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# Interaction with Choline and Pantothenic Acid with Carnitine in Humans and Rodents

James William Daily III
*University of Tennessee, Knoxville*

To the Graduate Council:

I am submitting herewith a thesis written by James William Daily III entitled "Interaction with Choline and Pantothenic Acid with Carnitine in Humans and Rodents." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Dileep S. Sachan, Major Professor

We have read this thesis and recommend its acceptance:

John W. Koontz, James W. Bailey

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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# INTERACTION OF CHOLINE AND PANTOTHENIC ACID WITH CARNITINE IN HUMANS AND RODENTS

**A Thesis**

**Presented for the**

**Master of Science**

**Degree**

**The University of Tennessee, Knoxville**

**James William Daily III**

**August, 1994**

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# Abstract

It had been shown previous to this research that supplementation with choline and pantothenic acid results in reduced plasma concentrations and urinary excretion of carnitine in adult humans. This research demonstrated similar effects in younger human subjects and developed an animal model for future study of the interaction of choline and pantothenic acid with carnitine.

Rats were first used as an animal model but neither choline, pantothenic acid, nor both combined had any effect on urinary or plasma carnitine concentration. It was concluded that rats were not an acceptable model, possibly because of the high choline oxidase activity present in rat liver.

Guinea pigs were then tested as an animal model and were found to have reduced urinary excretion of carnitine in response to choline supplementation, but not pantothenic acid supplementation. Plasma concentrations of carnitine in guinea pigs were unaffected by either choline, pantothenic acid or both combined. It was concluded that choline was the supplement responsible for reducing plasma and urinary excretion of carnitine in guinea pigs, and that guinea pigs provide an acceptable model for future study of interactions of choline and pantothenic acid in humans.

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## List of Abbreviations

Acid Insoluble Acyl Carnitine.....AIAC

Acid Soluble Acyl Carnitine.....ASAC

Alanine Transaminase.....ALT

Analysis of Variance.....ANOVA

Aspartate Transaminase.....AST

Choline + Pantothenic Acid.....CH/PA

Non-Esterified Carnitine.....NEC

Pantothenic Acid.....PA

Phosphatidylethanolamine-N-methyltransferase.....PEMT

S-Adenosylmethionine.....SAM

Total Carnitine.....TC

# Chapter 1

## Introduction

Carnitine and choline are both recognized as essential for metabolic processes; neither, however, are considered essential nutrients as they are synthesized in the human body. The possibility that under certain conditions, suboptimal quantities may be synthesized by some individuals and that supplemental levels of intake may confer benefits has resulted in the use of both as dietary supplements (1,2). Much of the interest in supplementary choline has focused on its lipotropic action and on its effects on brain function as a result of increased acetylcholine synthesis (3-5). Choline is usually one of the ingredients in the "smart drinks" that are currently popular as an alternative to alcoholic beverages. Choline has long been known to be essential for membrane phospholipids, as a source of labile methyl groups, and for the synthesis of acetylcholine. Recently, however, several choline derivatives have been recognized as essential for intracellular second messenger systems. Phosphatidylcholine products resulting from the action of phospholipase C, D, and $A_2$, and products of sphingomyelin degradation by sphingomyelinase are important for intracellular signaling (for a brief review of choline's involvement in intracellular signalling see reference 6).

Platelet activating factor also contains choline and is involved in such diverse functions as blood clotting, inflammation, pulmonary regulation, and allergic reactions (6).

Carnitine is structurally very similar to choline, but quite different in function. Carnitine is best known for its role in transporting long-chain fatty acids into the mitochondria of cells, but also is useful in shuttling fatty acids between different tissues and in removing short chain fatty acids from the mitochondria thereby maintaining a pool of free coenzyme A inside the mitochondria (1,7-9).

Our laboratory first became interested in choline when doing a survey of carnitine excretion and plasma concentrations in college students. One subject had significantly lower plasma carnitine concentration and very low urinary excretion of carnitine. The subject was questioned about dietary habits and it was discovered that she was taking supplementary choline and pantothenic acid (10). A study was undertaken giving choline and pantothenic acid supplements to subjects and comparing plasma carnitine concentrations and urinary excretion of carnitine to controls. The subjects given choline and pantothenic acid, as expected, had decreased carnitine concentrations in the plasma and excreted less carnitine in the urine, thus confirming the single observation. We had no basis to judge the mechanism of the alteration in carnitine concentrations

by choline or even to judge whether the effect was harmful, beneficial, or of no consequence. We also were uncertain if the observed effect was a result of the choline and/or the pantothenic acid.

The purposes of the studies reported here were to develop an animal model to study the reduced plasma concentration and urinary excretion of carnitine by choline and pantothenic acid effect in humans, to determine if the effect was mediated by choline or pantothenic acid, and to determine the mechanism involved. Studies in our laboratory and others using rats as an animal model found no effect of choline on either urinary excretion or plasma concentrations of carnitine. We concluded that this was probably due to the high activity of choline oxidase in rat liver, and that using an animal with similar choline oxidase activity to humans should be used. The guinea pig was chosen because the liver choline oxidase activity is most similar to that of humans of any common laboratory animal (11).

# Chapter 2

# Literature Review

## Choline

Choline is a quaternary trimethylated amine as is carnitine, another nutrient of primary importance in this research. Choline is synthesized de novo in humans but also is widely available in the diet. Choline deficiency has been induced in animals resulting in liver damage, renal hemorrhage, and inability of the liver to export triglyceride with hepatic fat accumulation resulting (12). There has never been a case of choline deficiency syndrome observed in humans, probably because of it's ubiquitous nature in the human diet.

***Choline in the diet:***

Daily dietary intake of choline in humans is estimated to be between 600 and 900 mg per day (12). Choline is available in the diet as both choline chloride and phosphatidylcholine (lecithin). The best sources of choline include meat, eggs, oatmeal, soybeans, wheat germ, peanuts, and other nuts and grains (13). The most abundant form of choline in the diet is lecithin, but many foods contain significant amounts of choline chloride, especially liver and soybeans. Choline is also obtained in the diet from sources other than naturally occurring foods. Lecithin is a common food additive because of its ability to emulsify

fats, is an ingredient in frying pan sprays, and is available as a vitamin supplement as lecithin or choline chloride. Lecithin is usually the preferred source of supplementary choline because intestinal bacteria can degrade free choline to trimethylamine which produces an unpleasant body odor and breath (13).

***Absorption of choline:***

Free choline is absorbed in the upper small intestine, primarily in the jejunum in rats and hamsters (14). The absorption appears to utilize an unidentified carrier at low concentrations that is not sodium dependent but that is saturable at concentrations above 4 mM. At concentrations above 4 mM choline is absorbed by passive diffusion and proportional to intraluminal choline concentrations (15). Once absorbed choline enters the portal circulation as do other water soluble molecules (13).

The majority of choline in the diet is in the form of lecithin which is absorbed more like a fat. Pancreatic juice contains phospholipase $A_2$ which cleaves the middle fatty acid from the phosphatidylcholine molecule forming one lysolecithin and one free fatty acid from each molecule. Approximately half of the absorbed phosphatidylcholine is absorbed in the form of lysolecithin which is reacylated in the intestinal mucosa and exported to the lymphatic system as chylomicrons or very low density lipoproteins. The other half of the phosphatidylcholine is completely hydrolyzed to

free fatty acids and free choline and the choline absorbed as described above (16,17,18).

***Non Dietary Sources of Choline:***

Lecithin is formed in mammals by three different pathways; the base exchange pathway, CDP-choline pathway, and the methyltransferase pathway (13). The base exchange pathway simply exchanges choline for another base on a phospholipid, the CDP-choline pathway uses diacylglycerol and free choline to form phosphatidylcholine. The methyltransferase pathway is the only pathway that actually synthesizes new choline. In the methyltransferase pathway phosphatidylethanolamine is sequentially methylated by phosphatidylethanolamine-N-methyltransferase (PEMT) using S-adenosyl methionine (SAM) as the methyl source (19,20). PEMT is most concentrated in the liver but is also found in kidney, testes, heart, lung, adrenal gland, erythrocytes, spleen, and brain (21,22,23). In the rat about 15% of the choline requirement is supplied by the methylation pathway with the remainder obtained from the diet (13,24,25). The percentage of choline that must be obtained from the diet in humans is not something that is well understood currently, but human dietary requirements for choline will be discussed later.

PEMT is actually two enzymes that catalyze the methylation of phosphatidylethanolamine. The first methylation is the rate limiting step and is facilitated by

an enzyme located on the cytoplasmic side of the plasma membrane in erythrocytes, and is followed by two subsequent methylations by an enzyme located on the exterior side of the plasma membrane (22,26). The first methyltransferase, which methylates phosphatidylethanolamine to phosphatidylmethylethanolamine, is $Mg^{2+}$ dependent, has an optimum pH of 6.5 and a high affinity for SAM (Km of 1.4 $\mu$M). The second methyltransferase is not $Mg^{2+}$ dependent, has an optimum pH of 10 and has a much lower affinity for SAM (Km of 100 $\mu$M). Choline deficiency and ethanol exposure increase the activity of the methylation pathway of lecithin synthesis (27, 28,29).

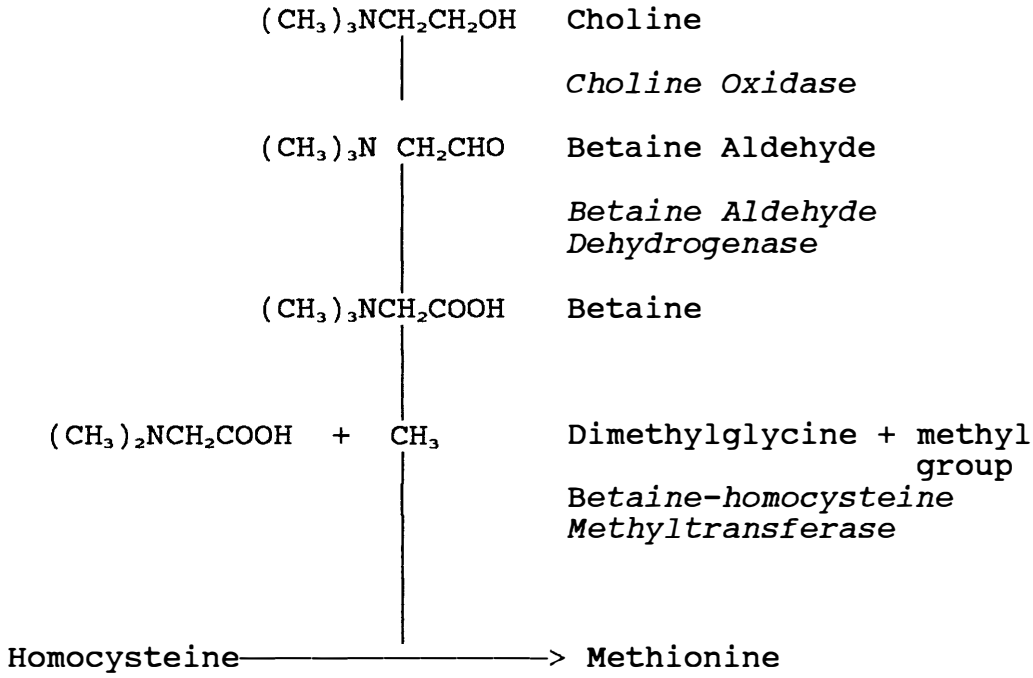
***Choline in single carbon metabolism:***

Choline is frequently referred to as an important source of labile methyl groups (2). S-adenosyl methionine is the active agent in many synthesis reactions requiring addition of a methyl group (30). When SAM donates its methyl group it is hydrolyzed to form homocysteine and adenosine. The methionine may be regenerated by the transfer of a methyl group to homocysteine. The methyl group may be obtained either from N5-tetrahydrofolate in a reaction catalyzed by homocysteine methyltransferase, or from betaine in a reaction catalyzed by betaine-homocysteine methyltransferase (31). Both reactions appear to be of about equal importance in the rat (32,33).

Methotrexate is a commonly used cancer therapeutic

agent which is a competitive inhibitor of dihydrofolate reductase thereby blocking the regeneration of methionine by the folate pathway. When rats are treated with methotrexate the liver is rapidly depleted of SAM, choline, phosphocholine, and betaine; the liver becomes fatty and liver function is impaired. Supplementation with choline can prevent the above symptoms in rats, at least for up to three months (34,35). Clearly choline is able to largely replace folate in methyl metabolism in rats.

Whether choline is a significant source of labile methyl groups in humans is uncertain (31). The following pathway is the only way that methyl groups from choline may be utilized (36).




Choline oxidase, however, is present in very low concentrations in the human liver compared to most animals.

Table 1 shows choline oxidase activity in several animals and man. Studies investigating choline as a methyl donor have used rats as the model and may not apply to humans since rats have 60 times the choline oxidase activity of humans. It is quite possible that choline is not able to significantly contribute to the labile methyl pool in humans even though humans do have active liver betaine-homocysteine methyltransferase (37). To suggest that choline may not be an important methyl donor in humans does not suggest that choline does not have important functions in humans, because there are other critical functions for choline in humans which are discussed in the next sections.

***Choline and acetylcholine:***

The central nervous system communicates between nerve cells and other cells using chemical messengers called neurotransmitters; one of the most important of these is

## Table 1

### Liver Choline Oxidase in Selected Species

| Species | Choline Oxidase Activity |
|---|---|
| Rat | 2408 ± 121 |
| Chicken | 1311 ± 86 |
| Mouse | 895 ± 72 |
| Hamster | 361 ± 63 |
| Monkey | 144 ± 21 |
| Guinea Pig | 136 ± 43 |
| Human | 40 ± 7 |

Taken from Sidransky and Farber, reference 11. Activity units are in microliters of $O_2$ uptake/hr/g wet liver ± SEM.

acetylcholine. Acetylcholine is one of the neurotransmitters between neurons in the central nervous system; the neurotransmitter between neurons and somatic motor neurons at the neuromuscular junction, and between parasympathetic nerve endings (38). Acetylcholine is synthesized by the transfer of an acetyl group from acetyl CoA to choline in a reaction catalyzed by choline acetyltransferase (39). Enzyme kinetics studies of choline acetyltransferase in brain tissue suggest that the enzyme is unlikely ever to be saturated and that choline and/or acetyl CoA availability determine the rate of acetylcholine synthesis (34,40).

