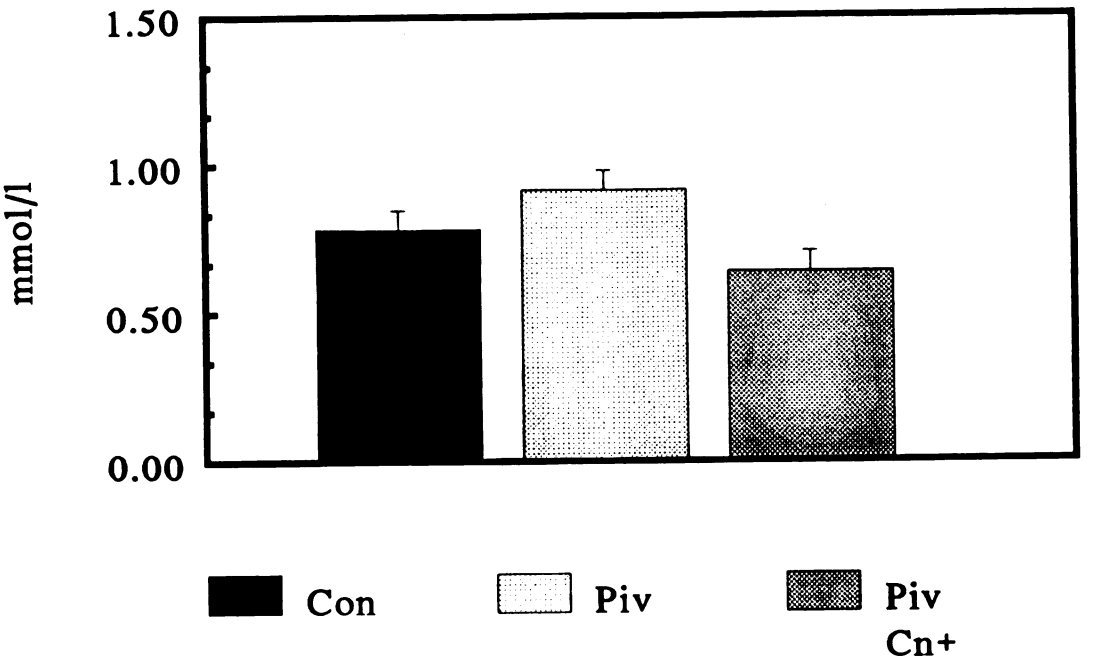


**FIGURE 8** Experiment 3. Plasma FFA concentrations of weanling rats given the following solutions in their water for two weeks: **Con** 20 mmol/L sodium bicarbonate, n=4; **Piv** 20 mmol/L sodium pivalate, n=6. **Piv/Cn+** 20 mmol/L sodium pivalate in the water and .65 mmol/day L-carnitine in the diet, n=6. Concentrations were not significantly different ($P < 0.05$), Student-Newman-Keuls test.

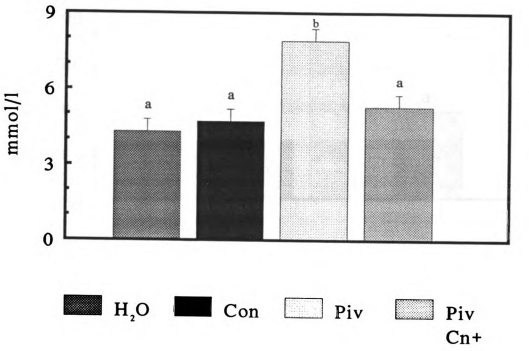


**FIGURE** 9 Plasma ketone (ß-hydroxybutyrate + acetoacetate) concentrations of weanling rats given the following solutions in their water for two weeks: **$H_20$** tap water, n=5; **Con** 20 mmol/L sodium bicarbonate, n=4; **Piv** 20 mmol/L sodium pivalate, n=6. **Piv/Cn+** 20 mmol/L sodium pivalate and 0.65 mmol/day L-carnitine in the diet, n=6. Bars with common superscripts are not significantly different (P > 0.05), Student-Newman-Keuls test.

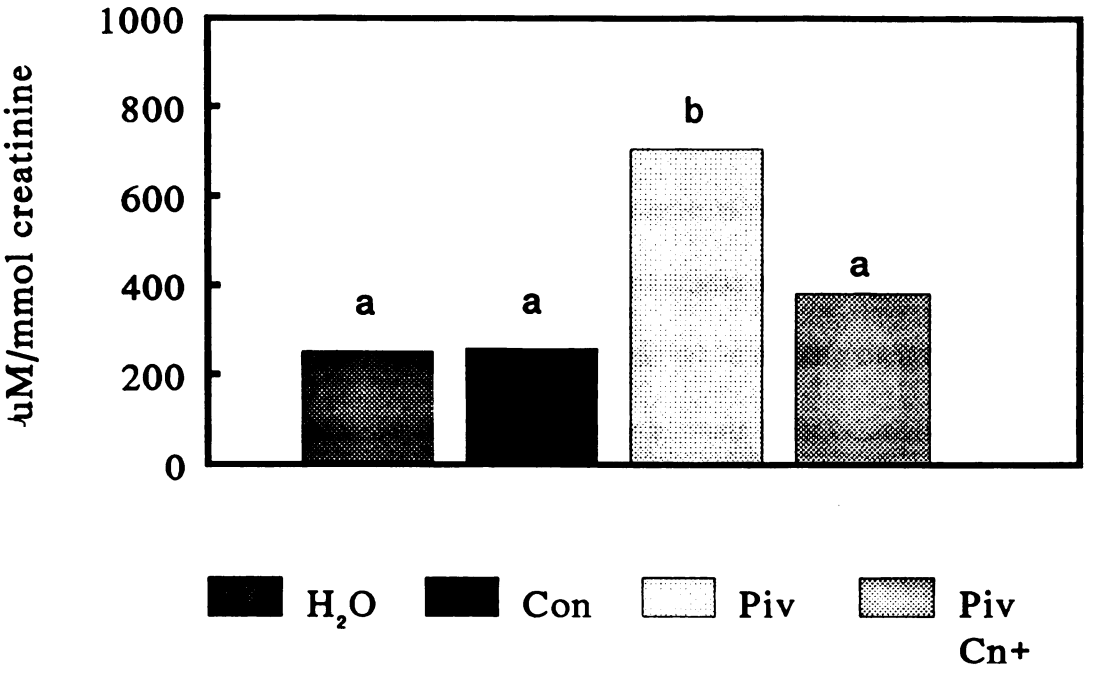


**FIGURE 10** Urine dicarboxylic acid concentrations of weanling rats given the following solutions in their water for two weeks: **$H_2O$** tap water, n=4; **Con** 20 mmol/L sodium bicarbonate, n=4; **Piv** 20 mmol/L sodium pivalate, n=5. **Piv/Cn+** 20 mmol/L sodium pivalate and 0.65 mmol/day L-carnitine in the diet, n=5. Bars with common superscripts are not significantly different ($P > 0.05$), Kruskall-Wallis test followed by Dunn's procedure.

As hypothesized, weanling rats appeared more susceptible to carnitine depletion with pivalate treatment than the older rats (12). Although plasma concentrations were reduced similarly to 35-36% of control values in the weanling and older rats, tissue carnitine levels were lower in the younger rats. Total carnitine in the heart was reduced to 39% of control values, compared to a 54% reduction in older rats. Muscle and liver levels were 36% and 68% of control levels, as opposed to 56% and 74% in the previous study. There is no evidence for a compensatory increase in carnitine synthesis when plasma and tissue carnitine concentrations are low (26). Thus, the young rats could not maintain tissue concentrations while tripling their weight. Carnitine may be an essential nutrient in growing animals (27).

Even though the tissue carnitine concentrations in pivalate-treated rats are not considered low enough to limit long chain fatty acid entry into the mitochondria for oxidation (28), carnitine supplementation ameliorated the effects of the pivalate. Both the hepatic steatosis and ketosis were reduced to control levels by providing carnitine at a molar concentration approximately 160% of pivalate intake. These results may indicate carnitine needs of rats are greater in the presence of pivalate. However, plasma and tissue carnitine concentrations were similarly increased with both levels of supplementation, whereas only the higher level of supplementation fully attenuated the fasting ketosis and steatosis. The mechanism by which carnitine is acting is not clear. However, rats receiving the greater carnitine supplementation may have excreted more acyl moieties, including pivaloyl carnitine, in their urine. Loss of these acyl groups may have permitted more normal metabolism.

The plasma ßOHB concentration is determined by the rate of production, the rate of utilization, the rate of excretion, and the balance between acetoacetate and ßOHB. The latter two mechanisms are not responsible for the exaggerated plasma ketone response to fasting by pivalate-treated rats. Control and treated rats excreted similar levels of urinary ßOHB in 24-hours. In addition, data shown in **Table 4** demonstrate that the total plasma ketone concentrations were also higher in treated rats, hence the greater ßOHB levels reflected greater ketosis, not merely a redistribution between the two moieties. Increased ketogenesis and or decreased utilization remain as possible causes of the hyperketonemia found in pivalate-treated rats.

In the fasted individual, ketogenesis is generally proportional to the free fatty acid delivery to the liver (29). Di Donato, et al (30) attributed the exaggerated fasting ketosis of a patient with myopathic carnitine deficiency to his elevated plasma FFA concentration. The patient's muscle carnitine concentrations were low, raising the possibility that reduced peripheral fat oxidation made more fatty acids available for liver uptake.

Results from the first study with pivalate-treated, weanling rats (Experiment 1) suggested that their pronounced plasma ketone response to fasting might also be a function of enhanced substrate supply to the liver. Low muscle carnitine

**TABLE 4 Comparison of ketones in pivalate-treated and control rats[1]**

| Group | Plasma ßOHB | Plasma Ketones[2] |
|---|---|---|
| | *mmol/L* | *mmol/L* |
| Pivalate, fed | 0.60 ± 0.04[a] | Not done |
| Control, fed | 0.60 ± 0.07[b] | Not done |
| Pivalate, unfed | 4.83 ± 0.78[b] | 6.75 ± 1.02[a] |
| Control, unfed | 3.22 ± 0.44[b] | 4.27 ± 0.50[b] |

[1]N=6 per group. Unfed rats had food withdrawn 1 day prior to data collection. Values are means ±standard error. Values in a column with unlike superscripts are significantly different from each other, $p < 0.05$, Student's t-test. Data are from Bianchi, P.B. Comparison of ketones and liver triglycerides of fed and unfed pivalate-treated and control rats. Dissertation, Appendix.

[2]ßOHB + acetoacetate.

concentrations and lipid droplets in the muscle were found, which suggested fatty acid oxidation may be diminished in that tissue. Even though carnitine levels in the treated rats were not reduced to a rate-limiting level for CPT, 23-30 nmol/g (28), one could speculate that the presence of pivalate or pivaloyl CoA in the muscle might provide an additional inhibitory effect.

There are number of mechanisms by which this might occur. Pivalate may sequester carnitine in ester form, reducing the amount available for transporting fatty acids into the mitochondria. However, the mean muscle free carnitine concentration of pivalate-treated rats was 115 nmol/g, still substantially above the rate-limiting concentration. Pivalate may have effects like valproate, another compound known to esterify with carnitine and induce carnitine deficiency. The CoA ester of valproate and other valproate metabolites inhibit enzymes in the hepatic ß-oxidation pathway (31). However, analysis of urinary carnitine metabolites showed no characteristic excretion pattern denoting a block in the pathway. Finally, though pivaloyl-CoA is a poor substrate for carnitine acetyl transferase (CAT), the pivaloylcarnitine, once formed, modestly inhibits hepatic mitochondrial CAT and carnitine octanoyltransferase (32). Should pivalate act similarly in muscle, there could be augmented fatty acid supply to the liver despite only a modest muscle carnitine depletion. Finding significantly greater dicarboxylicaciduria, a marker of peroxisomal fatty acid oxidation, in pivalate-treated rats than control rats is consistent with this hypothesis. Dicarboxylic acids derive from medium chain fatty acids which are chain-shortened in the microsomes by omega-oxidation and which subsequently undergo partial ß-

oxidation (33). This is a salvage pathway for fatty acid oxidaton. Dicarboxylicaciduria is frequently an indicator of impaired ß-oxidation (10). If pivaloylcarnitine inhibits muscle CAT and COT, the ability to maintain adequate levels of mitochondrial free CoA for maximal CPT2 activity would be impaired. Peroxisomal fat oxidation and urinary excretion of dicarboxylic acids would likely be enhanced, and there could be reduced peripheral uptake of FFA and with augmented hepatic FFA supply. Although hepatic enzyme inhibition would also lead to dicarboxylicaciduria, because of the vigorous ketone response to food deprivation by the rats, this seems less likely. After oral ingestion or injection with pivaloyl compounds, relatively little pivaloylcarnitine is found in the livers of rats compared with concentrations in the heart, fat, and muscle (34). In addition, fasting fatty acid oxidation rates measured in the peroxisomes are 10-30% of mitochondrial rates (35). Consequently, if hepatic mitochondrial fatty acid oxidation were significantly inhibited, ketone levels in pivalate-treated rats should be lower, not higher, than in controls, despite increased peroxisomal oxidation.

