5-2002

Carnitine, Choline and Caffeine Promote Fat Loss and Metabolism in Rats and Humans

Nobuko Hongu
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Nobuko Hongu entitled "Carnitine, Choline and Caffeine Promote Fat Loss and Metabolism in Rats and Humans." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

DiLeep S. Sachan, Major Professor

We have read this dissertation and recommend its acceptance:

Edward T. Howley, Naima Moustaid Moussa, Jean D. Skinner

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

To the Graduate Council:

I am submitting herewith a dissertation written by Nobuko Hongu entitled "Carnitine, Choline and Caffeine Promote Fat Loss and Metabolism in Rats and Humans." I have examined the final paper copy of this dissertation for form and content and recommend that it be accepted in partial fulfilment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

Dileep S. Sachan, D.V.M., Ph.D., Major Professor

We have read this dissertation
and recommend its acceptance:

Edward T. Howley, Ph.D.

Naïma Moustaïd Moussa, Ph.D.

Jean D. Skinner, Ph.D., R.D.

Accepted for the Council:

Vice Provost and Dean of
Graduate Studies

Carnitine, Choline and Caffeine Promote Fat Loss and Metabolism in Rats and Humans

A Dissertation
Presented for the Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Nobuko Hongu
May 2002

DEDICATION

This dissertation is dedicated to my parents

Mr. Teruhiko Kikugawa and Mrs. Yoshiko Kikugawa,

for ensuring the quality of my early education;

and my husband

Dr. Yuji Hongu,

for his encouragement, patience, and support throughout this study.

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to all those who contributed to the success of this study. First and foremost, I must mention my committee members. Dr. Dileep S. Sachan, my major professor, guided me throughout my graduate career and provided numerous opportunities for practical training and financial support. He introduced me to the importance of nutrient-nutrient interaction. Through his class, he has taught me the value of nutrition under normal and disease conditions. It was a privilege to work under the direction of such a talented scientist.

Dr. Naïma Moustaïd Moussa showed great interest in our research and spend many hours discussing the importance of fat metabolism, always asking questions that forced me to go back and reevaluate our perspective. Dr. Jean D. Skinner provided valuable insights regarding the meaning of nutrient anlayses. Dr. Edward T. Howley, for his knowledge of exercise gave me direction in my research. Their professionalism and commitment to a high quality of research have been an inspiration for me to keep searching for truth in my study.

I owe many thanks to the faculty of the Nutrition Department: Dr. James W. Bailey, Dr. Betty Ruth Carruth, Dr. Betsy Haughton, Dr. Jay Whelan and Dr. Michael B. Zemel. They managed to keep graduate school exciting and challenging through many years. Additionally, I owe many thanks to the members of the Sachan's laboratory, Dr. James W. Daily III, Dr. Mei-Shin Mong, and Dr. Ayub M. Yatim who taught me how to run carnitine assays and their own skills and techniques in the lab. I am extremely grateful for the time we spent together and their friendship.

I am also grateful to the former professors and the special friends, Dr. Ralph O. Blackwood, Dr. Nancy Paisley, Dr. Tuyoshi Shimizu, Mrs. Julia A. Eckard, Ms. Karen Sprang and Ms. Denise Serrano, whose long-standing beliefs in my ability to complete the study and produce a dissertation. They made this study possible by their friendship and emotional support.

I can not forget to thank all men and women who came to participate our study. Their efforts to follow the experimental procedures, which were tedious and time consuming, were greatly appreciated. Without their help the project could not have accomplished.

Finally, I am grateful to my parents Teruhiko Kikugawa and Yoshiko Kikugawa, and my sister Yasuyo Hayashi, for their unwavering support through the years. My husband, Yuji Hongu, who is a great chemist taught me basic organic chemistry day and night without any hesitation, which greatly helped build my foundation of chemistry. He took many responsibilities so that I could focus my time and effort on the completion of this dissertation. His encouragement kept me going even when things appeared bleak and slow.

This dissertation would not have been possible without assistance of those many individuals. With all of my heart I thank them.