Cohen and Wurtman (41) found that brain (particularly the caudate nucleus) acetylcholine varied on a day to day basis with dietary intake, though remaining within the normal range for rats. Others, however, have not found steady state acetylcholine concentrations to be affected by dietary variations but only found increases in acetylcholine under induced conditions of increased cholinergic neuronal activity (42). Cognitive benefit from using choline supplements (as in "smart drinks") has yet to be demonstrated, but benefits have been demonstrated in some diseases involving cholinergic neurons. Choline and lecithin have been used most successfully in treatment of tardive dyskinesia, but some benefit have also been reported in the treatment of Huntington’s chorea, Alzheimer’s disease, and ataxia (36).

***Choline in membrane phospholipid:***

Choline in the forms of phosphatidylcholine and sphingomyelin is a major structural component of mammalian cell membranes. Phosphatidylcholine makes up approximately 50% of the phospholipid content of cell membranes and sphingomyelin 5-20%, depending on the cell type (43). It has recently become clear that choline phospholipids in membranes are much more than just structural components, but are essential for intracellular signal transduction (33,43,44).

One of the best understood mechanisms of intracellular signalling occurs when a hormone binds to a receptor protein activating a G protein which in turn activates phospholipase C. Phospholipase C hydrolyses the phosphate from phosphatidylinositol generating inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (45). The hydrolysis of phosphatidylinositol results in the activation of protein kinase C which requires binding of diacylglycerol and $Ca^{2+}$ for activation. The diacylglycerol is supplied directly from the hydrolysis, and $Ca^{2+}$ indirectly; IP3 causes the release of stored intracellular calcium. It was reported in 1981 that the diacylglycerol released as a result of cholinergic stimulation of rat pancreas did not resemble the fatty acid composition of phosphatidylinositol (46,47). In 1985 the first evidence that hormones can promote phosphatidylcholine hydrolysis was reported (48). Hepatocytes stimulated with a

calcium mobilizing agonist were shown by high performance liquid chromatography to have two primary types of diacylglycerol. One type had higher concentrations of palmitic, oleic, and linoleic acids than normally found in phosphatidylinositol, which is typically rich in arachidonic and stearic acids. It was therefore suggested that phosphatidylcholine was the source of the diacylglycerols (46,47,49).

Apparently, a frequently occurring scenario is that diacylglycerol is initially released primarily from phosphatidylinositol as a result of the action of phospholipase C. The initial release is followed by a longer lived and greater release from phosphatidylcholine as a result of a phosphatidylcholine specific phospholipase C and/or D. Phospholipase D hydrolysis produces a phosphatidic acid from which the phosphate is cleaved by phosphatidic acid phosphohydrolase thus releasing DAG (44,46).

Studies of phosphatidylcholine hydrolysis show that calcium depletion in hepatocytes blocked diacylglycerol formation whereas addition of the calcium ionophore A23187 potentiated the hydrolysis (46,50). The requirement for $Ca^{2+}$ in the hydrolysis of phosphatidylcholine suggested a possible role for protein kinase C since binding of $Ca^{2+}$ to this enzyme is required for it's activation. This was demonstrated to be the case in rat brain where it was shown that purified protein kinase C can activate a

phosphatidylcholine hydrolyzing phospholipase D (51). A possible role for the hydrolysis of phosphatidylcholine subsequent to protein kinase C activation is to maintain the activation for a longer time. When protein kinase C is activated by $Ca^{2+}$ and diacylglycerol, the diacylglycerol binds for only a short time, but the calcium dissociates rather slowly. The addition of further diacylglycerol may be useful in maintaining the activation of protein kinase C (43), which may be made possible by activation of the phospholipase D & C by protein kinase C. Far more diacylglycerol can be released from phosphatidylcholine than from phosphatidylinositol hydrolysis since cellular phosphatidylcholine is several hundred times the concentration of phosphatidylinositol (52).

There are also instances in which phosphatidylcholine breakdown occurs without the prior breakdown of phosphatidylinositol; examples include the activation of T-lymphocytes by interleukin 1 and the simulation of fibroblasts by epidermal growth factor, both of which can result in sustained elevation of intracellular diacylglycerol for hours (46,53,54). It has been shown that diacylglycerol can activate some protein kinase C isotypes alone, which is interesting because during choline deficiency in rats hepatocytes accumulate triglyceride and diacylglycerol. The concentration of diacylglycerol can reach levels that exceed that in normal stimulation,

resulting in protein kinase C activation (43,55). It has been suggested that sustained activation of protein kinase C may explain the spontaneous development of hepatic carcinomas seen in choline deficient rats (55).

The study of phosphatidylcholine metabolites in intracellular signalling is still very recent and the mechanisms have not yet been clearly elucidated as with phosphatidylinositol. The release of arachidonic acid by the action of phospholipase $A_2$ resulting in eicosanoid formation is also an important mediator of metabolic events (56). This is not limited to choline phospholipids, however, and will not be covered here.

Another choline containing membrane phospholipid involved in intracellular signaling is sphingomyelin, which can be hydrolyzed to form ceramide and choline phosphate (43). The hydrolysis of sphingomyelin can be activated by several known factors including 1-α,25-dihydroxyvitamin D, tumor necrosis factor, and γ-interferon (57). Ceramide is a potent inhibitor of cell growth and sphingosine an inhibitor of protein kinase C (58,59). Choline itself has not yet been reported as a signal transduction molecule and its function, if any, when choline containing phospholipids are hydrolyzed, is unknown.

***Choline: an essential nutrient:***

Choline has been shown to be an essential nutrient in many animal species including rat, hamster, guinea pig, pig,

dog, monkey, trout, and chicken (33). Choline deficiency results in spontaneous liver cancer in rats without exposure to carcinogens and is the only nutrient known whose deficiency is known to result in malignancies (43,60). Choline deficiency, in species for which it has been shown to be an essential nutrient, can result in impaired hepatic, renal, pancreatic function, memory loss, and decreased growth (33). It has been difficult to demonstrate that choline is an essential nutrient for humans, however, it has been shown that human cells grown in culture have an absolute requirement for choline (2,61). Malnourished humans have decreased serum choline concentrations and parenterally fed persons without choline supplementation develop abnormal liver function, indicating that there may be a requirement for choline in the diet (62,63,64).

Zeisel et al. conducted a human study in which the study group was fed a choline free diet and a control group was fed an identical diet with added choline (2). They found decreased choline and phosphatidylcholine in plasma of the choline deficient group as compared to control as expected, but also the serum alanine aminotransferase activity increased consistently over the three weeks of the diet and the study was then terminated to avoid further risk to the subjects.

Impairment of liver function seems to be consistent among all species, including humans, during choline

deficiency. It has been difficult to find cases of choline deficiency disease in human populations because choline is very plentiful in foods, and it would be difficult to construct a diet of natural foods that is deficient in choline This does not mean however, that choline is not essential - just easily obtained. Studies with artificial diets indicate that choline is essential in human diets.

## Carnitine

Carnitine, L-ß-hydroxy-γ-N-trimethylaminobutyric acid, like choline is a quaternary, trimethylated amine. Carnitine, also like choline is synthesized de novo in humans, and is available in the diet - though not as plentifully as choline (65). Carnitine was originally called vitamin $B_T$ but this terms is no longer used since carnitine is not considered essential in the diet of higher organisms. Diseases of carnitine deficiency have been identified in humans (65) and will be discussed later. These diseases are usually very rare genetic disorders, and do not reflect a need for carnitine in normal human nutrition (65).

***Carnitine in the diet:***

Dietary carnitine is derived primarily from animal food sources, and the average nonvegetarian diet is estimated to supply 100-300 mg of carnitine per day (65). Vegetables, fruits, and grains are very poor sources of carnitine.

Ground beef has about 580 μmol of carnitine per 100 grams whereas asparagus (one of the richest vegetable sources) has about 1.2 μmol per 100 grams (65,66).

Dietary intake of carnitine has been shown to have a significant influence on carnitine status in humans. Children receiving total parenteral nutrition for 7.2 years ± 2.6 years from birth had total plasma carnitine concentrations approximately half that of other children of similar age serving as controls (66). When adults and children eat either strict vegetarian diets, lacto-ovovegetarian diets, or mixed diets (including meat) for long periods of time both plasma carnitine and urinary excretion of carnitine have been shown to be affected (67). Plasma carnitine differences between groups were small but significant. Differences in urinary excretion of carnitine were much greater, adult men on mixed diets, lactoovovegetarian diets, or strict vegetarian diets excreted 5.79 ± 3.08, 2.1 ± 0.76, and 1.36 ± 0.49 μmol carnitine per kg body weight per day, respectively. Similar trends were seen for male children as well as female adults and children. The urinary excretion of carnitine decreased proportionately with predicted carnitine intake. The subjects eating diets highest in carnitine had the highest excretion rate and those with diets lowest in carnitine (strict vegetarian) had the lowest excretion rate.

How the differences in plasma carnitine and urinary

carnitine excretion affect tissue concentrations is not certain It has been shown in rats that dietary differences do substantially affect tissue concentrations, but were roughly proportional to plasma concentrations which had a much greater variation than seen in the above mentioned human study (68).

To what extent humans rely on dietary carnitine is unknown. Cederblad and Lindsted report that synthesis contributes four times more carnitine than does diet in laboratory rats (69). In humans, however, dietary intake may be more important since diet typically contributes 100-300 mg per day. The functional consequences of variations in dietary intake of carnitine in humans are not known at this time, but it is clear that dietary intake can affect plasma and tissue concentrations in rats.

***Intestinal absorption and tissue uptake of carnitine:***

It has been difficult for researchers to determine the percentage of carnitine absorbed from the diet. It was assumed for many years that carnitine was nearly 100% absorbed because when labeled carnitine was given orally, little radioactivity showed up in the feces. Kinetic analysis in animals and humans, however, indicated that there was a higher rate of excretion than was reported (70,71,72). In the kinetic studies, the carnitine was given intravenously and no metabolites of carnitine were found in urine or feces. When radioactively labeled carnitine was fed

orally to rats, however, as much as 34% of the radioactivity was found in excreted metabolites of carnitine in urine and to a small extent in feces (73). When humans are fed radioactive carnitine 95% of the radioactivity is absorbed from the intestinal tract, but several metabolites show up in the urine, primarily trimethylamine N-oxide (70). Apparently intestinal bacteria degrade carnitine to other metabolites which are in turn absorbed and then excreted in the urine. Recent studies indicate that carnitine is 54-87% absorbed without degradation depending on the dosage (70).

Carnitine is absorbed both actively and passively across the intestinal mucosa (65). Active carnitine absorption is a sodium linked process similar to the absorption of phosphate (74,75,76). Carnitine enters the blood stream via the portal circulation and circulates as free or acyl carnitines (65). Most tissue cells transport carnitine against a concentration gradient resulting in a 10-100 fold greater intracellular concentration (65), suggesting an active transport mechanism there also.

***Carnitine biosynthesis:***

Carnitine is synthesized from two essential amino acids in all mammals including humans (9). The nitrogen and carbon backbone of carnitine are derived from lysine and the methyl groups attached to the nitrogen from SAM. Table 2 shows the steps in carnitine biosynthesis, and the nutrients required as cofactors (65,77).

## Table 2
## Steps in Biosynthesis of Carnitine And Nutrient Requirements

| Step | Reactant | Product | Required Nutrient |
|---|---|---|---|
| 1. | Lysine | N-trimethyl-lysine | Methionine, Lysine |
| 2. | N-trimethyl-lysine | N-trimethyl-3-hydroxylysine | Vitamin C, Iron |
| 3. | N-trimethyl-3-hydroxylysine | 4-N-trimethylamino butryaldehyde | Vitamin B6 |
| 4. | 4-N-trimethylamino butyraldehyde | 4-N-trimethylamino butyric acid | Niacin |
| 5. | 4-N-trimethylamino butyric acid | L-carnitine | Vitamin C, Iron |

In step 1 lysine is methylated as a post-translational modification in proteins including actin, myosin, and histones; and N-trimethyl-lysine is released as a result of enzymatic degradation of those proteins (65,78,79). Steps 2-5 in table 5 occur in both the liver and kidney in higher mammals (78,80). It is not clear how much effect diets deficient in the nutrients involved in carnitine biosynthesis have on carnitine status. Wheat gluten diets (deficient in lysine) have little effect on carnitine concentrations in adult rats (81), however, weanling rats fed a lysine deficient diet will become carnitine deficient (82,83).

Vitamin C deficiency has also been proposed to affect carnitine status since it is required for its biosynthesis.

Vitamin C deficiency has been shown to result in decreased activity of hepatic γ-butyrobetaine hydroxylase and hepatic and renal ϵ-N-trimethyllysine hydroxylase (87). It was not determined, however, if the effect on enzyme activity altered carnitine status.

Iron has an obligatory role in carnitine synthesis and a deficiency could impair the process. Nutritional iron deficiency in pregnant rats results in hypertriglyceridemia in both mother and pups, which can be reversed with iron supplementation (84). Pregnant rats continued on a iron deficient diet into the lactation period produced milk with normal carnitine concentrations but reduced iron concentrations (85). On day 2 after birth there was no difference in carnitine concentrations in the pups, but on day 16 liver carnitine was about half that of iron sufficient pups. Rat pups are not fully able to synthesize carnitine until 8 days after birth (86), so the effect of iron deficiency on their own carnitine synthesis would not be expected to be seen very soon after birth. This demonstrates that iron deficiency may result in impaired carnitine synthesis and may also explain the hypertriglyceridemia seen in the iron deficient pups.

***Carnitine and fatty acid metabolism:***

The transport of long chain fatty acids across the inner mitochondrial membrane into the matrix of the mitochondria is the best characterized function of carnitine

(65). This process involves the activation of a fatty acid by coupling to coenzyme A (CoA) via a thioester bond, followed by transfer of the acyl group to carnitine, translocation of the acylcarnitine across the inner membrane, and transfer of the acyl group to intramitochondrial CoA (9). The first step in carnitine mediated transport of fatty acids across the inner mitochondrial membrane is the cytosolic generation of acyl CoA which diffuses across the outer membrane into the inter membrane space.

Carnitine palmitoyltransferase I (CPT1) spans the outer mitochondrial membrane, with the transferase active site on the inside of the membrane and a malonyl CoA binding site on the cytosolic side. The active site transfers the acyl group of the acyl-CoA to carnitine to make an acylcarnitine (65).

Binding of malonyl CoA to the cytosolic side of the CPT1 inhibits the enzyme and assures that acyl groups will not be transported into the mitochondria for oxidation at the same time that fats are being synthesized (88). Carnitine-acylcarnitine translocase shuttles the acylcarnitine across the inner mitochondrial membrane where another CPT ($CPT_2$) transfers the acyl group back to intramitochondrial CoA after which it proceeds to oxidation (65). The reverse of the process for transporting long chain fatty acids into the mitochondrial matrix appears to

be used for removing short and medium chain fatty acids from the inside of the mitochondria (65).