The high plasma FFA concentrations and triglyceride data were also consistent with the hypothesis that peripheral fatty acid oxidation of pivalate-treated rats was reduced. Maximum ketone production by perfused livers from food-deprived rats has been reported with a perfusate fatty acid concentration of 1.7 mmol/L (15), a level less than the plasma level of pivalate-treated rats in Experiment 1. Additional FFA supply led to hepatic lipid accumulation. Similar findings are reported for diabetic rats (36, 37), isolated perfused rat livers of diabetic rats (15, 38), and hepatocytes from rats

previously fed a high fat diet (39). In each case high ketone production rates accompanied by elevated liver triglycerides were found. Meier el al (36) proposed that with a high FFA influx to the liver, fatty acid oxidative capacity is eventually exceeded, and triglycerides accumulate. Plasma triglyceride concentrations also rise, as the liver secretes triglyceride (38). All of the initial findings were consistent with this scenario.

Our supplementation study (Experiment 2) again showed similarly high plasma ketone concentrations in pivalate-treated rats, but a less marked hepatic steatosis, a non-significant plasma FFA elevation and no evidence of impaired muscle fatty acid oxidation. The milder steatosis accompanying ketosis of rats in the supplementation study could reflect the less pronounced elevation in the plasma FFA concentration. The correlation between plasma FFA concentrations and liver triglycerides was .78, $p<.001$. The correlation between the plasma FFA and plasma triglycerides was also strong, 0.65, $p<.002$.

The ketone data from the supplementation studies does not fit the above scenario well. Though with the greater level of carnitine supplementation both plasma FFA and ßOHB levels were reduced to control levels, the correlation between the free fatty acid levels and ßOHB levels was poor, 0.33, $P<.10$. In the follow-up supplementation study, Experiment 3, plasma FFA levels were, again, not significantly higher in the pivalate-treated group. As in Experiment 2, carnitine supplemented rats had lower plasma FFA concentrations, but the correlation between the FFA values and total ketone values was even poorer, 0.20, $P<0.45$. These findings concur with those

of Wolff et al (40), who found a lack of correlation between the plasma FFA concentration and ketone concentration of carnitine-deficient children with fasting ketosis. Based on these findings, it appears the degree of hepatic steatosis is related to the plasma FFA concentrations. However, the data did not indicate the high plasma ketone concentrations of the pivalate-treated rats or the reduction in level with carnitine supplementation was simply due to fatty acid supply to the liver. The simplest explanation for the ketosis and steatosis of pivalate-treated rats apparently does not tell the whole story.

On the other hand, the data could not eliminate this as a possible mechanism. FFA concentrations were measured in mixed arterial and venous blood obtained from the neck. Although data from such blood samples are often extrapolated to represent FFA delivery to the liver, the plasma concentration reflects the balance between the rate of release of FFA and their uptake by hepatic and peripheral tissues. Measurement of hepatic artery and portal concentrations would provide a more accurate picture of hepatic substrate inflow. Another potential confounding factor is that the plasma FFA concentration, particularly an isolated measurement, may not accurately reflect utilization. As demonstrated in the diabetic rat (36), an isolated plasma FFA level may not reflect the animal's metabolic state well. Although overall, plasma FFA concentrations of the diabetic rat correlated with plasma ketone levels, depending on the specific time blood samples were obtained, the correlation between the plasma FFA and ketone concentrations was poor or even inverse. Thus, despite the poor correlation between the plasma FFA and ketone concentrations, the data does

not preclude a role for differential fatty acid supply as one mechanism for the ketosis and steatosis of pivalate-treated rats.

There are other possible explanations for the exaggerated fasting ketosis by pivalate-treated rats and its amelioration by carnitine supplementation. Wolff and co-workers (40) proposed a number of mechanisms by which carnitine might diminish ketosis: by reducing fatty acid entry into the mitochondria, by reducing ß-oxidation, by enhancing fatty acid synthesis from acetyl CoA, by inhibiting 3-hydroxy-3-methylglutaryl (HMG) synthase, by modifying ketone utilization, and by increasing tricarboxylic acid (TCA) cycle oxidation of acetyl-CoA. The first proposed mechanism is at odds with the main function of carnitine, and I am aware of no data implicating carnitine with the next two possibilities. The final three proposals appear to be the more likely explanations, and will be addressed in the next paragraphs.

It is unlikely reduced TCA activity would be the sole explanation for the enhanced ßOHB response to fasting of the pivalate-treated rats. The primary fate of labeled palmitate in liver tissue slices (41) and oleic acid in isolated, perfused livers (42) taken from food-deprived rats is ketone production. $^{14}CO_2$ recovery from tissue slices from fed or food-deprived rats was similar at only 2-3%, whereas acetoacetate production was 10-fold higher in slices from 24 hour food-deprived rats (43). Therefore, it seems unlikely changes in TCA activity alone could produce the two-fold greater plasma concentrations observed. In addition, Ruff and Brass (43) showed no diminution in $^{14}$C-palmitate oxidation to $^{14}CO_2$ on hepatocytes incubated with pivalate. Since fat oxidation to $^{14}CO_2$ requires an intact TCA cycle, the pivaloyl CoA found in

the hepatocytes did not appear to inhibit cycle function.

There are data that suggests carnitine supplementation might influence the activity of HMGCoA synthase via its role in modulating acyl CoA levels. In liver homogenates and liver mitochondria, the enzyme can reversibly be inactivated by succinylation in the presence of physiological levels of succinyl CoA. The rate of reactivation is enhanced by raising the concentration of acetyl CoA, which desuccinylates the enzyme and inhibits resuccinylation (44). Addition of 5 mmol/L L-carnitine to liver mitochondria oxidizing pyruvate or octanoate leads to a signicant decrease in acetyl CoA levels without affecting succinyl CoA concentrations (45). Consequently, supplemental carnitine in the pharmacological range may shift the balance of HMG CoA synthase toward its inactivated form. Again, however, liver acyl carnitine/free carnitine ratios in supplemented and unsupplemented pivalate-treated rats do not point to such a shift.

The other mechanism Wolff et al (43) was a possible carnitine influence upon ketone utilization. Supplemental carnitine may enhance ketone utilization. The reactions by which extrahepatic tissues utilize ketone bodies are reversible reactions. Nosadini et al (46) hypothesized the reversal of ketone utilization could even play a significant role in peripheral ketogenesis. The first step in the utilization of acetoacetate (see **Figure 11**) is the formation of acetoacetyl CoA by 3-oxoacid CoA transferase in the mitochondria. The less active cytosolic enzyme is acetoacetyl CoA synthetase. In the second step, acetoacetyl CoA and CoASH are catalyzed by acetoacetyl CoA thiolase, to 2 Acetyl CoA. By reversing these steps, a significant

amount of peripheral ketogenesis could occur when the intracellular pool of acetyl-CoA is increased. Some have proposed (47) and demonstrated with high dose bolus injection of label in the dog (48) that this expansion of the acetyl-CoA pool would dilute the label with unlabeled acetyl-CoAs, resulting in pseudoketogenesis rather than true label dilution due to peripheral ketogenesis. However, recent studies of forearm ketone body metabolism of normal volunteers and diabetics using a steady-state,

$$\textit{βOHB} + \text{NAD+} \xleftrightarrow[\text{dehydrogenase}]{\text{βOHB}} \textit{acetoacetate} + \text{NADH}$$

$$\text{acetoacetate} + \text{succinyl CoA} \xleftrightarrow[\text{transferase}]{\text{3-oxoacid CoA}^{1}} \text{succinate} + \textit{Acetoacetyl CoA}$$

$$\textit{acetoacetyl CoA} + \text{CoASH} \xleftrightarrow[\text{thiolase}]{\text{acetyl CoA}} 2 \text{ acetyl CoA}$$

**FIGURE 11** Pathway of ketone utilization.

[1]In the cytosol, the 3-oxoacid-CoA transferase step is replaced by ATP + CoA <----> acetoacetyl CoA + AMP + pyrophosphate, but the activity of this enzyme is ten times less than of the mitochondrial enzyme shown above.

[3,4-$^{13}C_2$] acetoacetate infusion showed no exchange of label between carbon-3 and carbon-4 and carbon-1 and carbon-2 as would be expected if a significant exchange of carbon in acetoacetate and acetyl-CoA pools had occurred (49). Since mitochondrial hydroxymethylglutaryl (HMG) CoA synthase, the enzyme by which net synthesis of ketone bodies occurs in the liver, has not been proven to exist in muscle (50), the mechanism by which the forearm ketogenesis occurs remains in question. However, in theory, reversal of the steps in Figure 11 should accomplish this feat.

Results obtained thus far provide no evidence to implicate non-hepatic ketone production being important in this model of carnitine deficiency. The total acyl carnitine to free carnitine ratio was not significantly greater in muscle from pivalate-treated rats than from controls. Short chain acyl carnitine concentrations primarily reflect acetyl carnitine concentrations, hence the short chain acyl carnitine/free carnitine ratio may be a better indicator of the potential reversibility of these pathways. However, the short chain acyl carnitine to free carnitine ratio was also not significantly greater in these animals, even though the mean values were twice those of controls. The data, therefore, probably do not suggest an expansion of the acetyl CoA pool capable of reversing the ketone utilization pathway.

However, the data may indicate that ketone utilization is impaired under these conditions, since a smaller acetyl CoA pool expansion would be required to slow the forward ketone utilization reactions than to reverse them. Even though the elevation in the short chain acyl carnitine to free carnitine ratio was not statistically significant

in the pivalate-treated rats studied, the 100% increase in the ratio may reflect a physiologically significant process worthy of further investigation.

Carnitine supplementation was shown to reduce steatosis and ketosis in this pivalate animal model of secondary carnitine deficiency. The mechanism by which this was achieved is not clear, and is the subject of further studies in this laboratory.

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# CHAPTER 3

## Reduced ketone utilization and unaltered hepatic arteriovenous differences in in the carnitine-depleted, pivalate-treated rat.

Peri B. Bianchi and Alan T. Davis

## ABSTRACT

Ketone utilization and hepatic arteriovenous differences in ketones and free fatty acids were studied in male rats given 20 mmol/L sodium pivalate for 2 weeks to induce a secondary carnitine deficiency and in animals given a control solution. In the ketone utilization experiment, male rats were food-deprived for 24 hours and infused with the sodium salt of ß-hydroxybutyrate (ß-OHB) to maintain total plasma ketone concentrations between 6.0-10.0 mmol/L. After a bolus of 3-hydroxy[$^{3-14}$C]butyrate, the recovery of expired $^{14}CO_2$ collected in the ensuing 120 min. was significantly lower in the pivalate-treated rats than in the controls ($P<0.05$). Hepatic arteriovenous differences of free fatty acids, acetoacetate, and ß-OHB measured in the second experiment were not significantly different between pivalate-treated and control rats. The higher plasma ketone concentrations of food-deprived, pivalate-treated rats was due to their lower rate of ketone utilization. No evidence of increased ketone production with pivalate treatment was found.

Carnitine is a naturally occurring compound required for the mitochondrial oxidation of long chain fatty acids (1). Although fat oxidation is the primary and best-studied function of carnitine, research suggests it plays a role in several other processes including modulation of the acylCoA:free CoA ratio (2) and elimination of toxic metabolites (3).