***Other possible functions of carnitine:***

Carnitine has been shown to be able to inhibit the clustering of cells including erythrocytes (89) and the slime mold *Dictyostelium discoideum* (90). The carnitine concentrations required for 50% inhibition of aggregation are substantially higher than physiological concentrations of carnitine in plasma at 0.5 mM for erythrocytes and 5-10 mM for *Dictyostelium*. The normal concentration of carnitine in plasma is about 0.05 mM, so the significance of this finding is questionable.

The concentration of carnitine in the brain is lower than most tissues, but still significant (9). The function of carnitine in the brain is unknown since it does not utilize fatty acids for energy. Recent studies have investigated the use of acetyl carnitine for treatment of neurological disorders. In streptozotocin induced diabetic rats, treatment with acetylcarnitine was able to reverse neurological damage as determined by measurement of nerve conductance velocity (91). In aged rats acetylcarnitine was able to restore neuromuscular conduction velocity to that of young rats after 6 months of treatment (92). Human tests with Alzheimer's disease patients have shown some evidence that acetylcarnitine may ameliorate the symptoms of the disease when given orally (93). How acetylcarnitine may

exert the effects reported on neurological function is unknown.

The organ most sensitive to carnitine deficiency is the heart, because the heart relies on fatty acids for about 60% of its energy needs (8). Primary carnitine deficiency is a rare syndrome that has been described in children (94). In this disease plasma carnitine concentrations are very low resulting in congestive cardiomegaly which can be reversed by oral carnitine administration. Cardiomegaly can also develop as a result of carnitine being dialyzed out in hemodialysis patients and carnitine should be supplemented in such patients (95). The use of carnitine has been investigated for treatment of ischemic heart disease, but the results thus far have been contradictory so it is still under investigation (8).

Carnitine has also been shown to reduce the hepatic steatosis seen in chronic alcoholic rats (96). Rats fed a diet with 36% of the calories from alcohol will accumulate 3-4 times the hepatic lipid content of controls within 8 weeks. Rats supplemented with D,L-carnitine (1% by weight in the diet or 0.5% L-carnitine) accumulated less than half the lipids as did the non-supplemented alcoholic rats. The effect of carnitine on liver lipids was later shown to be dose dependent with the greatest effect at 0.8% D,L-carnitine in the diet (97).

Carnitine was also shown to reduce the rate of alcohol

disappearance from the blood (98). The effect was not seen with choline supplementation although choline is very similar in structure to carnitine (98). It was concluded that the attenuation of ethanol metabolism was specific for carnitine. The reduced rate of ethanol clearance was shown to be the result of reduced oxidation even though the activity of ethanol oxidizing enzymes was unaffected (99).

The hepatic steatosis resulting from aflatoxin $B_1$ administration has also been shown to be reduced by carnitine supplementation (100). Carnitine also reduced formation of aflatoxin adducts with RNA, DNA, and proteins in the liver.

***Carnitine and the immune system:***

The effect of carnitine on cells of the immune system has been investigated with uncertain results. Borum et al. reported in 1985 that erythrocytes contain 20-30% of the blood carnitine (101). In 1987,however, Katrib et al. reported that erythrocytes had virtually no carnitine and that high concentrations were found in polymorphonuclear leukocytes (neutrophils) and mononuclear cells (macrophages and monocytes) (102). After that, Furst and Gloggler reported that erythrocytes had substantial carnitine concentrations and polymorphonuclear cell and mononuclear cells had virtually non-detectable concentrations of carnitine (103). It is difficult to explain why the different laboratories found such extreme differences in

carnitine concentrations in blood cells. The group that found high concentrations of carnitine in immune cells demonstrated the next year that patients with inflammatory disorders have decreased concentrations of carnitine in plasma but increased concentrations in neutrophils (104). Polymorphonuclear cells were found to have increased acyl and total carnitine, mononuclear cells to have increased ratio of acyl to free carnitine but the same total carnitine, and plasma to have severely decreased free carnitine but increased acylcarnitines. It is possible that the activation state of immune cells can have a significant effect on carnitine concentrations which resulted in the discrepancies reported between laboratories.

***Carnitine excretion:***

At normal plasma concentrations about 90% of the carnitine filtered in the kidney is reabsorbed (105). The macronutrient content of human diets is known to affect urinary excretion of carnitine and plasma carnitine concentrations. High fat diets have been shown to increase both plasma concentration and carnitine clearance over low fat diets (106). Plasma carnitine concentrations tend to remain rather constant at a wide range of dietary intake. Renal adaptation to carnitine intake appears to be a major regulator of plasma carnitine concentration (102). Persons given a diet high in fat, protein, or both excrete significantly increased amount of carnitine (107). The

increased carnitine excretion appears to be the result of increased plasma carnitine resulting an increased filtered load. Carnitine intakes were constant between groups so the diet apparently resulted in increased carnitine absorption, biosynthesis, or release from tissue stores.

## Pantothenic Acid

Pantothenic acid is one of the B vitamins and is widely distributed throughout the food supply. Virtually all foods contribute significant amount of pantothenic acid to the diet, however, meats, legumes, and whole grain cereals are the richest sources (108). Although plentiful, much of the dietary pantothenic acid may be destroyed in food preparation, it is reported that cooking meat results in a 15-50% loss of the pantothenic acid (109).

***Absorption and transport of pantothenic acid:***

Most dietary pantothenic acid is in the form of coenzyme A, but during digestion it is released from the coenzyme A molecule and is absorbed as free pantothenate (110). Approximately 50% of the coenzyme A in the diet is absorbed (111). The mechanism for intestinal absorption of pantothenic acid is uncertain, but one recent report has demonstrated that pantothenate utilizes a sodium cotransport mechanism in the human placental brush-border membrane (112). It was also shown that biotin is transported by the

same mechanism and competes with pantothenic acid. Whether this mechanism is utilized in the intestinal mucosa is uncertain.

***Functions of pantothenic acid:***

Pantothenic acid has two primary functions, first it is part of the coenzyme A molecule and second it is the prosthetic group on acyl carrier protein. As part of the coenzyme A molecule pantothenic acid is essential to all energy releasing reactions in the body (113). As part of the acyl carrier protein, pantothenic acid is essential for the synthesis of fatty acids (114).

***Excretion of pantothenic acid:***

Pantothenic is excreted as the free vitamin in the urine. Most is derived from the hydrolysis of coenzyme A to produce pantothene which is further hydrolyzed to pantothenic acid (114). When excretion of pantothenic acid drops below 1 mg/d (an amount corresponding to an intake of < 4 mg/d) a deficiency of the vitamin should be expected (113).

## Interactions Between Nutrients

The concept of a nutrient interacting with another nutrient in ways that effect the tissue concentrations or dietary requirement for that nutrient is not new. It was reported in 1947 that dietary tryptophan reduces the dietary

requirement for niacin (114,115).

***Interaction between vitamin C and iron:***

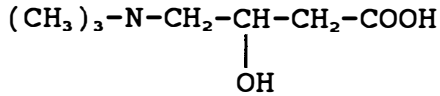
A classical nutrient-nutrient interaction is that between vitamin C and iron. Vitamin C is able to increase the absorption of iron by keeping it in the reduced ($Fe^{2+}$) form and by chelating it and keeping the iron in a more soluble form (116). Vitamin C is also important in other aspects of iron metabolism such as synthesis of hemoglobin and the removal of iron from ferritin (116).

***Interaction between calcium and magnesium:***

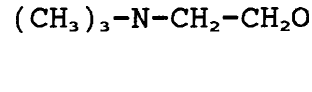
Similar nutrients often affect each other, such is the case with the two divalent cations calcium and magnesium. It has been reported that increasing the calcium consumption in humans can reduce absorption of dietary magnesium (117). In rats fed a parenteral nutrition diet with graded doses of calcium, urinary excretion of magnesium increased as calcium supplementation increased (118). Calcium, therefore, may decrease absorption and increase excretion of magnesium simultaneously.

***Interaction between choline and carnitine:***

Choline and carnitine are also very similar in structure as shown here.



Carnitine



Choline

With such striking similarity it would not be surprising if interactions were found. Rats that are fed a choline deficient diet have been shown to have reduced carnitine concentrations in liver, heart, and skeletal muscle, but increased plasma carnitine concentrations compared to animals fed a choline supplemented diet (119). Comparisons were not made to a control diet in this study.

Choline deficiency results in a decrease in lipid oxidation in heart and liver cells that can be reversed in vitro by the addition of carnitine (120). The difference in carnitine levels in this study were even more dramatic after the animal were fasted for 48 hrs. Carnitine concentrations increased almost four fold in liver and significantly in heart and muscle after fasting of the choline supplemented animals. In the choline deficient animals carnitine increased in the liver about 3 fold but decreased slightly in heart and muscle. It would appear that increases in carnitine concentrations are a part of the adaption to fasting that is impaired when there is choline deficiency in rats.

It was shown in another study that the decreased hepatic carnitine in choline deficient rats results in a four fold decrease in oxidation of $^{14}C$ palmitate and an increased incorporation into triglyceride in liver homogenates (120). Addition of carnitine, but not choline, in the liver homogenates restored palmitate oxidation to

control levels and decreased the rate of esterification palmitate to glycerol. This suggested that the fatty liver seen in choline deficient rats may be due in part to a lack of carnitine.

Choline and pantothenic acid supplementation has also been shown to reduce carnitine concentrations in plasma and 24 hr urine of humans (121,122). Follow-up studies demonstrated that the effect on carnitine was due to choline and not pantothenic acid (123). The results of these studies raised a number of intriguing questions which could not be adequately answered by reasonable experiments in humans. The questions included 1) Are the decreases in plasma and urine carnitine concentrations a reflection of altered partitioning of carnitine among tisues? 2) Is it mediated via the carnitine transporters of tissues? 3) What are the consequences of the decreased carnitine concentrations in plasma and urine?

Thus a need for an animal model was clearly realized and the primary objective of this research was to find a good animal model to study the choline-carnitine interactions.

# Chapter 3

# Materials and Methods

The human research protocol presented here was approved by the Committee on Research Participation of the University of Tennessee. The animal research protocols were approved by the animal care and use committee of the University of Tennessee. All animals were housed in a AAALAC accredited animal facility maintained at a constant temperature and relative humidity of 70° F and 50% respectively.

## Experiment in Humans

The human study was conducted on college students age 20-35. Choline only was used in this study because it was suspected to be causing the effect on carnitine status in the previous studies. Six female students volunteered to take the choline supplement (experimental group). The control group consisted eight female students.

Each subject in the experimental group was provided a choline supplement (Rexall 300 mg ea., Ft Lauderdale, Fl.) and instructed on the number of tablets to take per day to obtain 0.165 mol/kg body weight (20 mg/kg). All subjects were instructed to eat their normal diets and report any significant deviations from their normal pattern of eating

and exercise. After five days of supplementation blood and urine samples were collected.

Each subject was provided a container containing thymol crystals and instructed to collect 24 hr urine samples by voiding and discarding the urine upon rising on the morning of day 4, and collecting all subsequent urine voids until the morning of day 5 when the final void was collected at the exact same time as the discarded first void of the previous morning. The volume of all urine collections was measured and a 10 ml aliquot saved and frozen at -20°C until assayed.

A professional phlebotomist was hired to draw blood from all subjects. The blood was drawn into vacutainer tubes containing EDTA (Becton Dickinson, Rutherford, NJ), centrifuged and the plasma stored at -20°C until assayed.

## Experiment Using Rats as an Animal Model

Twelve Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were purchased and individually housed in stainless steel wire mesh cages for the duration of the study except when urine was collected at which time they were housed in stainless steel metabolic cages. All animals were fed Purina Rat Chow #5001 (Ralston Purina Co., St Louis, MO) ad libitum for one week prior to the beginning of the study.

The twelve animals were randomly divided into four groups of three and assigned to one of four dietary supplement treatments. Dietary supplements were either choline chloride (Mallinckrodt, Paris, KY), or pantothenic acid (Sigma Chemical Co, St Louis, MO), or both. All groups were provided food and water ad libitum. Diets were prepared by grinding rat chow and adding the appropriate supplement. The groups and supplements received were:

| | |
|---|---|
| Control | No Supplement |
| Choline (CH) | CH 21 mmol/kg (2.5g/kg) diet |
| Pantothenic Acid (PA) | PA 0.5 mmol/kg (100 mg/kg) diet |
| CH/PA | CH 21 & PA 0.5 mmol/kg diet. |

The choline supplement was approximately 1.5 times the amount inherent in the chow and the pantothenic acid about two fold greater. There were only trace amounts of carnitine detectable in the chow.

The blood collection procedure was too labor intensive to allow blood to be drawn from all animals on the same day. For this reason the groups were paired and staggered with the control group and the CH/PA groups beginning the diet and samples collected on the same days. Likewise the CH and PA groups were paired.

Animals were kept on the experimental diet for 21 days and blood and urine collected before the study began and at 5 day intervals thereafter. The day of the study was counted form the day the animals began the experimental diets, which was a different day for each group. The schedule was not strictly adhered to because of problems in obtaining

samples, but when a day was missed samples were taken again as soon as possible. Blood was collected by inserting an intravenous catheter (Insyte intravenous catheter placement unit, Deseret Medical, Inc, Sandy UT) in a lateral tail vein. Blood was drawn into a 1.5 ml microcapillary tube containing EGTA (Sigma Chemical Co, St Louis, MO), centrifuged and the plasma frozen at -20°C until assayed. Urine was collected by placing the animals in stainless steel metabolic cages with the urine draining into 250 ml Erlenmeyer flasks containing 5 thymol crystals. After 24 hr the urine was measured, centrifuged and a 10 ml aliquot frozen at -20°C for future assays.

At the end of the experimental period the animals were anesthetized with open drop methoxyflurane and sacrificed by decapitation. Blood was collected by draining from the neck into a 10 ml tube containing EGTA. Blood was centrifuged and the plasma frozen at -20°C until assayed.

## Experiments Using Guinea Pigs as an Animal Model

There were three studies using guinea pigs with the only difference being the blood and urine collection times and methods. In each trial 12 guinea pigs were purchased from SASCO, Inc; Omaha, NE. All animals were individually housed in stainless steel wire mesh cages except when urine was being collected. All animals were provided water and Purina

Purina Guinea Pig Chow #5025 ad libitum for one week prior to the beginning of the study.