Fasting hypoketonemia is commonly associated with primary carnitine deficiency or with secondary carnitine deficiencies due to congenital defects in the ß-oxidation pathway. However, hospitalization for episodes of ketoacidosis is a common problem encountered by patients with organic acidurias with secondary carnitine deficiency (4). Saudubray et al (1) stated "[with propionic and isovaleric acidemias] hyperketosis is thought to be mainly related to an excess of ketone body production, although this assumption is not always relevant and many mechanisms of hyperketosis still have to be elucidated." The pivalate-induced animal model of carnitine deficiency shows several similar features to the carnitine deficiency induced by genetic lack of propionyl-CoA carboxylase or isovaleryl-CoA dehydrogenase. In each case there is an accumulation of a compound capable of binding to free CoA and forming a conjugated CoA: pivaloyl CoA, propionyl CoA, isovaleryl CoA. Some of the acyl groups are then conjugated to carnitine, which can restore the ratio of free CoA to acyl CoA in the tissues toward normal but results in a loss of conjugated carnitine in the urine (5). A high acylcarnitine/free carnitine ratio in the urine and muscle (6, 7, 8), fasting ketosis (9), and fat accumulation in the liver (8, 14) are findings common to the human and animal model carnitine deficiency states. The pivalate-treated rat is a useful animal model of secondary carnitine deficiency due to organic acidurias.

The goal of these studies is to determine the cause of the exaggerated ketosis observed in food-deprived, pivalate-treated rats. Pereira et al (10) attributed increased fasting ßOHB levels in a carnitine deficient patient to subnormal hepatic carnitine levels. Free CoA inhibits hepatic mitochondrial acetyl CoA acetyltransferase (11). When carnitine concentrations are low, the acetyl CoA:free CoA ratio rises. Lower free CoA concentrations might limit regulation of ketogenesis via this reaction. Another mechanism could be via reduced peripheral utilization of lipid, leading to greater free fatty acid delivery to the liver. Evidence supporting such a mechanism are a report of lipid vacuoles in the muscle of a patient with propionic aciduria (8, 14), and of higher free fatty acid (FFA) levels in a patient with isovaleric acidemia in crisis (12). Both muscle lipid vacuolization and elevated plasma free fatty acid concentrations were found in food-deprived, pivalate-treated rats in one experiment, but not in follow-up studies. Still another way by which carnitine may influence ketosis is via ketone utilization. Reports of the effect of carnitine upon ketone utilization are conflicting (13, 14). Therefore, experiments were designed to test the following hypotheses: 1) In the pivalate-treated rat with moderate carnitine depletion ketone utilization is diminished due to reduced CoASH and/or succinyl CoA concentrations; 2) In the pivalate-treated rat with moderate carnitine depletion ketogenesis is enhanced, and may be accompanied by increased free fatty acid (FFA) flux to the liver.

## MATERIALS AND METHODS

The protocols of these experiments were approved by the All-University Committee on Animal Use and Care of Michigan State University and the GRAMEC

Animal Use and Care Committee.

***Experiment 1***

***Animals and experimental design.*** Fifteen male Sprague-Dawley, weanling rats (21 d old, weight 40 grams) were fed a low carnitine, purified diet (AIN-76A; Teklad, Madison, WI) for 14 days. Half received 20 mmol/L sodium bicarbonate in their drinking water, while the other half received 20 mmol/L sodium pivalate.

***Ketone infusion.*** After 24 hours of food deprivation, rats were restrained and had a catheter with a three-way stopcock inserted into the tail vein for infusion of the sodium salt of ß-hydroxybutyrate (ßHOB). Because ketone utilization is proportional to the plasma ketone concentration, the infusion was necessary to equalize ketone concentrations in the pivalate-treated and control rats. A pH neutral sodium salt of ßHOB was infused at rates determined in preliminary experiments to raise the plasma ßOHB concentration to approximately 7.0 mmol/L in a manner analogous to the procedure used by Keller (15). This concentration exceeded, by a small margin, all plasma levels previously obtained in food-deprived, pivalate-treated rats. Subsequently the infusion rate was adjusted over the following 180 minutes to maintain a total KB concentration of 6.0-10.0 mmol/L. Blood samples, 0.2 mL, were obtained at 30-60 minute intervals from the dorsal foot veins for ßOHB and acetoacetate analysis.

***Label administration and sample collection.*** Once consecutive stable plasma KB concentrations were achieved over a 20-30 minute interval, 3-hydroxy[3-$^{14}$C]butyrate was injected via the 3-way stop-cock. Initially 3.81 µCi/rat was given. This dose proved more than adequate for measuring $^{14}CO_2$ in the gas traps. Consequently, due to the cost of the labelled compound, the dose was reduced to 1.41 µCi/rat. The rats

were transferred to respiration chambers through which room air was pumped at 1.0 L/min, and the expired $CO_2$ was collected in two traps containing 100 mL 0.1 M monoethanolamine-ethylene glycol monomethyl ether (1:2). Aliquots were removed for scintillation counting, and the percent recovery of the $^{14}C$ dose as $^{14}CO_2$ during 90-120 minutes was estimated from the sum of expired $^{14}CO_2$/time. Rats were removed from the chamber, decapitated, and a sample of red gastrocnemius muscle was rapidly freeze-clamped in liquid nitrogen for subsequent determination of free, pivaloyl, and acyl Co A levels.

***Chemical analyses.*** ßOHB was measured using a Kit No. 310-UV from Sigma Chemical Company. Acetoacetate was determined by a modification (16) of the method of Mellanby and Williamson (17). Coenzyme A esters in muscle were measured by a high performance liquid chromatography (HPLC) method described by Corkey (18). Samples were homogenized in ice cold 10% trichloroacetic acid to which 10 $\mu$L of ß-methlycrotonyl CoA, the internal standard, was added. After centrifugation the extracts were neutralized by 4 ether extractions, concentrated using a Speed-Vac Centrifugal Evaporator, and stored at -80°C. Dried samples were resuspended in 0.075M $KH_2PO_4$, pH 5.0, filtered, and separated by gradient reversed-phase chromatography using a Spherisorb S5 ODS2 column. The mobile phases were 0.075M $KH_2PO_4$, pH 5.0, and 0.075M $KH_2PO_4$ containing 40% acetonitrile, pH 5.0.

***Statistical analyses.*** The $^{14}CO_2$ data were evaluated with Student's unpaired *t*-test using $P \leq 0.05$ as the limit for accepting the null hypothesis. CoA data were more variable in the pivalate group. Because of unequal variances between treatment groups, the data for all but succinyl CoA concentrations and the succinyl CoA/free

CoA ratio were analyzed after log transformation. Values are reported as means ± SEM.

***Experiment 2.***

***Animals.*** Sixteen male Sprague-Dawley, 75-125 gm rats were fed a low carnitine, purified diet (AIN-76A; Teklad, Madison, WI) for 14 days and were given sodium bicarbonate and sodium pivalate as in experiment 1.

***Sampling procedures and hepatic blood flow measurements.*** After a 24 hour fast, the rats were placed under general anesthesia with 1.5% isoflurane and an oxygen flow of 1 L/min. Hepatic blood flow was measured with labeled *p*-aminohippuric acid (PAH) by a modification of the method of Rémésy and Demigné (19). A mesenteric vein was exposed following a midline excision, and a 24 gauge over-the needle Insyte catheter was inserted into the vein for infusion of $^{14}C$ para-amino hippurate (PAH) (New England Nuclear, Boston), 0.2 μCi/mL at 1.9 $mL \cdot hr^{-1} \cdot 100$ g body $weight^{-1}$, after giving a priming dose of 0.08 μCi/100 gm body weight. Another catheter was inserted into the right femoral artery and threaded into the abdominal aorta for blood collections. Rats with significant blood loss from the catheter insertions were given 0.9% sterile saline to increase circulatory volume before starting label administration. Preliminary experiments indicated label equilibration was usually achieved after 20 minutes. Blood samples, 0.1 mL, were collected at 10 and 20 minutes after starting the infusion to verify equilibration of the label had been achieved. Blood samples, 0.3 ml each, were collected into heparinized syringes from the abdominal aorta, the portal vein, and hepatic vein for $^{14}C$, acetoacetate, ßOHB, and FFA determination. Samples were obtained over a 90 second interval and were held on ice. Assays for ketones

were completed the day of collection. Duplicate plasma samples, 0.020 mL each, were mixed with Picofluor for scintillation counting. Additional plasma was stored in a -80° C freezer for subsequent FFA analysis. Rats were killed with 1.0 mL sodium pentobarbital, 390 mg/mL.

***Chemical analyses.*** FFA and ßOHB were measured using Kit No. 1383 175 from Boehringer Mannheim and Kit No. 310-UV from Sigma, respectively. Acetoacetate was determined by a modification (16) of the method of Mellanby and Williamson (17). Plasma samples mixed with Picofluor were measured for $^{14}C$ with a Minaxi scintillation counter.

***Calculations.*** Hepatic and portal blood flows and hepatic balance were calculated as follows (19):

$$Hepatic\ flow\ (HBF) = \frac{Infusion\ rate[^{14}C]PAH_{(uCi/min)}}{[PAH]_{Hepatic\ Vein(uCi/ml)} - [PAH]_{Hepatic\ Artery(uCi/ml)}}.$$

$$Portal\ flow\ (PBF) = \frac{Infusion\ rate[^{14}C]PAH_{(uCi/min)}}{[PAH]_{Portal\ Vein(uCi/ml)} - [PAH]_{Hepatic\ Artery(uCi/ml)}},$$

Hepatic arterial blood flow (HABF) = hepatic blood flow - portal blood flow.

$$Hepatic\ Balance = [(Hepatic\ Vein_{(mmol/l)} - Portal\ Vein_{(mmol/l)}) x\ PBF] +$$
$$[(Hepatic\ Vein_{(mmol/l)} - Artery_{(mmol/l)})\ x\ HABF].$$

***Statistical analyses.*** The data were evaluated with Student's *t* test, or for non-normally distributed data, the Mann-Whitney U test. $P \leq 0.05$ was used as the limit for

accepting the null hypothesis. Values in the text are means ± SEM.

## RESULTS

***Experiment 1.***

***Body weight.*** At the end of the study, mean weights of rats in both groups were similar, 114 ± 8 g for pivalate-treated rats, and 126 ± 10 g for control rats.

***Expired $^{14}CO_2$.*** Percent recovery of exhaled $^{14}CO_2$ are shown in **Figures 1 and 2.** Data in Figure 1 are expressed as the percent $^{14}CO_2$ collected at 20 minute intervals•label administered$^{-1}$. Maximal evolution of $^{14}CO_2$ by control rats occured at 60 minutes. At both 60 and 80 minutes, expired $^{14}CO_2$ by control rats was significantly greater than by pivalate-treated rats. Values for cumulative exhaled $^{14}CO_2$ shown in Figure 2, expressed as the sum of each $^{14}CO_2$ collection•label administered$^{-1}$, were significantly lower in the pivalate-treated animals. Muscle concentrations of CoASH, CoA esters and acyl CoA:CoASH ratios are shown in **Figures 3 and 4.** There were no significant differences in total CoA, free CoA, or succinyl CoA between the two groups. The acetyl CoA concentration and acetyl CoA/free CoA ratio were significantly greater in the pivalate-treated rats. No pivaloyl CoA was detected in muscle tissues.

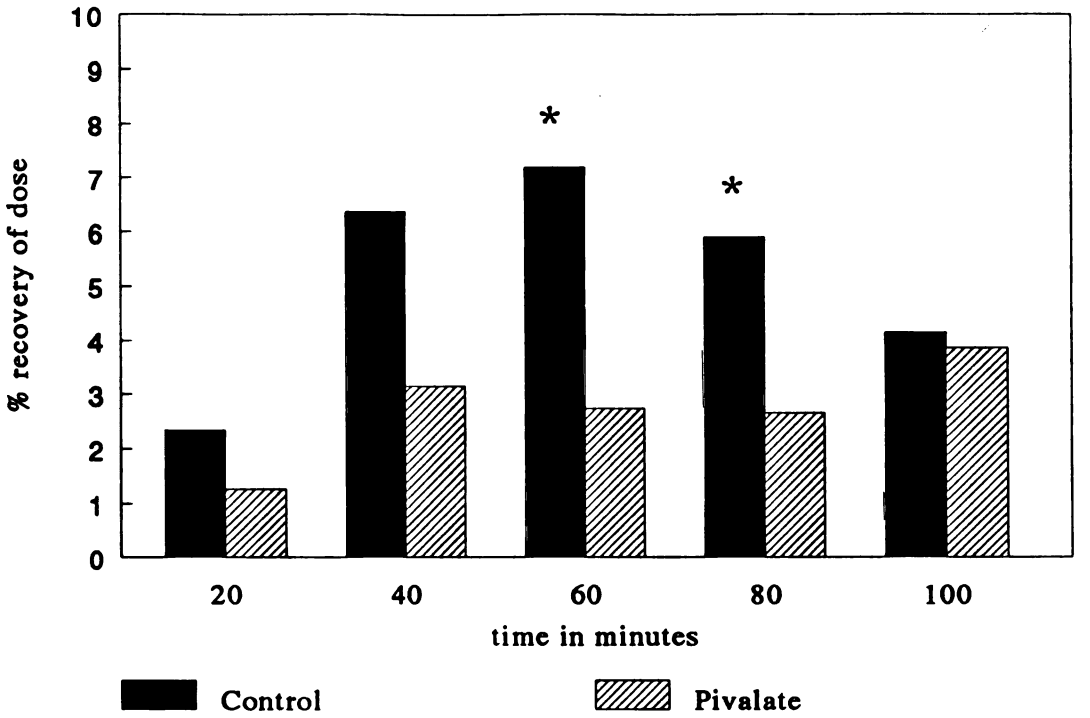


**FIGURE 1** Percent recovery $^{14}CO_2$ from rats given 20 mmol/L sodium bicarbonate, Control, or 20 mmol/L sodium pivalate, Pivalate, in their water for two weeks followed by 24 hours food deprivation. Data from 7 control and 8 treated rats are expressed as medians. Data points showing significant differences between control and treated rats, ($p<.05$) by the Mann-Whitney U test, are designated by an asterisk.

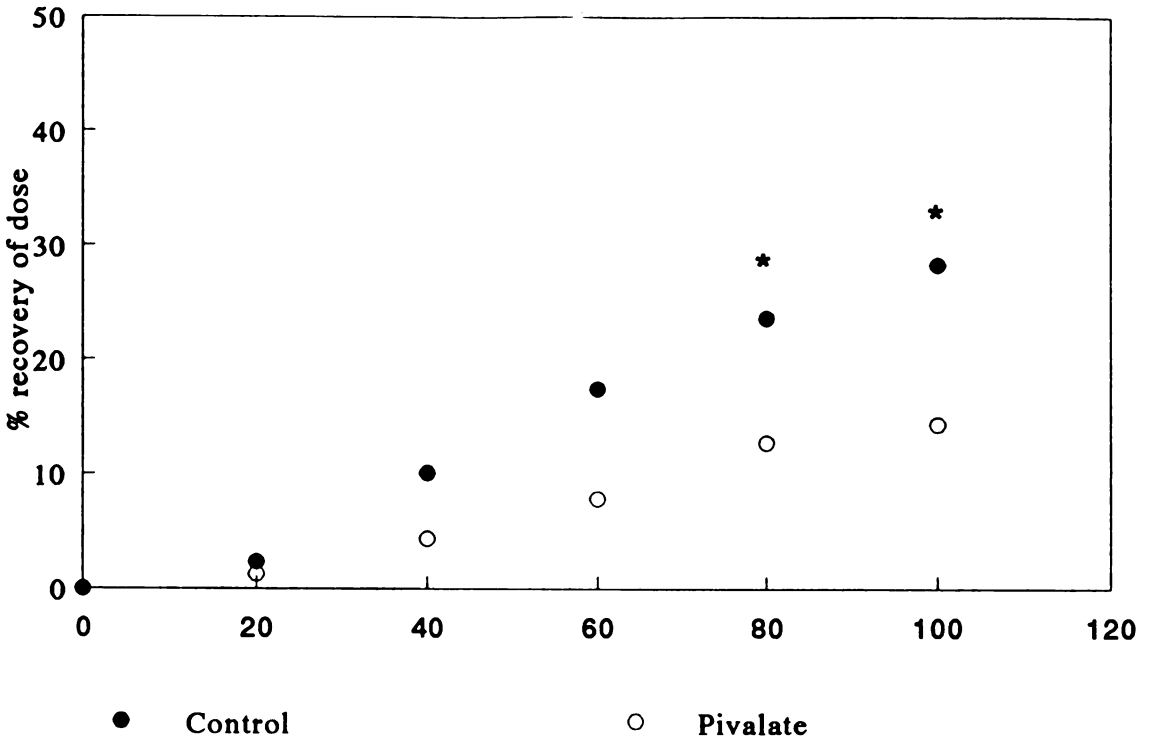


**FIGURE 2** Cumulative percent recovery $^{14}CO_2$ from rats given 20 mmol/L sodium bicarbonate, Control, or 20 mmol/L sodium pivalate, Pivalate, in their water for two weeks followed by 24 hours food deprivation. Data from 7 control and 8 pivalate-treated rats are expressed as the sum of each $^{14}CO_2$ colletion•label administered$^{-}$. Data points showing significant differences between control and treated rats, ($p<.05$) by the Mann-Whitney U test, are designated by an asterisk.

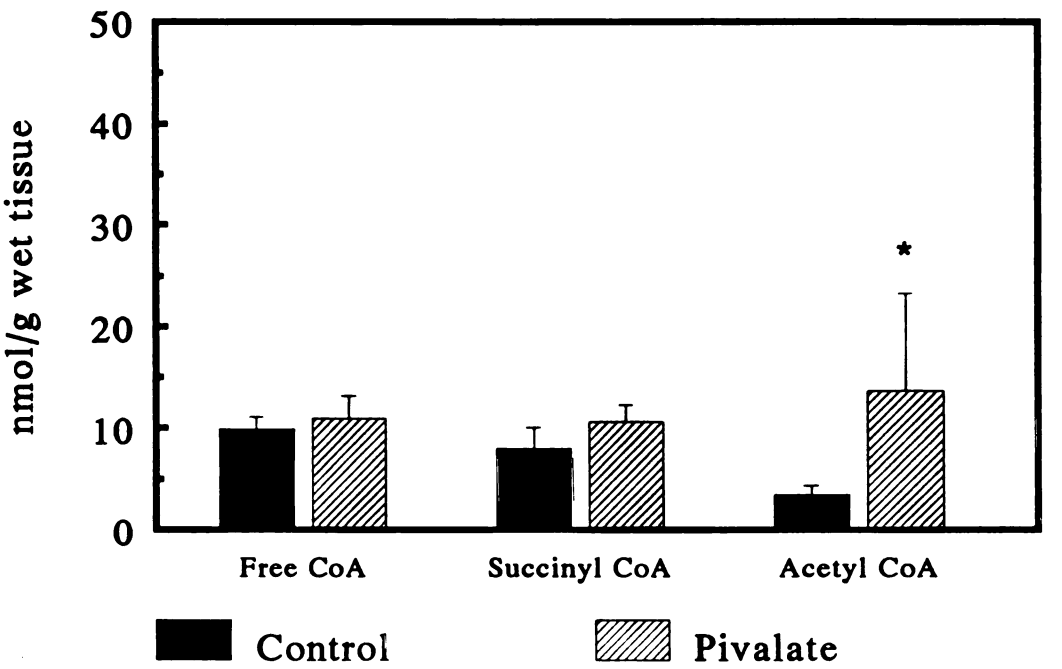


**FIGURE 3** Muscle CoA and CoA esters of rats given 20 mmol/L sodium bicarbonate, Control, or 20 mmol/L sodium pivalate, Pivalate, in their water for two weeks followed by 24 hours food deprivation. Bars showing significant differences in data from 7 control and 8 pivalate-treated rats, ($p<.05$), are designated with an asterisk.

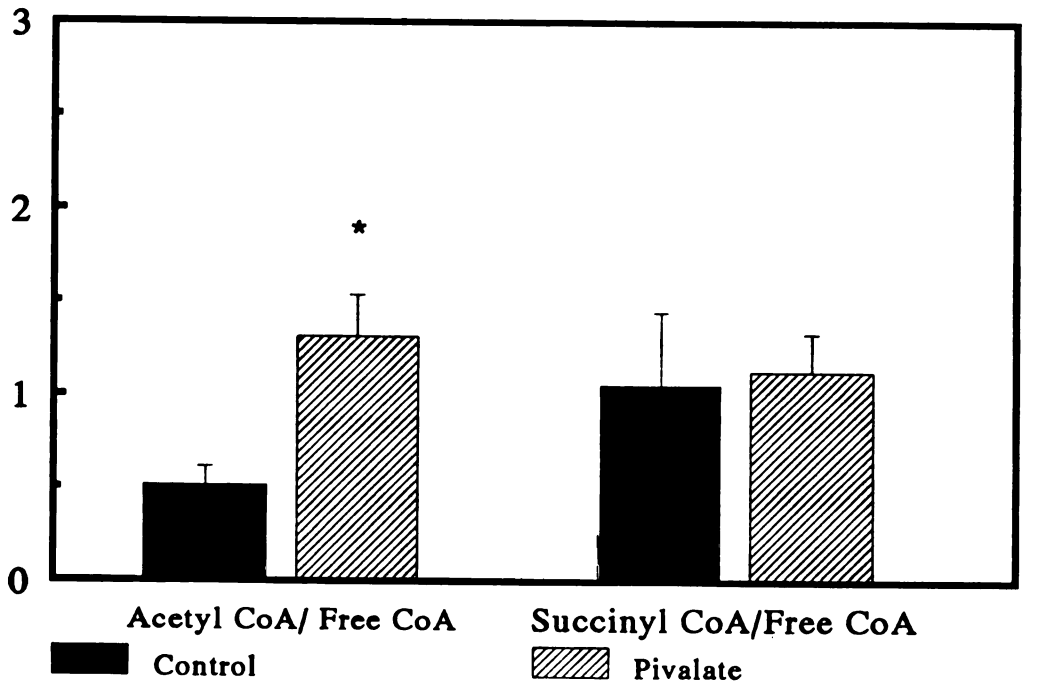


**FIGURE 4** Muscle acyl CoA to free CoA ratios of rats given 20 mmol/L sodium bicarbonate, Control, or 20 mmol/L sodium pivalate, Pivalate, in their water for two weeks followed by 24 hours food deprivation. Bars showing significant differences in data from 7 control and 8 pivalate-treated rats, ($p<.05$), are designated with an asterisk.

***Experiment 2***

We were able to obtain stable plasma $^{14}$C-PAH concentrations and all necessary blood samples from 5 control and 6 pivalate-treated rats.

***Body weight.*** Mean weights of rats are shown in **Table 1**. Pivalate treatment had no effect on body weight.

***Ketone and FFA levels.*** Plasma concentrations of acetoacetate and ßOHB, presented in **Table 2**, were significantly greater in the pivalate-treated rats. However, neither the ßOHB/acetoacetate ratio of the hepatic vein nor arterial blood were significantly different. Values for FFA from the hepatic vein and portal vein were higher in the pivalate-treated rats than the control rats, but not significantly so.

***Arteriovenous differences.*** Hepatic arteriovenous differences in FFA concentrations and ketone concentrations of pivalate-treated and control rats, were not significantly different, Table 1. There was a significantly greater portal and hepatic vein flow in the pivalate-treated rats. Saline administration to the two groups was similar.

## DISCUSSION

These data indicate plasma ketone concentrations of pivalate-treated rats are higher than those of control rats because their degree of ketone utilization is lower. Peak $^{14}CO_2$ evolution by control rats occurred at the 60 min collection interval, which indicates the period of collection to 100 minutes was adequate. The curve for $^{14}CO_2$ evolution by treated rats was flat except for a high value at the first collection, due to high values by one rat who may have been excited or stressed by being placed in the metabolic chamber. There was no indication of a rise in $^{14}CO_2$ evolution late in the

**TABLE 1 Hepatic blood flow and hepatic balance of FFA and ketones by the livers of pivalate-treated and control rats[1]**

| | *Experimental group* | |
|---|---|---|
| | Control | Pivalate |
| Blood flow, *mL·min$^{-1}$·liver$^{-1}$·100g$^{-}$ body wt.$^{1}$* | | |
| Portal vein | 1.6 ± 0.0 | 3.0 ± 0.5* |
| Hepatic vein | 2.7 ± 0.2 | 5.0 ± 0.7* |
| Hepatic artery | 1.1 ± 0.2 | 1.9 ± 0.3 |
| Hepatic balance, *μmol·min$^{-1}$·100 g body wt.$^{-1}$* | | |
| Total ketones | 3.7 ± 1.1 | 3.9 ± 1.3 |
| Free fatty acids | -2.1 ± 0.5 | -2.1 ± 0.7 |
| Body weight, g | 212 ± 12 | 208 ± 11 |
| Saline given, ml | 1.2 ± 0.4 | 1.4 ± 0.3 |

[1]Values are means ± SEM for 6 rats in the pivalate group and 5 rats in the control group. Hepatic blood flow was determined by an indicator method using $^{14}$C *p*-aminohippurate infusion into a mesenteric vein. Values for pivalate-treated rats that are significantly different from those of controls, $P<0.05$, are shown by *.