In each study twelve animals were divided into four groups of three and assigned to one of the four dietary supplement treatments. All groups were provided food and water ad libitum. Experimental diets were prepared by grinding guinea pig chow and adding the appropriate supplement(s). The groups and supplements received were:

| | |
|---|---|
| Control | No Supplement |
| Choline (CH) | 25 mmol/kg (3mg/kg) diet |
| Pantothenic Acid (PA) | 0.7 mmol/kg (150mg/kg) diet |
| CH/PA | CH 25 & PA 0.7 mmol/kg diet |

The choline supplement was 1.75 times the amount contained in the diet and the pantothenic acid about two times the amount in the diet. There was no detectable amount of carnitine in the guinea pig chow.

In the first guinea pig trial the experimental diets were begun one week after arrival. Blood was collected at the end of the study in the same manner as with the rats. Urine was collected before the experiment began, and on days 6 and 11 of the experimental diets and processed in the same manner as with the rat urine samples. The day 11 urines contained no carnitine after being stored in the refrigerator over night prior to processing. In the next trials urine was immediately centrifuged and frozen and 1 ml 0.1 N HCl was used as a preservative.

In the second trial the experimental diets were begun after one week. Urine was collected before the experimental

diet began and on days 6 and 11, and processed the same as previously except as noted above. Blood samples were collected at the end of the study in the same manner as with the rats.

The third guinea pig trial was conducted exactly as the first except that urine was collected on days 0, 3, 6, and 11. Blood was collected by cardiac puncture on days 3, 6, and 11, centrifuged and the plasma frozen at -20°C until assayed.

Urine was collected for 24 hr in acrylic metabolic cages with thymol crystals added to the urine collection bottle in the first trial, and 1 ml of 0.1 M HCl added in the second and third trials. All urine volumes were recorded, centrifuged and a 10 ml aliquot frozen at -20°C.

## Assays

***Carnitine:***

Carnitine was assayed in all blood and urine samples by a modified radioisotopic method of Cederblad and Linstedt (124,125). In this assay, acid insoluble long-chain acyl carnitines (AIAC) are precipitated using perchloric acid, leaving the short-chain acid soluble acyl carnitines (ASAC) and the non-esterified carnitines (NEC) in the supernatant. An aliquot of the supernatant is assayed to determine the NEC concentration and another hydrolyzed with 0.5 N KOH to

assay all acid soluble carnitines. ASAC is the difference between the total acid soluble carnitines and the NEC. The pellets are drained, washed, suspended in 0.5 N KOH, and hydrolyzed in a hot water bath at 60°C for 60 minutes and assayed for carnitine.

In each case carnitine is assayed by using carnitine acetyl transferase (Sigma Chemical Co., St Louis, MO) to esterify carnitine to a $^{14}$C-acetate from 1,$^{14}$C-acetyl CoA (Moravek Biochemicals, Brea, CA). Radioactivity of samples and standards was determined by a Beckman LS3801 liquid scintillation counter.

***Creatinine:***

Creatinine assays in urine were performed by the Jaffe reaction according to the method of Taussky and Henry (126,127). Plasma creatinine was determined using another modification of the Jaffe reaction by Raffael (128). Creatinine clearance was calculated using the equation: Clearance = (U/P)V where U = urine creatinine concentration, P = plasma creatinine concentration, and V = urine flow in ml per minute.

***ALT and AST:***

In the human study, plasma alanine amino-transferase (ALT) and aspartate amino-transferase (AST) were determined using Sigma kits 59-UV and 58-UV respectively (Sigma Chemical Co, St Louis, MO).

## Statistical Analysis

The human study had only two groups and they were tested for significance using the Student's t test ($P < 0.05$). The rat study had four groups but only two groups were compared directly, therefore the t test was used to test significance in this experiment also ($P < 0.05$).

In the guinea pig studies there were four groups. Differences in the means were determined by analysis of variance (ANOVA) and the Fisher's least significant difference test. Guinea pig data was also expressed as two groups (choline supplemented or not supplemented) and the means compared using a two tailed Student's t test ($p \leq .05$).

# Chapter 4

# Results

## Experiment in Humans

No subjects reported any adverse effects associated with choline supplementation and none reported any illness during the study. Serum ALT and AST remained within the normal range for all subjects which indicated no liver damage due to choline treatment. In choline deficiency these enzymes have been shown to be elevated (64).

Urinary and plasma carnitine and urinary creatinine concentrations are summarized in table 3. Urinary excretion of creatinine was significantly lower in choline supplemented subjects compared to controls (figure 1). Plasma carnitine concentrations were lower in the choline supplemented subjects than in controls, but the difference was not statistically significant (figure 2). Urinary excretion of carnitine in the choline group was one third of the excretion in the control group and the difference was statistically significant (figure 3). When expressed as μmol carnitine/gram creatinine per day, the choline group still excreted less than half the carnitine of the control group. Carnitine clearance was also significantly lower in the choline group than the control group (figure 4).

## Table 3
## Effect of Choline Supplementation on Carnitine and Creatinine in Humans

| Parameter | Control Group | Choline Group |
|---|---|---|
| Plasma Carnitine (μmol/L) | 27.7 ± 3.1 | 23.7 ± 3.1 |
| 24 Hour Urine Carnitine (μmol/day) | 174.6 ± 35.8[a] | 55.6 ± 8.9[b] |
| Renal Clearance of Carnitine (ml/minute) | 4.5 ± 0.9[a] | 1.8 ± 0.4[b] |
| 24 Hour Urine Creatinine (grams/day) | 2.23 ± 0.30[a] | 1.60 ± 0.12[b] |

Values are the group mean ± SEM. Different subscripts indicate statistical significance at $P < 0.05$ by Student's t test (control n = 8, choline n = 6).

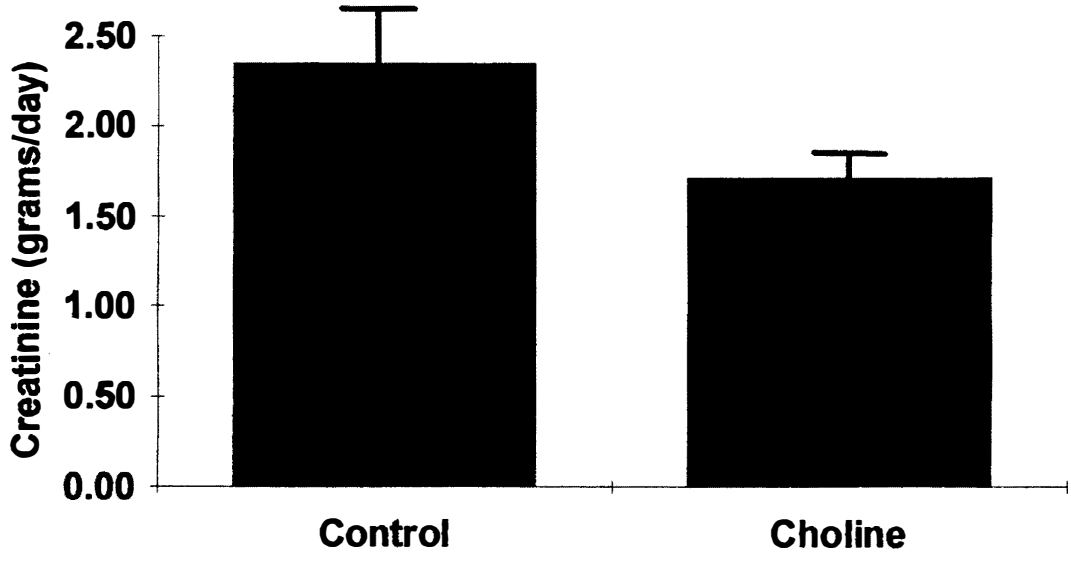



**Figure 1: Effect of choline supplementation on creatinine excretion in humans.**
Creatinine was determined in samples from 24 hr urine collections. Values represent the group mean ± SEM. The means differed statistically at $P < 0.05$ by Student's t test (control n = 8, choline n = 6).

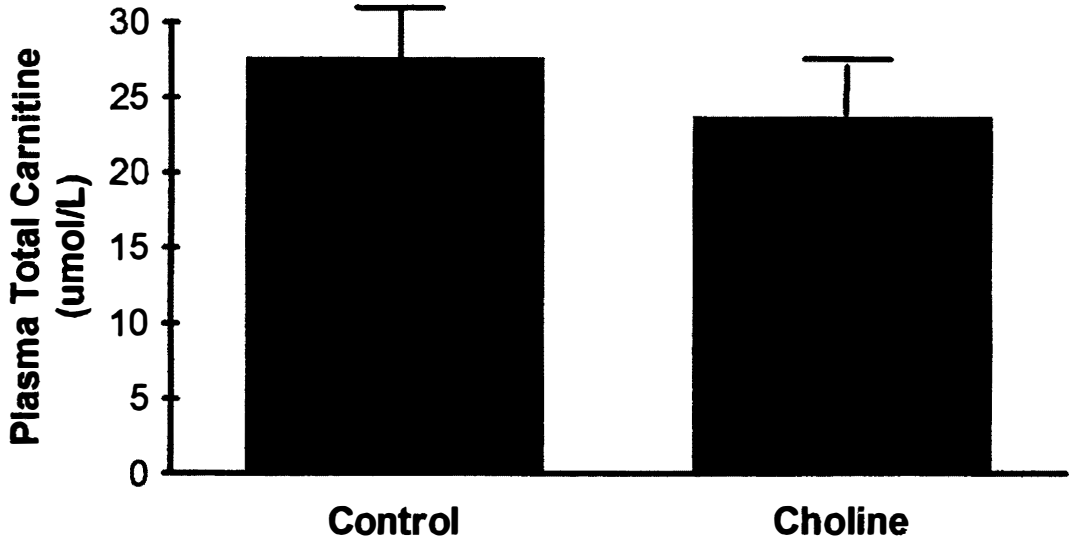



**Figure 2: Effect of choline supplementation on plasma carnitine in humans.**
Values represent the means for the groups ± SEM. The group means did not differ significantly at $P < 0.05$ by Student's t test (control n = 8, choline n = 6).

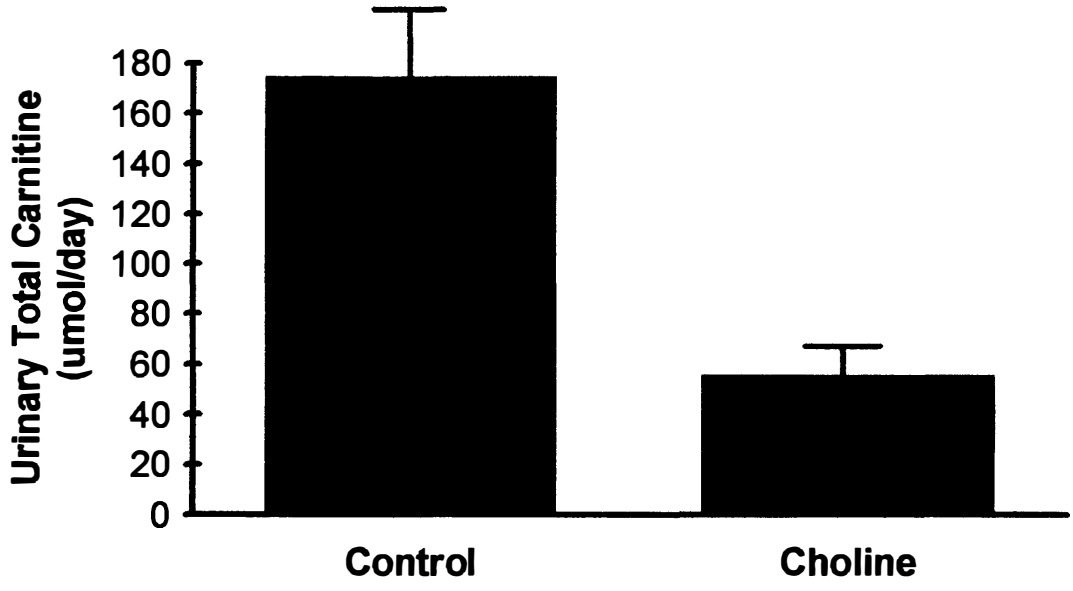



**Figure 3: Effect of choline supplementation on urinary excretion of carnitine in humans.**
Values represent the group means ± SEM. The values differed statistically at $P < 0.01$ by Student's t test (control n = 8, choline n = 6).

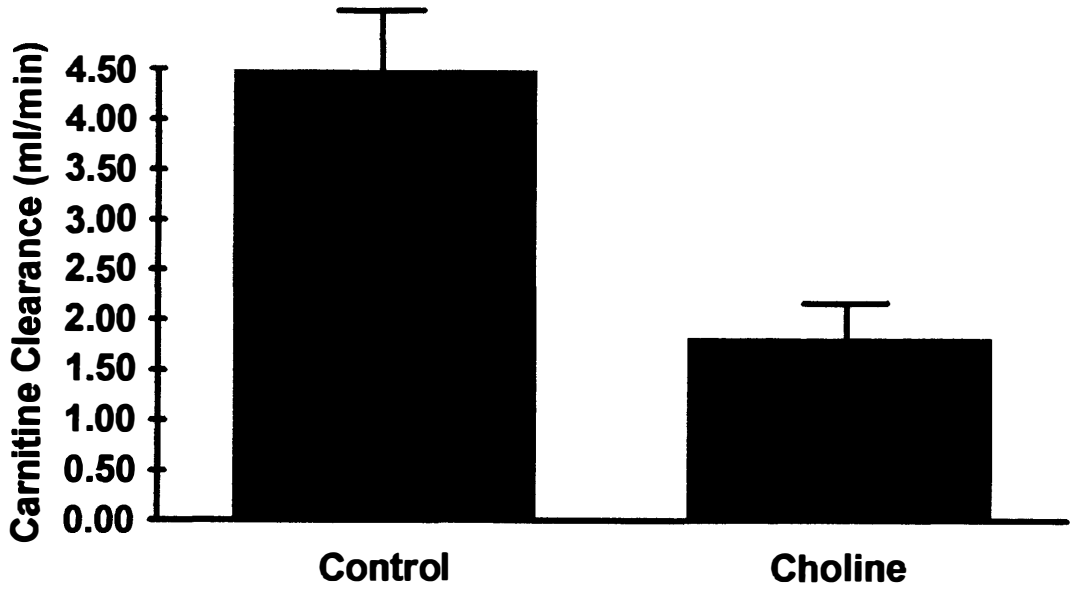



**Figure 4: Effect of choline supplementation on renal clearance of plasma carnitine in humans.**
Values represent the means for the groups ± SEM. For the choline supplemented group n=6, and the control group n=8. Values differed statistically at P = 0.01 using Student's T test.