**TABLE 2 Blood concentrations of free fatty acids and ketone bodies[1]**

| Group | Acetoacetate | ß(OH)butyrate | Free Fatty Acids | AcAc/ßOHB[2] |
|---|---|---|---|---|
| | *mmol/L* | *mmol/L* | *mmol/L* | |
| Control | | | | |
| Artery | 1.61 ± 0.29 | 2.71 ± 0.64 | 1.11 ± 0.10 | 1.11 ± 0.38 |
| Portal | 1.38 ± 0.26 | 2.23 ± 0.35 | 1.29 ± 0.11 | |
| Hepatic vein | 2.19 ± 0.51 | 3.32 ± 0.39 | 0.46 ± 0.07 | 1.64 ± 0.25 |
| Pivalate | | | | |
| Artery | 3.27 ± 0.52* | 5.16 ± 0.40* | 1.44 ± 0.14 | 1.70 ± 0.22 |
| Portal vein | 3.43 ± 0.48* | 5.32 ± 0.49* | 1.74 ± 0.26 | |
| Hepatic vein | 3.80 ± 0.50 | 5.64 ± 0.52* | 0.94 ± 0.22 | 1.60 ± 0.30 |

[1]Results are means ± SEM for 5 control and 6 pivalate-treated rats. Values for pivalate-treated rats that are signifiantly different from those of controls, P > 0.05, are shown by an *.

[2]Acetoacetate/ß(OH)butyrate

collection period, again verifying adequacy of the $^{14}CO_2$ collections. Cumulative recovery of $^{14}CO_2$ by pivalate-treated rats was only 57% of control levels, and was statistically significant by 80 minutes. No additional role for enhanced ketogenesis as a factor contributing to their hyperketosis was demonstrated.

The steps in ketone body metabolism are shown below:

Acetoacetate + CoASH -*acetoacetyl CoA synthetase*- Acetoacetyl CoA

Acetoacetate + succinyl CoA -*3-oxoacid CoA transferase*- acetoacetyl CoA + succinate

Acetoacetyl CoA + CoA -*acetoacetylCoA thiolase*- 2 acetyl CoA

Acetyl CoA - Tricarboxylic acid cycle

The acetoacetyl CoA synthetase reaction occurs primarily in the cytosol, whereas the more active 3-oxoacid CoA transferase enzyme is mitochondrial. The reactions catalyzed by 3-oxoacid CoA transferase and acetoacetyl CoA thiolase are reversible (20), and ketone utilization is regulated by the concentrations of ketones, cofactors, and products of the reactions. Pivalic acid could reduce ketone utilization by inhibiting the enzymes of ketone utilization, by influencing the concentrations of cofactors, substrates, or products, or by depressing tricarboxylic acid (TCA) cycle activity.

Ketoacidosis due to hereditary deficiencies of 3-oxoacid CoA transferase and acetoacetyl-CoA thiolase (AAT) have been reported (21), and may provide clues for elucidating the actions pivalate treatment has on rats. The ketoacidosis of patients with deficiency of the cytosolic synthetase and 3-oxoacid CoA transferase differs from that of pivalate-treated rats in that patients' ketonemia was present after eating as well as after fasting. Succinyl CoA concentrations of pivalate-treated rats measured in this study do not suggest reduced activity of 3-oxoacid CoA transferase. Succinyl CoA is required for the forward reaction. Lack of this co-factor would limit ketone utilization. Direct inhibition of the enzyme would decrease usage and could cause a

buildup of the compound. However, muscle concentrations of pivalate-treated rats were not different from those of controls. Some difference in metabolism between people and rats could be the reason for these discrepancies. Nevertheless, the discrepancies leave us with little ground for suspecting that pivalate or pivaloyl CoA inhibit these enzymes.

In contrast, similarities in presentation between human and rat responses imply that pivalate treatment may influence the activity of mitochondrial AAT. Deficiency of mitochondrial AAT is characterized by episodic ketoacidosis triggered by fasting, catabolism, infections, or a high protein intake (21). The food deprivation protocol used fits that scenario. Competitive or non-competitive inhibition of the enzyme by pivalate is possible but not probable. Indirect inhibition of ketone utilization by modulation of the acyl CoA/Free CoA is a more likely etiology. Free CoA is needed for the reaction to proceed. Although muscle free coenzyme A concentrations were similar in control and treated animals, the acetyl CoA concentrations of the carnitine-depleted rats were almost 3 times greater. The acetyl CoA/CoASH ratio was 2.5 times greater. Such an increase would promote enzyme activity in the ketone generation direction, not ketone utilization (22). The data are compatible with inhibition of ketone utilization under conditions in which carnitine buffering of acyl CoAs may be reduced. Conclusions must be tempered, however, by an awareness that CoA measurements of homogenized muscle may not reflect concentrations in the mitochondria, where the enzyme occurs. However, in the heart, 90% of CoAs are intramitochondrial (23). Hence, measurement of whole tissue fractions may provide a good estimate of mitochondrial levels.

These data do not rule out some influence of pivaloyl CoA on TCA activity. Reduced TCA cycle activity in skeletal muscle could lead to the accumulation of

acetyl CoA in muscle, which was observed, and would also reduce the amount of $^{14}CO_2$ recovery from $^{14}$C-ßOHB. In hepatocytes, pivalate does not appear to inhibit the TCA cycle activity. Hepatocytes incubated with various concentrations of pivalate showed no diminution in their ability to oxidize $^{14}$C labeled palmitate to $^{14}CO_2$ (24). Fat oxidation to $CO_2$ requires an active TCA cycle. There are no reports of similar studies in muscle, but pivalate inhibition of the enzymes of the TCA cycle appears unlikely. Such inhibition would limit metabolism of carbohydrates and amino acids as well as ketones, and should cause observable aberrations in fed as well as fasting animals.

However, the reduced carnitine concentrations of pivalate-treated rats could lead to changes in co-factors required by enzymes of the TCA cycle. The ratio of CoASH to succinyl CoA is a key regulator of 2-oxoglutarate dehydrogenase activity (25). Reduced carnitine buffering capacity would lead to insufficient free CoA and a rise in the succinyl CoA to CoASH ratio to inhibiting levels. However, this ratio was similar in control and treated rats.

Another co-factor that may play a role regulating the TCA cycle is calcium. Three enzymes of the TCA cycle, pyruvate dehydrogenase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase, are activated by $Ca^{2+}$ concentrations (26). There are data linking carnitine with calcium metabolism. Palmitoyl carnitine promotes extracellular $Ca^{2+}$ influx in muscle by activating voltage dependant calcium channels. This increase in myoplasmic $Ca^{2+}$ can be relayed to the mitochondrial matrix, and is associated with increased $O_2$ uptake (27). In contrast, propionyl-L-carnitine prevented the rise in mitochondrial calcium observed from rabbit hearts exposed to ischemia (28). Propionyl-L-carnitine protects ischemic heart in a manner resembling the action of dihydropyridine calcium channel blockers, and has been shown to modulate binding

at L-type calcium channels competitively with calcium (29). Cultured endothelial cells incubated with propionyl-L-carnitine or with acetyl-L carnitine had reduced calcium concentrations (30). L-carnitine limits long-chain acyl CoA evoked calcium efflux from liver mitochondria by modulating the acyl CoA/CoASH ratio (31). The above in vitro results indicate that the balance of carnitine and carnitine esters in the cell have significant effects on calcium metabolism. However, because these studies examined the effects of carnitines upon a variety of endpoints, were conducted under different conditions, and used different cell types, and may not reflect the effect of physiological concentrations of carnitines, it is difficult to draw conclusions regarding carnitine modulation of TCA activity. Whether carnitine may influence TCA activity by a calcium-dependent mechanism has not been examined. Consequently, at the present time, it is more plausible to attribute the higher acetyl CoA/CoASH level of pivalate-treated rats to reduced carnitine buffering of CoA esters than to a reduced TCA activity.

There were no significant differences between control and treated rats for hepatic balance of free fatty acids or ketone bodies. Similar ratios of the ßOHB:AcAc in plasma taken from the hepatic vein of control and pivalate-treated rats suggests the presence of similar mitochondrial NADH:NAD levels as well, and is consistent with similar rates of ß-oxidation. The data provided no evidence to substantiate increased free fatty acid delivery to the liver as a cause of the high plasma ketone concentrations in pivalate-treated rats. In addition, since there was no difference in hepatic release of ketone bodies, the hypothesis that subnormal liver carnitine levels enhance ketogenesis via acetyl CoA/CoASH regulation of hepatic acetyl CoA acetyltransferase could not be substantiated. This finding is consistent with previous studies of pivalate-treated weanling rats that failed to find either an elevated acylcarnitine/free carnitine ratio (32)

or an elevated short chain acylcarnitine/free carnitine ratio (unpublished results) in the liver. The preponderance of short chain acylcarnitines is acetyl carnitine. Since there is an equilibrium between the acylcarnitine/free carnitine ratio and the acyl CoA/free CoA ratio, this implies there was no elevation in the acetyl CoA/free CoASH ratio as well, although conclusions must be tempered by acknowledging the actual acetyl CoA/free CoASH ratio was not measured. Nevertheless, from the data obtained, the model apparently does not produce conditions that promote excessive ketone production.

One limitation of this study was the blood collection at a single time period. Measurements were made after approximately 24 hours of food deprivation. Previous studies showed that ketone levels in pivalate-treated rats of this size which were either food-deprived for 48 hours, or food-deprived for 24 hours and subjected to 4 hours of cold exposure, would have plasma ßOHB levels approximately twice that of the controls. One could anticipate after 24 hours of food deprivation without the cold exposure, the plasma ßOHB response would be less. However, that was not the case. The arterial plasma ßOHB concentration of pivalate-treated rats was 5.16 ± 0.40 mmol/L and that of control rats was 2.51 ± 0.70 mmol/L, values very similar to those observed previously. This suggests that the plasma ßOHB levels had reached a plateau at a new steady state. Because a high ßOHB concentration reduces the rate of lipolysis in adipose tissue and increases the sensitivity of adipose tissue to the effects of insulin (33), free fatty acid release to the circulatory system may have been lower at the time data was collected than earlier in the food deprivation period. Were this to be true, a transient period of enhanced ketogenesis may have been missed..

The control group values for hepatic blood flow (the sum of portal and arterial flows), obtained in this study are somewhat lower than those reported by Rémésy and

Demigné, 6.8 $mL \cdot min^{-1} \cdot 100\ g^{-1}$ body wt. (19) and Niewoehner and Nuttal, 5.4 ± 0.7 $mL \cdot min^{-1} \cdot 100\ g^{-1}$ body wt. (34), though similar methods to measure hepatic balances were used. However, there were methodological differences including type, and potentially depth, of anesthesia, both of which are known to influence hepatic flow rates (36). The rats were a different size and strain that used by Rémésy and Demigné. In addition, hepatic blood flow and arteriovenous differences were measured in the same animals. This required catheter insertion into a mesenteric vein, which empties into the portal vein. As distal a site as possible was selected, because blood flow distal to the catheter would be lost. Portal flow of both groups of rats was ~61% of hepatic flow. Normal portal flow is 70% in starved rats, hence adequate flow should have been maintained for the purposes of this study.

Although portal flow of both groups of rats constituted ~61% of hepatic flow, the portal and hepatic vein blood flows in the control rats were significantly lower than the pivalate-treated rats. This was not anticipated. One explanation might be that the control group lost more blood during the catheter placements than the other group. Although some report a blood pressure change of 45% had no effect on hepatic flow (35), others report hemorrhage equivalent to 30% of estimated blood volume does decrease liver blood flow (36). However, saline administration to control and pivalate-treated rats was the same. Another possible explanation comes from differences in liver weight, since portal blood flow is proportional to liver weight (19). Livers were not weighed in this study. However, livers of pivalate-treated, food-deprived rats have lipid accumulation visible to the naked eye. It is possible that, when pivalate-treated rats were food deprived, their livers lost less weight than did control livers, and thus, maintained a higher portal blood flow. Since in fasted rats portal flow is typically ~70% of total hepatic blood flow, this would explain why pivalate-treated rats had a

higher hepatic vein flow as well.

In summary, the pivalate animal model of secondary carnitine deficiency shares several features with the carnitine deficiency experienced by patients with organic acidurias. Ketone utilization was impaired in these carnitine deficient rats, probably due to insufficient buffering of acetyl CoA in muscle tissues, with a resultant acetyl CoA/CoASH ratio unfavorable for ketone metabolism. These data demonstrate a mechanism by which episodes of ketoacidosis may occur in patients.

Despite anecdotal reports of the efficacy of carnitine treatment for patients with organic acidurias, including reducing the incidence of ketoacidosis (37), the value of carnitine supplementation has been challenged (38). Carnitine supplementation was promoted to enhance excretion of toxic acyl CoAs in these patients (39), but propionylcarnitine excretion was found to play a minor role in total propionate metabolism (40). Since skeletal muscle carnitine concentrations as low as 1.5% of normal may not lead to a clinical skeletal myopathy (10), some have questioned the value of simply treating a low carnitine level when clear symptoms of deficiency are lacking (40). Data from this study, combined with the previous finding that carnitine supplementation concurrent with pivalate administration could attenuate fasting ketosis (32), provide support for carnitine supplementation of patients with organic acidurias. Treating these patients with carnitine should improve modulation of CoASH and CoA esters, which should improve their ketone utilization. Study of the effects of carnitine supplementation on ketone utilization is necessary to verify whether this is the mechanism by which carnitine supplementation did act.

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# EXECUTIVE DISCUSSION

## EXECUTIVE DISCUSSION

Sodium pivalate administration proved to be a useful means of inducing a secondary carnitine deficiency in rats. The mechanism by which this has been achieved has not been fully delineated. It appears to act in the same way that propionic aciduria induces carnitine deficiency. Both pivalate and propionate are conjugated to carnitine for urinary excretion. After four days of pivalate treatment, acylcarnitine excretion by the rats was significantly elevated. Up to 90% of the acylcarnitines excreted were in the form of pivaloylcarnitine. Pivampicillin administration has been shown to increase total carnitine excretion in children (1). Significantly greater losses of total carnitine in the urine have not been documented for this model. However, the same is true for some patients with propionic aciduria (2). The experiment which measured carnitine excretion probably lacked sufficient power to confirm or deny this mechanism. Though after 4 days of pivalate treatment the median total carnitine excretion by the treated rats was four times greater than that of control rats, the difference was not statistically significant because of wide variability in the data. Valproate, another compound that increases acylcarnitine excretion and induces carnitine deficiency, also inhibits fibroblast carnitine uptake (3). This provides an additional mechanism for inducing a carnitine deficiency. Pivalate effects on carnitine uptake have not been studied. However, we found equimolar

carnitine supplementation concurrent with pivalate administration produced tissue levels ≥200% of normal, which indicates that if pivalate does inhibit tissue uptake, the inhibition can be relatively easily overcome.

The carnitine deficiency in these rats shares several other features with the deficiency seen in children with organic acidurias. Diminished muscle carnitine concentrations, hepatic steatosis, fasting ketosis, greater acyl to free carnitine ratios in the plasma and urine, and greater acylcarnitine clearances are all common features. Consequently, the model proved advantageous for exploring the seemingly anomalous finding of fasting ketosis in a carnitine deficient state. In children with propionic aciduria, the ketosis has generally been attributed to enhanced ketogenesis. For ethical reasons, the etiology has not been fully explored.

When radiolabelled ß-hydroxybutyrate was given to control and treated rats, ketone metabolism to $^{14}CO_2$ was diminished in rats receiving pivalate. Carnitine supplementation ameliorated the ketosis in pivalate-treated rats. Presumably, the additional carnitine provided the means for modulating the acetyl CoA:CoASH ratio in the muscle to a degree that ketone utilization was promoted. However, because plasma free fatty acid concentrations were lower in carnitine-supplemented animals, we cannot rule a role for decreased ketogenesis as an alternate or supplementary mechanism.

The study has clinical implications. The value of carnitine supplementation of patients with secondary carnitine deficiencies has been questioned, despite anecdotal reports of benefit (4). When it was reported that the additional carnitine promoted elimination of toxic propionyl groups to only a minor extent (5), enthusiasm for

routine carnitine supplementation of these patients waned. Because patients tissue levels are often not reduced to concentrations rate-limiting for CPT activity, whether or not patients are truly carnitine deficient has been a subject of controversy. Demonstrating that ketone utilization is impaired in our model of secondary carnitine deficiency may provide the impetus for clinical research looking beyond the classical role of carnitine in regulating fatty acid oxidation rates. Although ferrying fatty acyl groups into the mitochondria for ß-oxidation is the most well-studied function of carnitine, another important role for the compound is modulating the acyl CoA/CoASH ratio. Carnitine acyl transferases transesterify acyl groups from CoA to carnitine, which is an effective method of reducing excess acyl CoAs from the mitochondria, where their buildup may inhibit other reactions. When carnitine availability is limited, this mechanism of maintaining CoASH levels in the appropriate range may be impaired. This apparently was the case in the pivalate-treated rats. With pivalate administration for two weeks, muscle free carnitine concentrations were reduced to 32% of control values, and their muscle acetyl CoA/CoASH ratio was significantly elevated. The increased acetyl CoA/COASH ratio is unfavorable for ketone metabolism. A high ratio inhibits activity of the following step in ketone metabolism:

acetoacetyl CoA + CoA <--*Acetoacetyl CoA thiolase*--> 2 acetyl CoA.

Why pivalate-treated rats have high liver and plasma triglyceride concentrations has not been adequately examined. Except for the one study described in Appendix A, plasma and liver triglycerides were significantly elevated in fasted, pivalate-treated weanling and young adult rats. This occurred in animals with total liver carnitine

concentrations 67-76% of control values. Free carnitine concentrations were 66-88% of the controls. These levels are not deemed sufficiently low to limit fatty acid oxidation via carnitine palmitate transferase activity. Nevertheless, carnitine supplementation attenuated the steatosis in a dose-dependent manner. A similar amelioration of steatosis and hypertriglyceridemia by carnitine administration was reported in rats chronically exposed to alcohol (6). Like pivalate-treated rats, liver total and free carnitine concentrations of rats on the unsupplemented alcohol diet were only moderately reduced, being 77% and 73% of rats receiving a control diet, respectively. Nevertheless, the researchers hypothesized chronic alcohol intake induces a functional carnitine deficiency which induces fatty liver and hyperlipemia via still to be defined mechanisms. Current knowledge of hepatic metabolism of the pivalate-treated rat is at a similar vague state.

Steatosis could result from an increased supply of lipids to the liver from dietary intake, adipose mobilization or endogenous synthesis. On the other hand, fatty liver could occur if lipid disposal via oxidation, hepatic lipolysis, or secretion were impaired (7). Finally, some combination of these factors could be involved. None have been fully explored in the pivalate-treated rat. Each will be discussed briefly below.

***Increased lipid supply.*** In treated rats, hepatic free fatty acid (FFA) uptake after a 24 hour fast was similar to control levels, hence exogenous fatty acid supply of adipose origin was similar for the two groups at that time. It is conceivable that prior to the time of study, when plasma ketone concentrations were lower and consequent inhibition of adipose lipolysis was less, higher levels of FFA influx occurred.

Currently, we have no data for such an event.

Drug administration can alter hepatic uptake of dietary lipids. Acute administration of alcohol increases intestinal lymph flow and output of dietary lipids due to enhanced splanchnic circulation, but the effect diminishes after chronic alcohol intake (8). Pivalate-treated rats did have a higher portal blood flow than control rats, but after a 24 hour fast delivery of FFA from the gut should not be great enough to account for the steatosis of these rats, and the hepatic balance of FFA was similar between treatment groups. Whether increased portal flow occurred prior to fasting is not known. We have one study of lipid levels in fed rats showing steatosis, which would be consistent with this theory.

Although during starvation the partitioning of fatty acid metabolism primarily promotes fatty acid oxidation, the delivery of FFA to the liver is sufficient to maintain high rates of triglyceride synthesis (9) and lipoprotein secretion (10). The rate of synthesis is regulated primarily by the hormonal and nutritional state. Additional influences include the supply of α-glycerol, the glycerol precursor, and the supply of fatty acyl CoA (11). A high NADH/NAD ratio, such as is generated by the oxidation of alcohol, favors synthesis of α-glycerol phosphate, hence would promote formation of triglycerides. However, the data from these experiments does not point in that direction. In treated rats, similar ratios of the ßOHB:AcAc in plasma taken from the hepatic vein of control and pivalate-treated rats suggest the presence of similar mitochondrial NADH:NAD levels as well. FFA supply was addressed above. In isolated, perfused livers (12) or hepatocytes from fasted rats approximately 13-15% of total fatty acid synthesis has been demonstrated to come from ketones. Since

pivalate-treated rats had higher plasma ketone concentrations, this could have been a source of extra substrate supply. However, in the perfused liver studies (12), total fatty acid synthesis by livers from fasted animals was only 2.85-4.72 μmoles of acetyl/g of liver (dry weight) in 90 min. The difference in liver triglyceride concentrations between fasted control and pivalate-treated rats in the supplementation study was much greater, 0.7 mmol/g (wet weight). Thus, though higher ketone levels may have played a role contributing to hepatic steatosis, the data provide no springboard for proposing substrate supply for triglyceride synthesis is enhanced to a significant extent by pivalate administration.

***Decreased lipid disposal.*** Little is know about factors regulating mobilization of lysosomal triglyceride lipase in the liver. Phagocytosis of fat droplets has been implicated, especially since glucagon and insulin have opposing effects upon formation of autophagic vacuoles (13). If the glucagon action is mediated by $Ca^{++}$ mobilization, the relative concentrations of palmitoyl carnitine, propionyl carnitine and acetyl carnitine, could conceivably modulate its action. These compounds, discussed in Chapter 3, have been shown to influence cellular calcium concentrations (14, 15), and hepatic carnitine concentrations of pivalate-treated rats were lower than control values. However, a greater understanding of both the basic mechanism of the lipase activation as well as the influence carnitine esters may have upon calcium concentrations needs to be established before seriously speculating a carnitine influence upon hepatic lipolysis.

Impaired hepatic fatty acid oxidation could occur at the site of fatty acid entry into the mitochondria, the ß-oxidation pathway, tricarboxylic acid (TCA) cycle, or

oxidative phosphorylation. Because hepatic carnitine levels are not reduced to rate-limiting levels in the liver, and plasma ketone concentrations are high in pivalate-treated rats, inhibition at carnitine palmitate transferase or the ß-oxidative pathway is unlikely. The subsequent finding of diminished ßOHB utilization by pivalate-treated rats as well as increased excretion of dicarboxylic acids left open the possibility that fatty acid oxidation may have been impaired despite higher plasma ketone concentrations. In addition, though pivaloyl-CoA is a poor substrate for carnitine acetyl transferase (CAT), the pivaloylcarnitine, once formed, modestly inhibits hepatic mitochondrial CAT and carnitine octanoyltransferase (16). CAT inhibition could limit excess acetyl unit transfer out of the mitochondria via carnitine, and thereby force them towards either utilization, in the TCA cycle or the ketone synthesis pathway, or exit in the form of citrate. Hence, compared to fatty acid oxidation rates, ketone synthesis might be relatively greater in CAT-inhibited animals. Under these circumstances, finding no differences in ßOHB and AcAc release by control or pivalate-treated rats after 24 hour fast would not demonstrate equal rates of fatty acid oxidation. However, the concentration of pivaloyl carnitine required to inhibit CAT activity by 50% is high, around 2 mM. Profound hepatic CAT inhibition in the conditions of our study are not likely.

Depressed TCA cycle activity was a possible mechanism to explain both hepatic steatosis and plasma ketosis. Like pivalate-treated rats, rats fed ethanol also exhibit fasting ketosis (17). Hepatic mitochondria from rats chronically consuming ethanol showed impaired metabolism of 2-carbon fragments to $CO_2$, whereas ß-oxidation rates were slightly above control levels (18). This implied reduced TCA or

oxidative phosphorylation activity led to diversion of acetyl units to the HMG CoA and triglyceride synthesis pathways. However, the hepatic balance of ketones in pivalate-treated rats was similar to that of control rats. Hence our data does not support the hypothesis of impaired TCA activity leading to increased ketogenesis and triglyceride synthesis. Funneling of acetyl units primarily to triglyceride synthesis remains a possibility. Acetyl CoA exits the mitochondria as citrate when it is formed faster than it can be used in the TCA cycle (19). High levels of citrate activate acetyl CoA carboxylase, which synthesizes malonyl CoA, the first compound committed to fatty acid synthesis. Although malonyl CoA inhibits carnitine palmitoyl transferase (CPT) activity, high levels of palmitoyl CoA partially desensitize CPT to this effect, permitting fatty acid synthesis under conditions of a high acetyl CoA load (19). One could further speculate if the presence of pivalate or pivaloyl CoA diminished binding of malonyl CoA to CPT, there could be an additional propensity for triglyceride synthesis in the face of high malonyl CoA levels. Both are short chain acyl CoA compounds.