## Experiment Using Rats as an Animal Model

No overt toxic effects due to choline or pantothenic acid supplementation was observed during the study. All animals ate normally and gained normal weight during the experimental period (47 grams average weight gain).

Plasma concentration of carnitine did not differ significantly for either pair at any day during the experiment (table 4 & figure 5). Urinary excretion of carnitine was significantly lower in the choline supplemented group than for the pantothenic acid group on day 21. There were no other days with statistically different excretions of carnitine for either group (table 5 & figure 6).

## Table 4
## Comparison of Group Means of Plasma Total Carnitine in Rats

| Group | Day of Study | Total Carnitine μmol/L |
|---|---|---|
| Control | 0 | 34.47 |
| Choline | 0 | 31.97 |
| PA | 0 | 35.43 |
| Choline/PA | 0 | 34.13 |
| Choline | 3 | 39.60 |
| PA | 3 | 50.17 |
| Control | 6 | 33.83 |
| Choline/PA | 6 | 34.53 |
| Choline | 7 | 41.40 |
| PA | 7 | 50.33 |
| Control | 9 | 44.40 |
| Choline/PA | 9 | 39.40 |
| Choline | 10 | 44.93 |
| PA | 10 | 51.60 |
| Control | 13 | 49.40 |
| Choline/PA | 13 | 43.40 |
| Choline | 14 | 46.00 |
| PA | 14 | 58.33 |
| Control | 16 | 47.10 |
| Choline/PA | 16 | 44.80 |
| Choline | 17 | 53.43 |
| PA | 17 | 60.40 |
| Control | 20 | 65.83 |
| Choline/PA | 20 | 60.90 |
| Choline | 21 | 53.53 |
| PA | 21 | 52.15 |
| Choline | 22 | 60.47 |
| PA | 22 | 64.33 |

Choline/PA = Choline plus pantothenic acid supplemented group. PA = Pantothenic acid supplemented group
None of the compared means differ significantly by students t-test at $p \leq 0.05$.

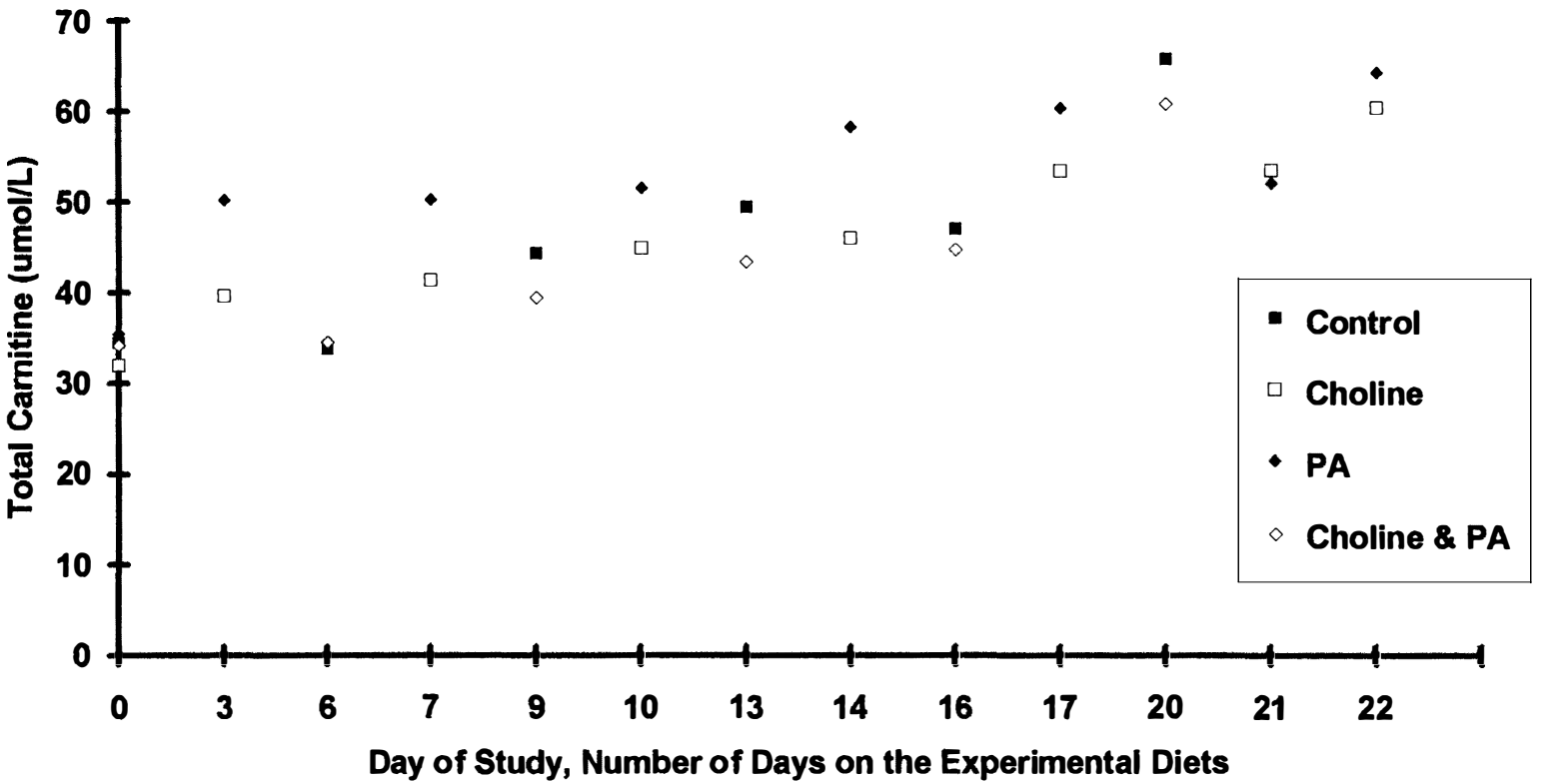



**Figure 5: Effect of choline, pantothenic acid (PA), or both on plasma carnitine in rats.** Each value represents the group mean for that time point. For each group n=3. There were no significant differences between paired groups at any time point at $P < 0.05$ using Student's t test.

## Table 5

### Comparison of Group Means of Urinary Excretion of Total Carnitine in Rats

| Group | Day of Study | Total Carnitine nmol/d |
|---|---|---|
| Choline | 0 | 635 |
| PA | 0 | 777 |
| Choline | 3 | 564 |
| PA | 3 | 682 |
| Control | 6 | 491 |
| Choline/PA | 6 | 437 |
| Choline | 7 | 520 |
| PA | 7 | 835 |
| Control | 8 | 692 |
| Choline/PA | 8 | 735 |
| Choline | 10 | 492 |
| PA | 10 | 852 |
| Control | 12 | 582 |
| Choline/PA | 12 | 689 |
| Choline | 13 | 655 |
| PA | 13 | 1069 |
| Control | 15 | 804 |
| Choline/PA | 15 | 813 |
| Choline | 17 | 885 |
| PA | 17 | 869 |
| Control | 19 | 1080 |
| Choline/PA | 19 | 583 |
| Choline | 21 | 756[a] |
| PA | 21 | 1430[b] |

Choline/PA = Choline plus pantothenic acid supplemented group.PA = Pantothenic acid supplemented group.
Different superscript letters beside values indicates significant difference between the group means at $P \leq 0.05$ by Student's t test.

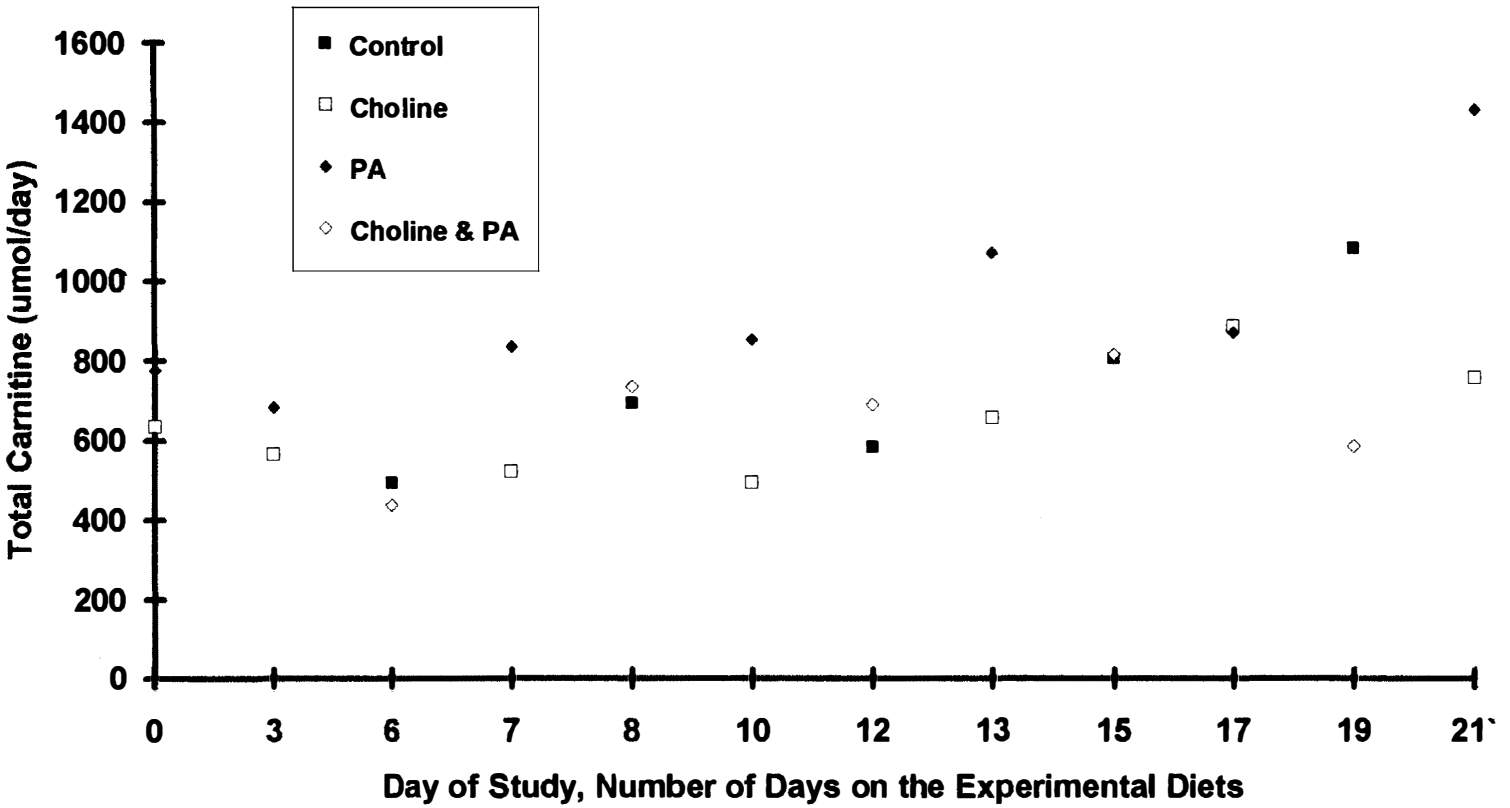



**Figure 6: Effect of choline, pantothenic acid (PA), or both on urinary excretion of carnitine in rats.** Each value represents the group means for that time point. For each group n=3. Only the day 21 difference was statistically significant at $P < 0.05$ using Student's t test.

## Experiments Using Guinea Pigs as an Experimental Model

**First guinea pig study:**

There were no apparent adverse effects of choline or pantothenic acid supplementation. All animals gained weight, average of 45 grams, and ate normally throughout the study period. Urine was collected on the day prior to beginning the experimental diet, and on days 5 and 10 of the of the experimental diet. Because the urine from the final collection was lost, only the pre diet and first post diet urinary excretions were able to be presented. Plasma was collected on day 10 when the animals were sacrificed. Plasma concentrations and urinary excretion of carnitine are shown in table 6 with all values expressed as means for the group ± the standard error of the mean (SEM). Urinary excretion of carnitine is depicted for all groups in figure 7. Figure 8 shows the results when both choline supplemented groups are combined and both groups not supplemented with choline are combined.

Plasma total carnitines were higher in all experimental groups than in the control group. The choline and pantothenic acid groups were not significantly different from each other but the choline/pantothenic acid group was significantly higher than all other groups. The higher value was due primarily to one animal with an exceptionally high acid soluble acyl carnitine (ASAC) concentration.

## Table 6

## Comparison of Group Means of Urinary Excretion and Plasma Concentration of Carnitine in Guinea Pigs (First Study)

| Group | Carnitine μmol/L(plasma) or μmol/d (24 h urine) | | | |
|---|---|---|---|---|
| | NEC | ASAC | AIAC | Total |
| | **Pre Experimental Diet Carnitine Excretion** | | | |
| Control | 0.88 ± 0.07 | 0.85 ± 0.31 | 0.00 ± 0.00 | 1.74 ± 0.39 |
| Choline | 2.04 ± 0.15 | 1.50 ± 0.26 | 0.09 ± 0.09 | 3.63 ± 0.21 |
| PA | 2.11 ± 0.21 | 1.91 ± 0.47 | 0.03 ± 0.00 | 4.05 ± 0.32 |
| CH/PA | 1.43 ± 0.02 | 1.00 ± 0.26 | 0.02 ± 0.00 | 2.45 ± 0.30 |
| | **Post Experimental Diet Carnitine Excretion** | | | |
| Control | 1.59 ± 0.70 | 0.56 ± 0.01 | 0.00 ± 0.00 | 2.71 ± 0.88 |
| Choline | 0.20 ± 0.08 | 0.25 ± 0.04 | 0.02 ± 0.00 | 0.69 ± 0.02 |
| PA | 1.78 ± 0.05 | 1.14 ± 0.32 | 0.00 ± 0.00 | 4.05 ± 0.68 |
| CH/PA | 0.48 ± 0.13 | 0.29 ± 0.04 | 0.00 ± 0.00 | 1.07 ± 0.20 |
| | **Post Experimental Diet Plasma Carnitine** | | | |
| Control | 37.93 ± 2.3 | 4.57 ± 0.3 | 0.80 ± 0.1 | 43.30 ± 2.2 |
| Choline | 45.13 ± 2.0 | 9.57 ± 1.7 | 0.70 ± 0.1 | 55.40 ± 1.5 |
| PA | 38.80 ± 4.1 | 4.10 ± 0.5 | 0.80 ± 0.1 | 43.70 ± 4.0 |
| CH/PA | 47.32 ± 3.4 | 16.90 ± 8.1 | 0.80 ± 0.0 | 63.93 ± 6.2 |

PA = Pantothenic acid supplemented group.
CH/PA = Choline + Pantothenic acid supplemented group.
NEC = non-esterified carnitine (free carnitine), ASAC = acid soluble acyl carnitine (short chain acyl carnitne), AIAC = acid insoluble acyl carnitine (long chain acyl carnitine), Total = total carnitine (NEC + ASAC + AIAC).
Values are the means for the group ± SEM (n = 3).