Although impaired hepatic synthesis or secretion of lipoproteins is a common mechanism of drug-induced fatty liver, studies thus far do not suggest such a mechanism in our rats. Hypolipemia is usually found when hepatic lipid secretion rates are reduced (8), whereas we found elevated plasma triglyceride concentrations in pivalate-treated rats. On the other hand, hyperlipemia would not reflect hepatic secretion rates if plasma lipid clearance were impaired. There is little data describing the effects of carnitine on this function. Richeter et al (20) found no effect of carnitine upon post-heparin lipoprotein lipase activity. Pivalate effects have not been

studied.

Hepatic liporetention would occur if high rates of production were not matched by equivalently high secretion rates. This has been observed in diabetic rats, in which uncontrolled FFA mobilization leads to steatosis and hyperlipemia (21). Although the major mechanism of alcohol-induced fatty liver is believed to be reduced fatty acid oxidation, ethanol also impairs secretion of export proteins (8). Thus hyperlipemia in the pivalate-treated rat does not preclude decreased release of serum lipoproteins as an etiology of the steatosis. Alcohol feeding reduces bile acid excretion by the liver and poses another unexplored means by which pivalate may induce fatty liver (8).

As the discussion above indicates, there are several areas left to explore in order to fully understand the effects of pivalate on fat metabolism. Our study of showing reduced ketone utilization by pivalate-treated rats did not distinguish between acetyl CoA inhibition of acetoacetyl-CoA thiolase as the major mechanism, as we hypothesized, or a more distal block preventing the metabolism of labelled ßOHB to $^{14}CO_2$ such as by diminished TCA cycle activity in periphery.

The etiology of the steatosis in these animals leaves many questions unanswered. A key question is: does it do any harm? Fatty liver does not necessarily mean liver damage. If liver enzyme studies or histology showed evidence of liver injury, then further investigative study would be warranted. Zammit and Moir (22) have recently described a method for measuring the relative partitioning of labelled VLDL to oxidative or synthetic pathways which might be useful for determining the direction of such studies.

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# SUMMARY AND CONCLUSIONS

## SUMMARY AND CONCLUSIONS

These studies have investigated the effects of administering the prodrug pivalate upon carnitine status and fat metabolism in male, Sprague-Dawley rats.

The initial studies described in "Sodium pivalate treatment reduces tissue carnitines and enhances ketosis in rats" showed that pivalate administration induces a secondary carnitine deficiency which mimics several features found in children with organic acidurias. Specifically, tissue and plasma carnitine concentrations of treated rats were significantly reduced, the acyl carnitine:free carnitine ratios in plasma and urine were significantly increased, and the compound being used to induce carnitine depletion was found in the urine esterified to carnitine in a manner analogous to that found in patients with propionic aciduria. Urinary carnitine excretion declined as the plasma carnitine concentration declined. In addition, indices of fat metabolism were altered in the treated rats. After 48 hours of food deprivation, liver lipid concentrations were greater in the pivalate-treated rats than in controls, and their plasma ß-hydroxybutyrate (ßOHB) concentrations were elevated as well. Plasma glucose concentrations were unaffected by pivalate treatment.

"Effect on indices of lipid metabolism in rats carnitine depleted with oral pivalate treatment for 4 days" showed that after short term pivalate administration, the plasma concentration of ßOHB of food-deprived, cold-exposed was as high as that found in previous experiments when 2 weeks of treatment were given. This implied

little tissue carnitine depletion was necessary to produce fasting ketosis.

The second chapter discusses three experiments which further characterized this animal model. The first experiment confirmed the alterations in lipid metabolism evidenced in the previous experiments and showed that a more pronounced carnitine depletion was achieved when pivalate treatment was initiated in weanling rats (weight ~40 g), rather than 100-125 g rats. The increase in liver lipids after fasting and cold exposure was also greater in the younger animals. In addition, plasma triglyceride concentrations and free fatty acid concentrations were found to be higher than in those of the control animals. Hindlimb muscle showed more staining of lipids in treated rats than in controls. These data suggested that peripheral fat metabolism was impaired, leading to enhanced substrate supply as the etiology of the increased liver and plasma lipids as well as the higher ßOHB concentrations.

The second experiment demonstrated that supplementing the diet of pivalate-treated animals with carnitine equimolar to or twice equimolar to the pivalate intake both prevented carnitine depletion and ameliorated the effects of pivalate treatment upon indices of fat metabolism of food-deprived rats. Plasma ßOHB concentrations and liver lipid concentrations were reduced to control levels in a dose-dependent manner. Although liver and muscle carnitine concentrations were similar in both carnitine supplemented groups, only rats receiving the higher level of supplementation showed complete attenuation of the steatosis and ketosis. This data, coupled with the results of the 4 day study suggest that the absolute concentration of carnitine in the tissues is not the sole determinant influencing the ketosis and steatosis, but rather the balance of carnitine relative to other compounds, presumably pivaloyl CoA and other

CoAs. Both levels of carnitine supplementation produced plasma triglyceride concentrations no different from those of control animals. The experiment failed to confirm higher plasma free fatty acid concentrations in pivalate-treated rats, and showed no correlation between free fatty acid concentrations and ßOHB concentrations. It did demonstrate plasma ßOHB concentrations were not high simply because of reduced excretion of ßOHB in the urine. Neither pivalate treatment or carnitine supplementation affected weight gain, final weights after food deprivation, or weight loss.

The third experiment confirmed that carnitine supplementation at approximately twice the molar intake of pivalate prevented a significant increase in plasma ßOHB and total plasma ketone concentrations. As in experiment 2 above, it confirmed the lack of effect of pivalate upon plasma free fatty acid concentrations. Additionally, no relationship was found between plasma ßOHB concentrations and plasma free fatty acid concentrations. Urine dicarboxylic acid concentrations were significantly increased in the unsupplemented pivalate-treated animals. This could mean mitochondrial ß-fatty oxidation is inhibited, because dicarboxylic acids are formed by ω-oxidation. However, no pattern of urine metabolites was found that would identify a block in mitochondrial oxidation, hence accelerated ß-oxidation of dicarboxylic acids such as is seen in starvation ketosis or diabetes could be implicated as a cause as well. The experiment further showed that plasma ketone concentrations in control rats given sodium bicarbonate in their water and those given plain water were similar. Hence, the sodium bicarbonate, used to maintain similar acid-base balance in control and treated rats, was not having an untoward effect on ketone metabolism.

"Comparison of indices of fat metabolism in pivalate-treated rats, fed or fasted" examined liver triglyceride levels, and plasma ketone concentrations in weanling rats after 2 weeks of pivalate administration. For the first time, no increase in liver triglyceride concentrations in food-deprived rats was observed. Triglycerides of treated, fed rats were significantly greater than those of controls. Acetoacetate and total ketone levels were also significantly higher.

The third chapter describes experiments which explored the etiology of the fasting ketosis in the pivalate-treated rats. The first experiment showed recovery of $^{14}$C-ßOHB as $^{14}CO_2$ by rats carnitine-depleted by pivalate was 51% of control rats, indicating their ketone utilization was impaired. CoASH and succinyl CoA concentrations in gastrocnemius muscle of treated rats were similar to control values, but their acetyl CoA concentrations were three times higher. These high values could inhibit the activity of acetoacetyl CoA transferase in metabolizing ketones, and are the likely explanation for the reduced ketone utilization. The second experiment examined arteriovenous differences across the liver of treated and control rats food-deprived for 24 hours. No differences in uptake of plasma free fatty acids or release of ketones were found.

Sodium pivalate administration proved to be a useful means of inducing a secondary carnitine deficiency in rats. The carnitine deficiency in these rats shares several features with the deficiency seen in children with organic acidurias. Diminished muscle carnitine concentrations, hepatic steatosis, fasting ketosis, greater acyl to free carnitine ratios in the plasma and urine, and greater acylcarnitine clearances are all common features.

For this reason, this model was used to examine a problem which causes repeat hospitalizations in patients with organic acidurias, hyperketosis after fasting. Patients' hyperketosis has generally been attributed to exaggerated ketogenesis, but for obvious ethical reasons has not been explored. The results have potential clinical implications. There are anecdotal reports of benefit in treating ketosis-prone organic aciduria patients with carnitine supplementation. Nevertheless, there has been concern expressed about treating low carnitine levels in these patients when it is not clear whether the low carnitine levels or the primary metabolic defect is responsible for the patient's clinical abnormalities. This research shows the fasting ketosis seen in patients with organic acidurias can be duplicated without duplicating their inborn error of metabolism. The data also shows that the secondary carnitine deficiency induced with this model leads to an actual impairment of a physiological function, ketone utilization. Furthermore, carnitine supplementation can prevent the fasting ketosis. The data provide evidence that the secondary carnitine deficiency per se deserves treatment. Further study is necessary to determine the reason why acetyl CoA concentrations in the muscle of the treated rats are elevated to the extent that they appear to inhibit ketone utilization. Additional research is also needed to clarify by what mechanism carnitine supplementation attenuates the fasting ketosis.

# APPENDIX A. Additional Results

# APPENDIX A. Additional Results

## A1. Effects on indices of lipid metabolism in rats carnitine depleted with oral pivalate treatment for 4 days.

In a previous study (1) 4 days of pivalate treatment to 100 gm rats significantly reduced their plasma and heart carnitine concentrations without significantly reducing total muscle carnitine levels. Free carnitine concentrations of the rats were significantly reduced in the muscle at that time, 383 ± 27 nmol/g vs. 518 ± 27 nmol/g (means ± SE), but still not to a rate-limiting range. The primary aim of this study was to determine whether using younger animals would produce a more profound muscle carnitine deficiency. Young animals should have an increased need for carnitine in order to maintain tissue carnitine concentrations while rapidly gaining weight.

Previous studies (1) also showed that after 2 weeks of pivalate administration, rats fasted 2 days had significantly higher plasma ß-hydroxybutyrate (ßOHB) concentrations than the control animals. The modest skeletal muscle carnitine depletion after 2 weeks of pivalate-treatment in combination with pivalate sequestration of CoASH may have impaired peripheral fatty acid oxidation, leading to higher circulating fatty acid concentrations and greater fat oxidation by the liver. However, because the degree of muscle carnitine depletion the rats experienced was not comparable to the degree of depletion necessary to limit fatty acid oxidation in vitro (2), the muscle carnitine deficiency observed in these animals may have been

coincidental. A secondary aim was to determine whether short-term pivalate-treatment would also cause an exaggerated plasma ketone response to fasting. Therefore, fasting ßOHB concentrations of male, weanling rats after 4 days of pivalate administration were measured in this experiment. Another aim was to determine whether plasma FFA concentrations correlated with plasma ßOHB concentrations.

## MATERIALS AND METHODS

**Animals and experimental design.** Thirteen (13) male, weanling, Sprague Dawley rats from Charles River (Portage, MI) were randomized to receive 20 mmol/l sodium pivalate (n= 6 ) or 20 mmol/l sodium bicarbonate (n= 7) in their water. Rats received a purified low carnitine diet, AIN-76A (Teklad, Madison, WI) for 3 days, and food cups were removed for 1 day. Rats were housed at 6° C. for 4 hours to further increase their rate of fatty acid oxidation. Their temperatures were monitored with a rectal probe to ascertain that none became hypothermic. All rats had a practice temperature taken on day one to aquaint them with this procedure. After the cold exposure on the fourth day, blood was collected from the tail vein. A feeding error prevented retesting the rats after 2 weeks of pivalate-treatment.

**Blood samples.** Blood was collected into heparinized capillary tubes and stored on ice until centrifugation and analysis. Plasma ßOHB was measured within 24 hours by Sigma Kit No. 310-UV. Insufficient blood was obtained to run planned FFA assays.

**Materials.** The pivalic acid, sodium salt, was obtained from Aldrich Chemical (Milwaukee, WI).

**Statistical analysis.** Differences between the control and pivalate-treated groups were analyzed using Student's *t*-test.

## RESULTS

After 4 days of pivalate, ßOHB levels in rats were significantly greater than control levels, $p<0.05$. Plasma concentrations were 5.34 ± 0.92 mmol/l (mean ± standard deviation) for treated rats and 3.76 ± 2.21 mmol/l for the controls. No rats became hypothermic during the cold exposure.