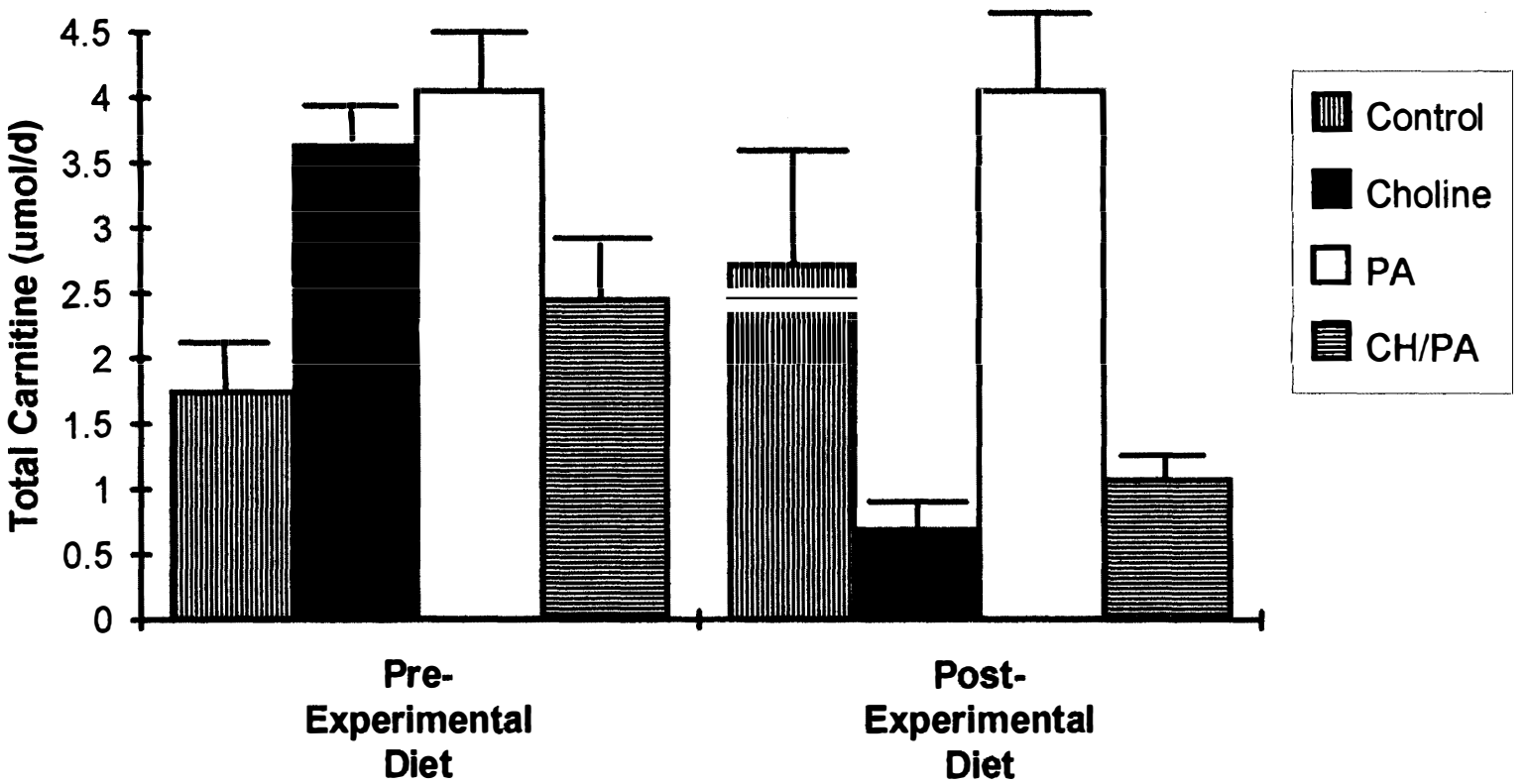



**Figure 7: Effect of choline, pantothenic acid (PA), or both (CH/PA) on urinary excretion of carnitine in guinea pigs.** Each value represents the group means ± SEM. For each group n = 3. Differences were not statistically significant at $p \leq 0.05$ using Fisher's least sigfnificant difference test.

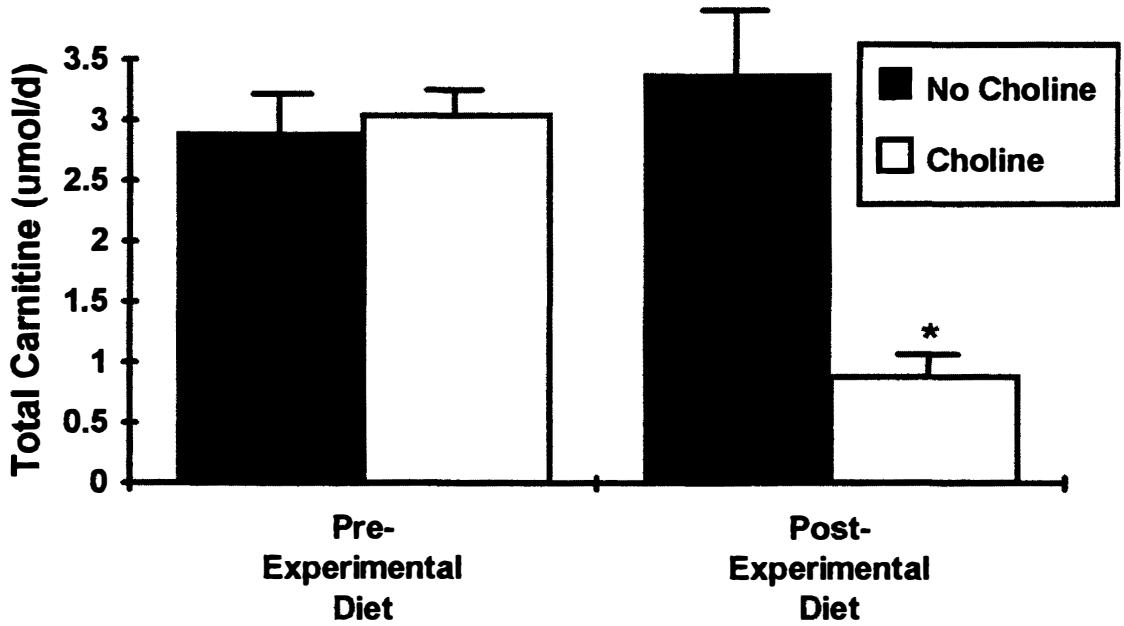



**Figure 8: Effect of choline on urinary excretion of carnitine in guinea pigs.** The pantothenic acid group was combined with the control group and the choline group was combined with the choline + pantothenic acid group. For each of the combined groups n = 6. Values are expressed as the group mean $\pm$ SEM. The asterisk indicates statistical difference from control at $p \leq 0.05$ by Student's t test.

No significant differences between groups were found in any of the carnitine fractions in the 24 hr urine collections prior to the experimental diets. When the results were expressed as two groups (choline and no choline), there was very little difference between groups in the first collection and again was not statistically significant.

In the second urine collection (day 5 of the experimental diet period) there was a clear trend. Urinary excretion of carnitine was lower in both of the choline supplemented groups compared to the control group, the latter being similar to the pantothenic acid supplemented group. Carnitine excretion was increased in the control group and remained the same in the pantothenic acid supplemented group. In spite of the differences, values were not statistically significant among the four groups by Fisher's least significant difference test. When the animals were divided into choline supplemented and non supplemented groups, the choline supplemented group had significantly lower urinary excretion of carnitine ($p \leq 0.01$) by Student's t test.

**Second Guinea Pig Study:**

Throughout the study animals no animals exhibited signs of adverse effects from the supplements; all ate normally, and gained weight with one exception. Animal #35 in the pantothenic acid supplemented group lost weight and ate very little during the last week of the study, indicating that the animal was sick. As a result, the final urine collection and the plasma collection data were eliminated.

Plasma was collected at the end of the study when the animals were sacrificed. Urine was collected on days 0 (pre diet) and on days 6 and 11 of the experimental diet period. Urinary excretion and plasma concentrations of carnitine are expressed in table 7 as the means for the groups. Mean urinary excretion of carnitine is shown for all groups in figure 9, and figure 10 shows the two choline supplemented groups combined and the two groups not supplemented with choline combined.

The choline supplemented group had significantly higher plasma NEC, ASAC, and total carnitine than did the control group. The pantothenic acid supplemented group also had significantly higher ASAC than did the control group. Urinary excretion of carnitine did not differ significantly among the groups prior to the experimental diets when expressed either as individual groups or as pooled groups of choline supplemented or non-supplemented. The urinary excretions of NEC and total carnitine were significantly

## Table 7

## Comparison of Group Means of Urinary Excretion and Plasma Concentration of Carnitine in Guinea Pigs (Second Study)

| Group | Carnitine μmol/L (plasma) or μmol/d (24 h urine) | | | |
|---|---|---|---|---|
| | NEC | ASAC | AIAC | Total |
| **Day 11 of Experimental Diet, Plasma Carnitine** | | | | |
| Control | $35.5^{a} \pm 2.2$ | $12.4^{a} \pm 5.0$ | $2.0^{a} \pm 0.4$ | $49.8^{a} \pm 7.2$ |
| Choline | $52.0^{b} \pm 3.1$ | $48.3^{b} \pm 5.5$ | $1.5^{ab} \pm 0.3$ | $101.7^{bc} \pm 7.9$ |
| PA | $37.5^{a} \pm 1.0$ | $41.9^{b} \pm 8.8$ | $0.6^{b} \pm 0.2$ | $79.8^{ab} \pm 9.5$ |
| CH/PA | $42.0^{ab} \pm 2.1$ | $36.1^{ab} \pm 3.8$ | $0.7^{ab} \pm 0.3$ | $78.7^{ac} \pm 5.3$ |
| **Day O (Pre-Experimental Diet), Carnitine Excretion** | | | | |
| Control | $0.9 \pm 0.2$ | $0.3 \pm 0.1$ | $0.1 \pm 0.0$ | $1.3 \pm 0.1$ |
| Choline | $1.1 \pm 0.2$ | $0.2 \pm 0.0$ | $0.2 \pm 0.1$ | $1.5 \pm 0.3$ |
| PA | $1.3 \pm 0.1$ | $0.5 \pm 0.1$ | $0.1 \pm 0.0$ | $1.8 \pm 0.2$ |
| CH/PA | $1.2 \pm 0.3$ | $0.6 \pm 0.3$ | $0.1 \pm 0.1$ | $1.9 \pm 0.6$ |
| **Day 6 of Experimental Diet, Carnitine Excretion** | | | | |
| Control | $1.5^{a} + 0.2$ | $0.5 + 0.2$ | $0.1 \pm 0.0$ | $2.0^{a} \pm 0.2$ |
| Choline | $0.3^{b} \pm 0.1$ | $0.1 \pm 0.0$ | $0.0 \pm 0.0$ | $0.5^{b} \pm 0.2$ |
| PA | $1.1^{ab} \pm 0.4$ | $0.4 \pm 0.1$ | $0.0 \pm 0.0$ | $1.5^{ab} \pm 0.4$ |
| CH/PA | $0.5^{b} \pm 0.2$ | $0.4 \pm 0.1$ | $0.1 \pm 0.0$ | $0.9^{ab} \pm 0.3$ |
| **Day 11 of Experimental Diet, Carnitine Excretion** | | | | |
| Control | $1.7^{a} \pm 0.3$ | $1.0 \pm 0.4$ | $0.0 \pm 0.0$ | $2.7 \pm 0.6$ |
| Choline | $0.6^{b} \pm 0.2$ | $0.5 \pm 0.2$ | $0.0 \pm 0.0$ | $1.1^{b} \pm 0.5$ |
| PA | $1.2^{ab} \pm 0.4$ | $0.5 \pm 0.2$ | $0.0 \pm 0.0$ | $1.7^{ab} \pm 0.5$ |
| CH/PA | $0.5^{b} \pm 0.0$ | $0.6 \pm 0.0$ | $0.1 \pm 0.1$ | $1.1^{b} \pm 0.1$ |

PA = Pantothenic acid supplemented group.
CH/PA = Choline + pantothenic acid supplemented group. NEC = non-esterified carnitine (free carnitine), ASAC = acid soluble acyl carnitine (short chain acyl carnitines), AIAC = acid insoluble acyl carnitines (long chain acyl carnitines), and Total = total carnitines (NEC + ASAC + AIAC).
Values are the means for the group ± SEM (n = 3).
Different superscripts indicate statitically significant differences by Fisher's least significant difference test ($P < 0.5$).

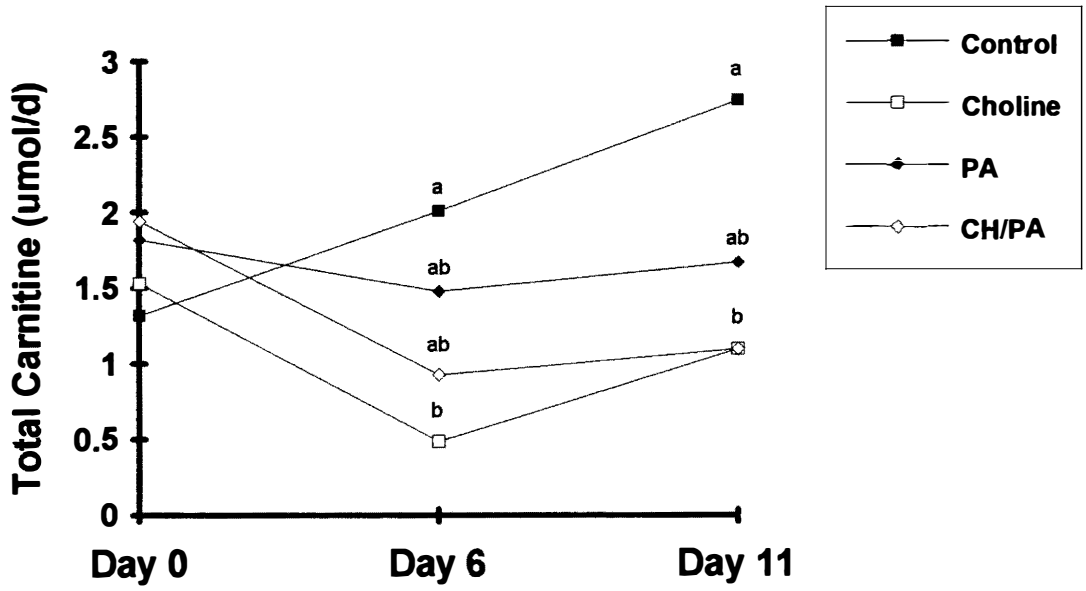



**Figure 9: Effect of choline, pantothenic acid (PA), or both (CH/PA) on urinary excretion of carnitine in guinea pigs (Second Study).** Each value represents the group means ± SEM. For each group n = 3. Different superscripts indicate statistical significance ( $P \leq 0.05$) by Fisher's least signifcant difference test.

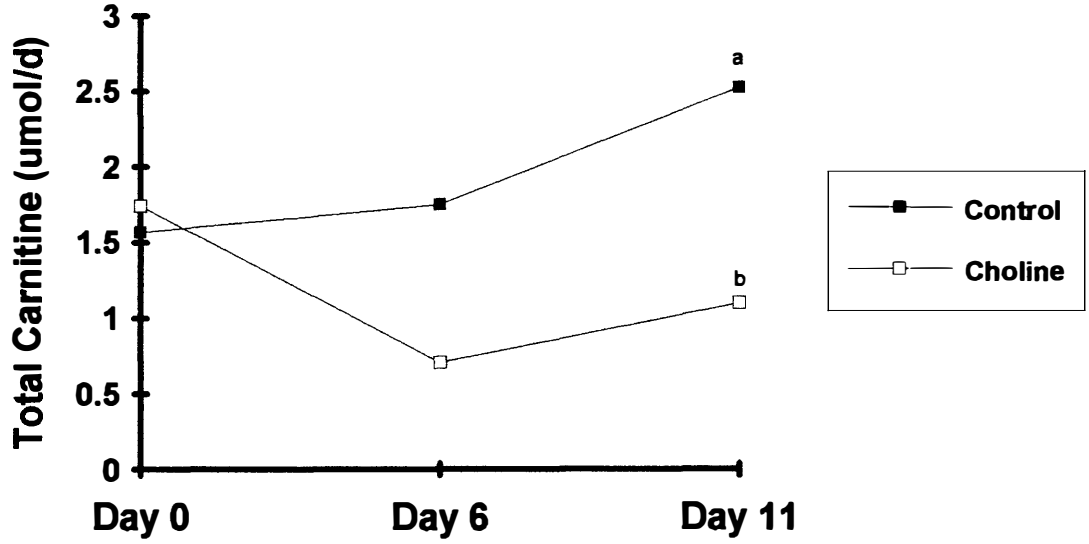



**Figure 10: Effect of choline on urinary excretion of carnitine in guinea pigs (Second Study).** The pantothenic acid supplemented group in this study was combined with the control group and both choline supplemented groups were combined. For each of the combined groups n = 6 except at day 11 when n = 5 for the control group. Values are the means for the combined group. Different superscripts indicate statistically different means at that time point ($P \leq 0.05$) by Fisher's least significant difference test.

lower for the choline supplemented group than control on day 6. When expressed as choline supplemented or non-supplemented pooled groups there was no significant difference between groups on day 6. Day 11 NEC and total carnitines, however, were significantly lower for the choline supplemented group and the CH/PA groups than for the control group, but not the pantothenic acid group. When expressed as pooled choline supplemented or non-supplemented groups, total carnitine excretion was significantly lower ($P < 0.01$) by Student's t test on day 11.

**Third guinea pig study:**

There were no apparent adverse effects dietary treatments during this study. All animals ate normally and gained weight (average 68 grams). Blood was collected on days 3 and 5 in addition to the end of the study. Plasma carnitine concentrations are shown in table 8 and urinary excretion of carnitine in table 9. Figure 11 shows the urinary excretion of carnitine by groups and figure 12 shows the pooled groups with both choline supplemented groups and both non-supplemented groups combined.

Plasma carnitine concentrations did not differ between groups significantly during the study. Urinary carnitine excretion was consistently lower for the choline supplemented animals than non supplemented. When the data was divided into the four groups, the differences were statistically significant only on day 11, the final collection. The choline group had significantly lower NEC and total carnitine excretion than the control group but was not significantly different from the groups supplemented with pantothenic acid or pantothenic acid and choline.

When carnitine excretion data was divided into choline supplemented or not supplemented, there was no significant differences on days 0 and day 11, although day 11 was almost significant ($P \leq 0.07$). Excretion of total carnitine was significantly lower on days 3 and 6 for choline supplemented animals (figure 12).

## Table 8
## Effect of Supplementation With Choline, Pantothenic Acid or Both on Plasma Concentrations of Carnitine in Guinea Pigs (Third Study)

| Group | Carnitine μmol/L | | | |
|---|---|---|---|---|
| | NEC | ASAC | AIAC | Total |
| | | Day 3 of Experimental Diet | | |
| Control | 16.1 ± 2.1 | 2.9 ± 0.1 | 1.3 ± 0.4 | 20.3 ± 3.6 |
| Choline | 18.2 ± 0.9 | 0.8 ± 0.0 | 0.4 ± 0.2 | 19.4 ± 0.4 |
| PA | 19.5 ± 2.0 | 4.9 ± 0.9 | 0.8 ± 0.1 | 25.2 ± 1.3 |
| CH/PA | 21.1 ± 0.4 | 8.5 ± 1.6 | 0.8 ± 0.4 | 29.6 ± 3.0 |
| | | Day 6 of Experimental Diet | | |
| Control | 17.8 ± 1.5 | 8.5 ± 1.6 | 0.5 ± 0.2 | 26.7 ± 0.2 |
| Choline | 18.6 ± 0.9 | 4.8 ± 0.2 | 0.4 ± 0.5 | 23.7 ± 1.7 |
| PA | 19.1 ± 1.6 | 3.3 ± 0.5 | 0.5 ± 0.3 | 23.0 ± 1.0 |
| CH/PA | 20.7 ± 0.3 | 5.7 ± 0.9 | 0.8 ± 0.1 | 27.2 ± 0.9 |
| | | Day 11 of Experimental Diet | | |
| Control | 18.6 ± 0.7 | 4.2 ± 0.8 | 0.6 ± 0.2 | 23.5 ± 1.3 |
| Choline | 20.7 ± 2.2 | 3.8 ± 0.7 | 0.6 ± 0.1 | 25.1 ± 1.8 |
| PA | 22.0 ± 7.5 | 6.0 ± 2.4 | 0.7 ± 0.1 | 28.7 ± 2.3 |
| CH/PA | 22.4 ± 2.5 | 4.8 ± 1.2 | 0.8 ± 0.2 | 28.0 ± 3.5 |

PA = patntothenic acid supplemented group. CH/PA = choline and pantothenic acid supplemented group. NEC = non-esterified carnitine (free carnitine), ASAC = acid soluble acyl carnitine (short chain acyl carnitines), AIAC = acid insoluble acyl carnitine (long chain acyl carnitine), and Total = total carnitine (NEC + ASAC + AIAC).Values are means for the group ± SEM. Means for the groups did not vary significantly at any time point.

## Table 9

### Effect of Supplementation with Choline, Pantothenic Acid or Both on Urinary Excretion of Carnitine in Guinea Pigs (Third study)

| Group | Carnitine μmol/Day | | | |
|---|---|---|---|---|
| | NEC | ASAC | AIAC | Total |
| | Day 0 of Experimental Diet, Pre Experimental Diet | | | |
| Control | 0.8 ± 0.2 | 0.4 ± 0.1 | 0.1 | 1.3 ± 0.2 |
| Choline | 0.6 ± 0.1 | 0.3 ± 0.1 | 0.3 | 0.9 ± 0.2 |
| PA | 0.8 ± 0.2 | 0.3 ± 0.1 | 0.1 | 1.2 ± 0.2 |
| CH/PA | 0.8 ± 0.1 | 0.5 ± 0.1 | 0.1 | 1.4 ± 0.3 |
| | Day 3 of Experimental Diet | | | |
| Control | 0.7 ± 0.3 | 0.4 ± 0.1 | 0.1 | 1.1 ± 0.4 |
| Choline | 0.2 ± 0.1 | 0.6 ± 0.1 | 0.1 | 0.9 ± 0.0 |
| PA | 0.9 ± 0.2 | 0.5 ± 0.1 | 0.1 | 1.3 ± 0.2 |
| CH/PA | 0.3 ± 0.0 | 0.7 ± 0.1 | 0.1 | 1.0 ± 0.1 |
| | Day 6 of Experimental Diet | | | |
| Control | 0.5 ± 0.1 | 0.5 ± 0.1 | ND | 1.0 ± 0.1 |
| Choline | 0.4 ± 0.1 | 0.5 ± 0.0 | ND | 0.9 ± 0.0 |
| PA | 1.7 ± 0.2 | 1.0 ± 0.1 | ND | 1.7 ± 0.2 |
| CH/PA | 0.4 ± 0.0 | 0.5 ± 0.0 | ND | 0.9 ± 0.0 |
| | Day 11 of Experimental Diet | | | |
| Control | $1.6^{a}$ ± 0.5 | 0.9 ± 0.2 | ND | $2.5^{a}$ ± 0.6 |
| Choline | $0.7^{b}$ ± 0.1 | 0.6 ± 0.1 | ND | $0.9^{b}$ ± 0.1 |
| PA | $1.0^{ab}$ ± 0.1 | 0.7 ± 0.1 | ND | $1.7^{ab}$ ± 0.3 |
| CH/PA | $0.6^{a}$ ± 0.2 | 1.0 ± 0.4 | ND | $1.7^{ab}$ ± 0.6 |

PA = pantothenic acid supplemented group. CH/PA = choline and pantothenic acid group. NEC = non-esterified carnitine (free carnitine), ASAC = acid soluble acyl carnitine (short chain acyl carnitine), AIAC = acid insoluble acyl carnitine (long chain acyl carnitine), Total = total carnitine (NEC + ASAC + AIAC), ND = non detectable. Values are means for the group ± SEM (n = 3). Different superscripts indicate statistical significance by Fisher's least significant difference test ($P \leq 0.05$).

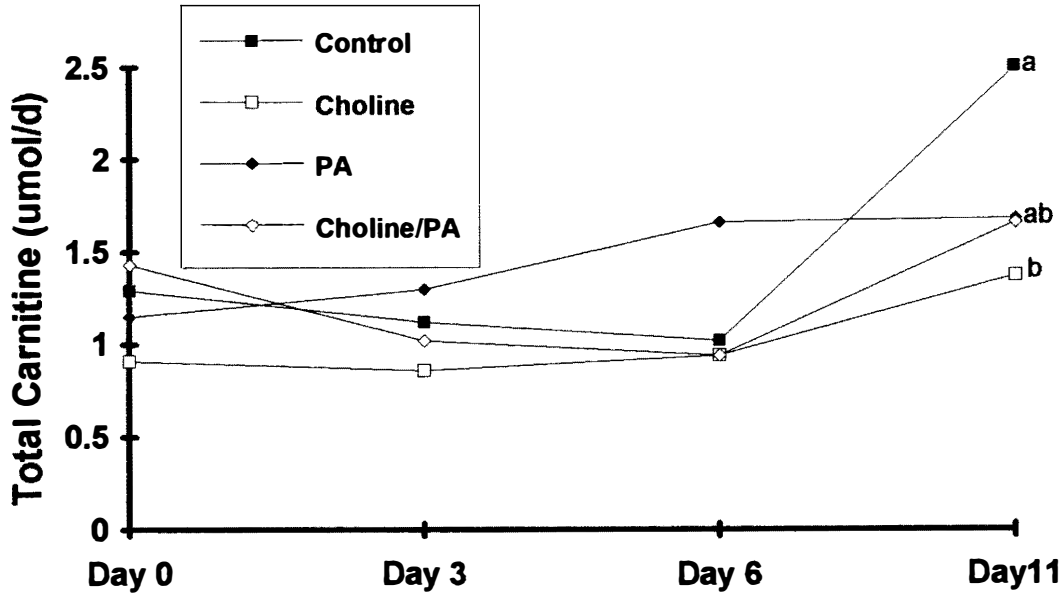



Figure 11: Effect of choline, pantothenic acid (PA), or both (CH/PA) on urinary excretion of carnitine in guinea pigs (third study). Each value represents the group means (n = 3). Different superscripts indicate statistical significance by Fisher's least significant difference test ($P \leq 0.05$).

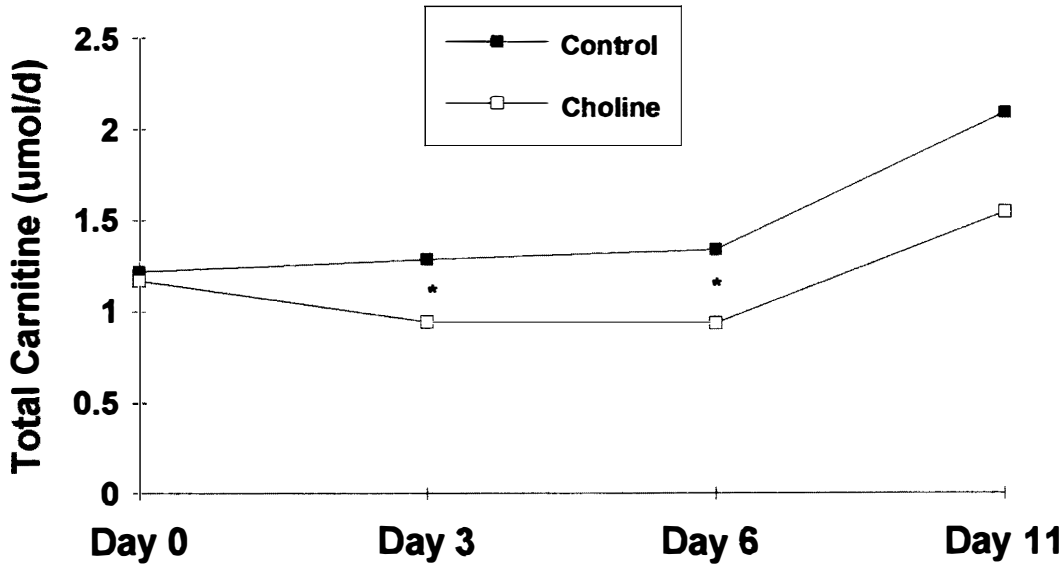



**Figure 12: Effect of choline on urinary excretion of carnitine in guinea pigs (third study).** Each value represents the mean for the group (n = 6). Asterisks indicates statistical significance by Student's t test ($P \leq 0.05$).