## DISCUSSION

It is unlikely that fasting ketosis in pivalate-treated rats was caused by reduced muscle fatty acid oxidation due to carnitine deficiency, since the ketosis was observed after only 4 days of pivalate exposure. Because of their planned re-testing after two weeks of treatment, muscle carnitine levels from these rats are lacking. However, reasonable estimates can be made of their degree of carnitine depletion from the data obtained with rats which started pivalate-treatment with body weights of 100-125 g (1). Muscle carnitine concentrations are low at birth and increase with age (3). Therefore, direct numerical comparisons of carnitine concentrations between the two age groups may be less useful than comparing the degree of depletion induced by pivalate in the two age groups. Because this experiment was not completed as planned, another experiment to achieve our primary aim was completed, and was described as Experiment 1 in chapter 2. Data from that experiment with weanlings, as well as data from study of older rats reported in Chapter 1, are presented in Table 1.

**TABLE A-1 Muscle carnitine levels in weanling and 100-125 g rats treated with pivalate**

| Age/size | Treatment Period | Pivalate Carnitine *nmol/g* | % depletion[1] | Control Carnitine *nmol/g* |
|---|---|---|---|---|
| Weanlings | 2 wks | 184 ± 68[2] | 64% | 508 ± 68 |
| 100-125 g | 2 wks | 509 ± 106[3] | 43% | 894 ± 141 |
| 100-125 g | 4 days | 665 ± 25[3] | none | 687 ± 28 |

[1]% depletion compared to control rats.

[2]Values are means ± pooles standard error.

[3]Data taken from J. Nutr. 121:2029-2036, 1991.

After 2 weeks of pivalate-treatment of the weanling rats, total carnitine depletion in the muscle was greater than that of the older rats so treated, 64% versus 43%. This difference is not great. Therefore, after only 4 days of treatment the degree of carnitine depletion between the two groups should be fairly comparable. After 4 days of pivalate-treatment, 100-125 g rats had total carnitine concentrations equal to 97% of control levels, a non-significant reduction. Thus, 4-day treatment of weanling rats should yield only a modest muscle carnitine depletion. Long et al (2) reported half-maximal rates of fatty acid oxidation in rat muscle are realized at 5-6% of normal muscle carnitine concentrations. In vivo measurements in rats confirmed that even a 48% depletion of carnitine in serum, muscle, and liver did not limit [1-$^{14}$C] palmitate oxidation (4). Consequently, muscle fatty acid oxidation in the studies described in this dissertation should not have been limited based on carnitine concentrations, and

there would be no reason to suspect undue FFA delivery to the liver.

Since results of this study suggested that impaired muscle fatty acid oxidation is an unlikely cause of the fasting ketosis, other mechanisms capable of producing excessive plasma ßOHB levels must be considered. The plasma ßOHB concentration is determined by the rate of production, the rate of utilization, the rate of excretion, and the balance between acetoacetate and ßOHB.

These possible causes of high plasma ßOHB in pivalate-treated rats are the subject of investigations in this laboratory reported in Chapter 3.

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## A2. Comparison of indices of fat metabolism in pivalate-treated rats, fed or fasted

A pivalate-induced animal model of carnitine deficiency has been described which reproduces several features of the carnitine deficiency observed in patients with organic acidurias (1). Total and free carnitine concentrations were reduced in the plasma, heart, and skeletal muscle after only 2 weeks of pivalate treatment. Excess liver triglycerides were present in fasted animals, analgous to the presence of lipid vacuoles in the liver of children with carnitine deficiency (2). The acylcarnitine:free carnitine ratio is elevated in the plasma, urine, and tissues, which suggests disturbed CoA homeostasis (3). In addition, the response to fasting is increased ketone production (4), unlike the hypoketonemia most often associated with primary carnitine deficiency (5).

Because the hepatic lipid accumulation occurred after only two weeks of pivalate-treatment when liver carnitine concentrations were not reduced to a level regarded as rate-limiting, it can be questioned why the steatosis was occurring. If it were present in unfasted rats, it might reflect profound pivalate inhibition of hepatic fat metabolism. If steatosis occured only with prolonged fasting, it would imply fat metabolism proceeded normally or close to normally until the liver was stressed by the higher influx of fatty acids associated with fasting. The primary focus of this experiment was to determine whether pivalate induced changes in lipid metabolism

under conditions of little or moderate stress.

A second aim of this study was to determine whether the exaggerated plasma ßOHB response to fasting in the pivalate-treated rats indicated a true fasting ketosis or merely a redistribution of ketones between acetoacetate and ßOHB. Thus, total ketone concentrations were measured in the fasted rats.

**Animals and research design.** Twenty-four (24) male, Sprague-Dawley, weanling rats were randomized to pivalate or bicarbonate solutions as in previous experiments. Rats received a purified low carnitine diet, AIN-76A (Teklad, Madison, WI). After 13 days, food was witheld from six (6) rats in each group for 1 day. Rats were killed by decapitation the following day, and blood was collected from the neck into heparinized tubes for measurement of ßOHB and acetoacetate. A piece of the median lobe of the liver was freeze-clamped in liquid nitrogen and stored at -20° C for subsequent measurement of liver triglycerides.

**Analyses.** The blood was held on ice until centrifugation, and the plasma was kept on ice until analyzed within 24 hours from the time blood was collected. Plasma ßOHB and liver triglycerides were determined using Sigma kits No. 310-UV and No. 405, respectively. Acetoacetate was measured with the method of Mellanby and Williamson (6) as modifided for unacidified plasma (7).

**Materials.** The pivalic acid, sodium salt, was obtained from Aldrich Chemical (Milwaukee, WI). All other chemical used were of reagent grade.

## RESULTS

As had been observed in previous studies of fed rats, there was no increase in the plasma ßOHB concentration in pivalate-treated rats. Consequently, blood was not assayed from the fed rats for acetoacetate. The mean plasma ßOHB concentration of food deprived rats, shown in **Table 1**, was 150% of the controls. This was not statistically significant. However, their mean total plasma ketone concentration was significantly higher. Liver triglyceride concentrations of the fed, pivalate-treated rats were significantly greater than those of the controls. There was no hepatic steatosis in food-deprived rats.

**TABLE A-2 Comparison of ketones and liver triglycerides of pivalate-treated and control rats, fed and unfed[1]**

| Group | Plasma ßOHB | Plasma Ketones | Liver Triglycerides |
|---|---|---|---|
| | *mmol/L* | *mmol/L* | *mg/g wet tissue* |
| Control, fed | 0.60 ± 0.07[a] | Not done | 22 ± 13[a] |
| Pivalate, fed | 0.60 ± 0.04[a] | Not done | 64 ± 10[b] |
| Control, fasted | 3.22 ± 0.44[b] | 4.27 ± 0.50[a] | 26 ± 10[b] |
| Pivalate, fasted | 4.83 ± 0.78[b] | 6.75 ± 1.02[b] | 34 ± 12[b] |

[1]N=6 per group. Unfed rats had food withdrawn 1 day prior to data collection. Values are means ± standard error (SE). Values in a column with unlike superscripts are significantly different from each other, $p<0.05$, Student-Newman-Keuls test.

[2]βOHB + acetoacetate.

## DISCUSSION

The plasma ketone data confirmed the exaggerated fasting plasma ßOHB response of pivalate-treated rats reflected true ketosis. Total plasma ketone body concentrations are a sum of ßOHB and acetoacetate concentrations, which establish an equilibrium with the following reaction: acetoacetate + H+ <--ßOHB dehydrogenase--> ßOHB. The equilibrium between the two moieties reflects the availability of reducing equivalents, which, in turn reflects the rates of reducing equivalent generation by fatty acid oxidation and use in oxidative phosphorylation and synthesis reactions. An imbalance in the rates of these reactions could lead to an increased ratio of ßOHB to acetoacetate without significantly increasing total ketone concentrations. The data showed plasma ketone levels were, indeed, higher in the pivalate-treated rats. The rats may have an enhanced rate of ketogenesis and/or a reduced rate of ketone utilization.

It is difficult to draw conclusions from the liver triglyceride results. This is the only study of fasted, pivalate-treated rats in which a significant increase in the liver triglycerides was not observed. However, in the other experiments rats were fasted 48 hours or 24 hours plus 4 additional hours with cold-stress. In this experiment, rats were fasted only 24 hours but not cold-stressed. Without the additional peripheral lipolysis that the longer period of fasting or the cold stress would induce, the fatty acid load to the liver may not have been sufficient to promote triglyceride synthesis concurrently with ketone synthesis. Van Harken et al (8) noted that hepatic triglyceride accumulation occured after the maximal rate of fatty acid oxidation had been achieved. Since the plasma ßOHB concentration of pivalate-treated rats in this study was not significantly elevated, ketone generation, though high, may not have

achieved a maximal rate.

A moderate fat increase in the livers of the fed rats was seen. This may denote impaired fatty acid oxidation or lipid secretion under low-stress, fed conditions. However, liver triglyceride sampies were three times for this study, raising the possibility that there was a problem with the assay. Both arms of the triglyceride phase of the study would need replication before attaching too much weight to its findings. Instead, the direction of this research turned towards the etiology of the plasma ketosis.

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# APPENDIX B. Tables

## APPENDIX B. Tables

### TABLE B-1 Rat Diet TD 86530[1]

| Component | g/kg |
|---|---|
| Casein, High protein | 200.0 |
| DL-methionene | 3.0 |
| Sucrose | 496.0 |
| Corn Starch | 150.0 |
| Corn Oil | 50.0 |
| Cellulose (fiber) | 50.0 |
| Mineral Mix, AIN-76 (170915) | 35.0 |
| Calcium carbonate | 4.0 |
| Vitamin Mix, AIN-76A (40077) | 10.0 |
| Choline bitartrate | 2.0 |
| Ethoxyquin (antioxidant) | 0.01 |

[1]This formula is a modification of AIN-76A obtained from TEKLAD.

**TABLE B-2 Food and water intake of rats receiving 2 dosages of pivalate[1]**

| Treatment | Food intake | Water intake |
|---|---|---|
| | g | ml |
| Con (n=4) | 14.1 ± 0.8[2] | 16.5 ±0.9 |
| 10 mM sodium pivalate (n=4) | 14.1 ± 0.2 | 13.9 ± 1.9 |
| 50 mM sodium pivalate (n=4) | 8.2 ± 1.2 | 8.0 ± 2.7 |

[1] Control rats drank plain water, treated rats received water containing sodium pivalate in the above concentrations. Data are an average of intake for two days.

[2]Data are shown as means ± standard deviation.

**TABLE B-3 Clearance of acyl and free carnitines[1,2]**

| | | Time Point | | |
|---|---|---|---|---|
| Urinary clearance | Group | 4 d | 2 wk | 8 wk |
| | | *x $10^{-3}$ ml/min* | | |
| Free carnitine | Pivalate-treated | 3.84 | 2.0 | 0.2 |
| | Control | 2.9 | 2.1 | 4.4 |
| Acyl carnitines | Pivalate-treated | 62.2 | 34.8 | 5.8 |
| | Control | 8.1 | 8.3 | 1.3 |

[1] Data are expressed as median clearances per 100 g of body wt.

2 Unpublished data from P.B Bianchi & A.T. Davis (1991) Sodium pivalate treatment reduces tissue carnitines and enhances ketosis in rats J. Nutrition 121:2029-2036, Chapter 1.

**TABLE B-4 Weights, food, and water consumption of pivalate-treated rats with and without carnitine supplementation[1]**

| Group | Initial wt. | Post-fed wt. | Wt. gain | Post-fast wt. | Wt. loss | Total food intake | Total water intake |
|---|---|---|---|---|---|---|---|
| | *g* | *g* | *g* | *g* | *g* | *g* | *ml* |
| Control | 46 ± 1 | 150 ± 4 | 105 ± 3 | 139 ± 3 | 12 ± 1 | 191.2 ± 10 | 269 ± 89 |
| Pivalate | 49 ± 2 | 155 ± 7 | 106 ± 5 | 140 ± 6 | 15 ± 1 | 194.4 ± 13.7 | 187 ± 82 |
| Piv + Cn | 51 ± 3 | 140 ± 10 | 89 ± 12 | 130 ± 10 | 11 ± 1 | 179.6 ± 12.9 | 178 ± 80 |
| Piv++Cn | 52 ± 3 | 156 ± 6 | 104 ±6 | 147 ± 8 | 12 ± 2 | 173.2 ± 2.3 | 198 ± 72 |

[1]Data from Experiment 2, Chapter 2.

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