**Combined Urinary Excretion Data From Guinea Pig:**

Figure 13 shows the results when the urinary excretion data are pooled from all three guinea pig trials and expressed as choline supplemented or control. Days 5 or 6 are combined from the trials as are days 10 or 11 and expressed as 5 and 10 respectively. This way of calculating data results in 18 animal per group on days 0 and 5. Because there was no data on day 10 of the first trial and one sample is missing from day 11 of the third trial, there are 11 animals per group on day 10.

There were no significant differences in carnitine excretion on day 0 of the study (pre-experimental diet). On days 5 and 10, however, carnitine excretion was much less for the choline supplemented animals and the difference significant ($P < 0.01$).

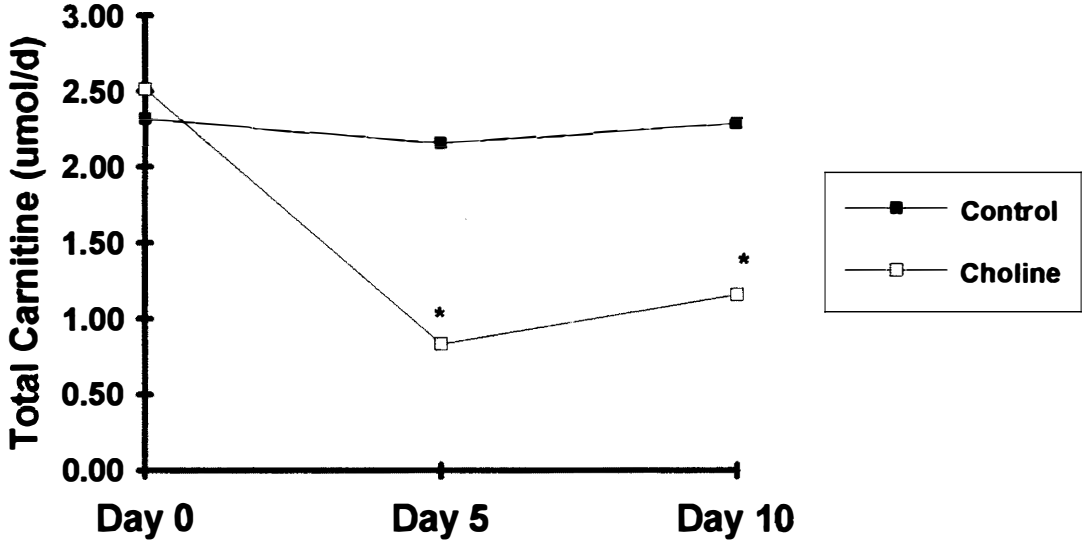



**Figure 13: Effect of choline supplementation on urinary excretion of carnitine in guinea pigs (pooled data).** Each value represents the pooled mean for the group from three trials. For days 0 and 5 n = 18, for day 10 n = 11. The difference on day 0 was not statistically significant. The differences on days 5 and 10,as indicated by asterisks, were statistically significant by Student's t test ($P < 0.01$).

# Chapter 5

# Discussion

**Human Study:**

The data presented here confirmed the results of our earlier experiments in humans (122,123). This study complimented earlier studies by looking at a younger population and by using a separate group as control (in previous studies each subject served as their own control by using pre-supplement values for control). As in the other studies, choline supplementation resulted in a statistically significant decrease in urinary excretion of carnitine. Plasma carnitine was also significantly decreased in the choline supplemented subjects in previous studies, but the decrease was not statistically significant in this study, possibly due to fewer subjects.

The decreased urinary excretion of creatinine was unexpected since it was not seen in our previous studies. We do not believe that the effect on creatinine excretion reflects impaired renal function that could explain decreased carnitine because it was much less than the decrease in carnitine excretion (28% and 68% respectively).

ALT and AST remained in the normal range for both

groups during the study (ALT 20.5 units/ml for choline group and 15.5 for controls; AST 25.5 units/ml for choline group and 16.6 for controls), indicating a lack of hepatotoxic effects for choline in the doses used in this study. Choline deficiency has been reported to cause liver abnormalities as evidenced by increased plasma ALT and AST activity (2). This has not been reported in choline supplementation, but neither could the possibility be ruled out prior to this study.

This study left many questions unanswered that can not be adequately addressed in experiments on humans subjects. For example, what are the physiological implications of the reduced plasma and/or urinary excretion of carnitine. Are the changes in carnitine homeostasis seen in these studies reflected in tissue concentrations of carnitine? What is responsible for the changes seen? Since urinary excretion of carnitine is reduced, and plasma concentrations of carnitine are either decreased or remain the same, either absorption of dietary carnitine is impaired, carnitine biosynthesis decreased, or carnitine is shifted to a different body pool and carnitine conserved as evidenced by the decreased urinary excretion.

Since many of these issues can not be adequately addressed in humans, an animal model was needed to investigate the mechanisms and consequences of the effects of choline and/or pantothenic acid on carnitine homeostasis.

## Development of an Animal Model

**Rats as an animal model:**

The laboratory rat was a natural choice as an animal model because it is the most commonly used animal in studies of carnitine, choline and pantothenic acid. It also has the advantage of being relatively inexpensive, easy to draw blood from, routinely maintained in our facility, and the animals that were previously used to study the effects of choline and carnitine on alcohol metabolism in this laboratory (81, 96, 97, 98, 98, 99).

While our study was in progress another investigator reported supplementing rats with only choline for 21 days after which no differences were found in plasma or liver concentrations of carnitine or in urinary excretion of carnitine (129). That study lasted for a longer time and samples taken only at the end of the study, therefore, if the effect of choline on plasma and urine is not permanent, it may have been missed. This study also did not supplement with pantothenic acid so its possible involvement was not determined in rats. We still had not determined if the effect was due to choline or pantothenic acid.

The results of the study in rats was disappointing. No significant differences could be seen in urinary excretion

or plasma carnitine during the course of the study except on the last day of the study. On that day we observed a significant difference between the pantothenic acid group and the choline group. It was difficult to tell if that was a real difference, however. Although statistically significant, at the previous sampling time (day 15) both groups had nearly the exact same carnitine excretion. The best that could be said is that there was a faint trend seen, choline supplemented rats usually had slightly lowered carnitine excretion.

It was obvious that a better animal model was needed to explain the observed effect of choline and pantothenic acid supplementation on carnitine status in humans. Previous studies of choline metabolism in rats have usually investigated the effects of choline deficiency. The high choline oxidase activity of rat liver probably makes it easy to induce choline deficiency by feeding choline deficient diets. Rats fed a choline free diet for only two days will have liver free carnitine reduced by 50% (130). The decreased liver free choline apparently had functional consequences as well since liver triglyceride was reduced four fold in the choline deficient animals. We were attempting to see the effect of choline supplementation rather than depletion and using similar dosages per kg body weight to that used in the human studies. It seems likely that using much higher dosages of choline could overcome the

effect of high choline oxidase activity in rats, but we felt that an animal model that responds similarly to humans at the same dosages of choline and pantothenic acid would provide a more suitable model.

The animal with the nearest reported choline oxidase activity to humans is the guinea pig. The guinea pig has choline oxidase activity about 3 times that of humans but the rat has about 60 times the activity. It was logical, therefore, that if the choline was responsible for the effect seen in humans, then guinea pigs should be a potential model for further investigation.

**Experiments using guinea pigs as an animal model:**

The first two guinea pig studies had blood taken only at the end of the study instead of throughout as in the rats. During the third and final guinea pig study blood was drawn by cardiac puncture throughout the study.

Three questions were addressed in the three guinea pig trials. The first trial clearly showed that as in humans, choline but not pantothenic acid supplementation causes decreased urinary excretion of carnitine. The second study provided a time course to look at the effect throughout the study. The third study addressed the question of plasma carnitine. The decreased plasma carnitine concentrations seen in humans were not seen in guinea pigs, and this was confirmed at three, six, and 11 days of supplementation in the third study.

The urinary excretion of carnitine in guinea pigs was more consistent with the observations in humans. In all three studies there was reduced excretion of carnitine in the choline supplemented animals. The differences were greatest in the first two studies, and statistically significant. Pantothenic acid did not affect urinary carnitine, but rather minimized the effect of choline in the groups supplemented with both choline and pantothenic acid.

The results of the urinary excretion of carnitine in the third study were not quite as clear as the first two studies. The same excretion trends were seen but to a much lesser degree and with statistically significant difference being obtained only on the last day, at which time the choline supplemented group had a significantly reduced urinary excretion of carnitine compared with the control group but no other groups. When the data was viewed as choline supplemented and non-supplemented groups, the differences were greater and statistically significant on days 3 and 5. The statistics were made more difficult throughout the study because of rather high variation within groups and only three animals per group.

The reason for smaller differences in total carnitine excretion between groups in the third study was due to higher excretion of ASAC in the choline supplemented animals. Excretion of NEC was reduced similarly to that seen in the first two studies but the ASAC excretion increased

in the third study, resulting in a much smaller change in total carnitine as opposed to the first two guinea pig studies.

Two primary conclusions were drawn from these studies. First, the guinea pig is a better animal model than the rat for studying choline/carnitine interactions in humans. Secondly, choline and not pantothenic acid supplementation is responsible for the decreased excretion of carnitine seen in humans and guinea pigs.

## Other Observations From The Studies

**Effect of age on carnitine excretion:**

In all of the animal studies young growing animals were used. Because of the rapid growth of young rodents it should be expected that age related changes in biological parameters, including carnitine, could be seen in a few weeks time. Rats reach sexual maturity at about 100 days of age and guinea pigs at 100-150 days (132).

In the rat and guinea pig studies there was a clear upward trend in urinary excretion of carnitine. The same upward trend in plasma carnitine concentrations was seen in rats. Since blood was collected only at the end of the first two guinea pig studies, it was not possible to look for a similar trend in those studies. The third guinea pig study was unlike the first two in this regard. Urinary excretion

of carnitine declined for the second and third urine collections and increased back to a concentration closer to the pre diet collection at the end of the study. Plasma concentrations remained rather constant throughout the study. It should be remembered that the guinea pig study lasted for about half the time of the rat study so age changes could be more important in the rats. Future studies should evaluate the effect of age on carnitine concentration in laboratory animals.

**Urinary Excretion versus plasma concentration of carnitine:**

Plasma concentration of carnitine appeared to be a less sensitive indicator of changes in carnitine status than did the urinary excretion in all of these studies. Other studies in humans also demonstrated a greater reduction in urinary excretion of carnitine than in plasma concentrations of carnitine (133). This same trend is apparent in data published by other laboratories as well. Table 10 shows plasma concentrations and urinary excretion of carnitine in humans who eat meat containing diets (high carnitine), lactoovovegetarian diets (medium carnitine), and strict vegetarian diets (low carnitine) (67).

## Table 10

### Plasma and Urinary Carnitine in Human Males With Diets of Varying Carnitine Content

| Carnitine Content of Diet | Plasma Total Carnitine | Urinary Excretion of Carnitine |
|---|---|---|
| | μmol/L | μmol/kg/d |
| High | 49.4 ± 7.3 | 5.79 ± 3.08 |
| Medium | 45.9 ± 8.2 | 2.10 ± 0.76 |
| Low | 46.7 ± 8.1 | 1.36 ± 0.49 |

Taken from reference 67 by Lombard et al.

In table 10 there was no significant effect of diet on plasma carnitine but urinary excretion of carnitine in the subjects with low carnitine diets was 18% that of those with a high carnitine diet. No data is yet available to correlate plasma and urinary carnitine data with tissue content of carnitine. Both our data that of others shown in table 10 strongly suggest that plasma carnitine is an inadequate index of carnitine status and should be combined with urinary excretion data.

**Changes in fatty acid oxidation:**

Carnitine performs an obligatory role in fatty acid oxidation, therefore it is appropriate to question if factors affecting carnitine metabolism are also affecting fatty acid oxidation. The third guinea pig study was particularly interesting because of the simultaneous increased acyl carnitines and decreased NEC. It has been

shown previously that increased acyl carnitines and increased ratios of acyl to free carnitines are positively correlated with plasma ß-hydroxybutyrate concentrations and therefore oxidation of fatty acids in rats (134). Table 11 shows the relationship of acyl carnitines to ß-hydroxybutyrate in that study. The acyl carnitine concentrations were highly correlated to ß-hydroxybutyrate concentrations (r = 0.99) as were the ratio of acyl to free carnitine (r = 0.96).

The increases in acyl carnitines seen in the third group of guinea pigs deserves further study because it may indicate that choline caused an increase in fatty acid oxidation that resulted in an increased need for carnitine. If this were the case, the decreased excretion of carnitine may indicate a conservation of carnitine.

## Table 11

### Effect of Diet on ß-hydroxybutyrate and Carnitine in Rats

| Diet | ß-hydroxybutyrate (mM) | Free Carnitine (μM) | Acyl Carnitine (μM) |
|---|---|---|---|
| High Carb. | 0.043 ± 0.007 | 39.2 ± 2.2 | 12.3 ± 1.0 |
| High LCT | 0.838 ± 0.73 | 20.0 ± 1.9 | 14.3 ± 1.3 |
| High MCT | 5.543 ± 1.13 | 19.4 ± 1.1 | 28.1 ± 1.6 |

Taken from Seccombe et al, reference 134.
High Carb. = high carbohydrate diet, High LCT = high long chain triglyceride diet, High MCT = high medium chain triglyceride diet.

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# Vita

James William Daily III was born on July 10, 1948. He graduated from Mount Pisgah Academy in 1966. In 1970 he graduated form Southern Missionary College with a Bachelor of Arts in History. After graduation he worked at various jobs including managing his father's stereo speaker factory, as a mountain climbing instructor, and real estate agent. During this time he was married to Ruth E. Gust and later had three children, Anne, Jackie, and Jimmy.

In 1980 he opened a health food store, Eden Way Natural Foods, which he owned and operated until 1991. While operating his store he began taking classes for self-improvement at the University of Tennessee and later enrolled as a graduate student.