ABSTRACT

Interaction of two nutrients, carnitine and choline, has been reported. Choline supplementation causes a significant conservation of carnitine in normal healthy humans and guinea pigs. The choline supplementation promoted tissue carnitine accretion, particularly in skeletal muscle of guinea pigs, and livers of rats. Also, choline supplemented guinea pigs had lower percentage of carcass fat and higher percentage of protein but the body weights or the respiratory quotient (RQ) were not affected.

Based on these observations, we hypothesized that a combination of choline and carnitine may further increase carnitine accretion by tissues, and if energy needs were increased by exercise and fat mobilization was stimulated by caffeine, there may be reduction in body fat. In other words, simultaneous availability of carnitine, choline, and caffeine may induce mobilization, transport and delivery of fat as the energy substrate of choice, and therefore, enhance utilization of fat. In a 2 x 2 factorial design, male Sprague-Dawley rats were assigned to nonsupplemented and supplemented groups and one-half of each group was exercised. Body weight was significantly reduced by exercise only, however, regional fat pad weights and serum leptin concentration were significantly reduced by the combination of carnitine, choline and caffeine supplements as well as by exercise. Regardless of exercise, supplements significantly lowered triglycerides in serum but increased triglycerides in the skeletal muscle.

We postulated that fat loss in rats was due to enhanced fat mobilization and fatty acid oxidation. To support this, we determined the RQ and several metabolic markers of fat oxidation in the rat model. No significant differences were found in the mean RQ

values of the groups at rest in all groups and at exhaustion between the two exercised groups. However, increased maximal oxygen uptake (VO_2max) and delayed exhaustion time was found in the supplemented rats. Post-exercise concentrations of serum triglycerides were decreased, but β-hydroxybutyrate, acylcarnitine and acetylcarnitine were increased in the supplemented rats. The changes in serum metabolites were complemented by the changes in the muscle and urinary metabolites. The magnitude of increase in urinary acylcarnitine (34 to 45-fold) of the supplemented rats is a unique effect of this combination of the supplements. Evidence indicates enhanced β-oxidation of fatty acids without a change in the RQ because acetyl units were excreted in urine as acetylcarnitine and not oxidized to carbon dioxide. For this phenomenon we proposed the term, "fatty acid dumping".

Finally, we determined fat loss and metabolic effects of this unique nutrient-nutrient interaction in humans. We asked, if the shift in tissue carnitine partitioning will result in enhanced fat oxidation in humans. Healthy adults aged 18-54 y were randomly assigned to a placebo or supplement groups. Supplementary choline and carnitine increased serum concentration of β-hydroxybutyrate. Another biochemical marker of fatty acid oxidation, acetylcarnitine, was elevated about 2-fold and 3-fold in serum and urine, respectively. Short-chain acylcarnitines were moderately elevated in serum but significantly increased in the urine by the supplementation. The observations in humans are consistent with those in rats. This research in rats and humans firmly established that the supplementation with carnitine, choline and caffeine promoted fatty acid oxidation to short-chain fatty acids and their disposal in urine as acylcarnitines.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF TABLES (continued)

LIST OF TABLES (continued)

APPENDIXES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AC	Acetylcarnitine
AIAC	Acid-insoluble acylcarnitine
ALT	Alanin amino transferase
ASAC	Acid-soluble acylcarnitine
ASC	Acid-soluble carnitine
AST	Aspartate amino transferease
ATP	Adenosine triphosphate
BIA	Bioelectric impedance analysis
BMI	Body mass index
cAMP	Cyclic adenosine 3', 5'-monophosphate
CAT	Carnitine acetyltransferase
CCC	Carnitine, choline, caffeine
CDP	Cytidine diphosphocholine
CNS	Central nervous system
CoA	Coensyme A
CPT I	Carnitine palmitoyltransferase I
CPT II	Carnitine palmitoyltransferase II
CS	Citrate synthase
CT	Computed tomography
DAG	1,2-sn-diacylglycerol
DDW	Double distilled water

LIST OF ABBREVIATIONS (continued)

DEXA	Dual energy X-ray absorptiometry
DTT	Dithiothreitol (Cleland's Reagent)
FDA	Food and Drug Administration
Fe2+	Ferrous iron
GABA	Gamma-aminobutyric acid
GFR	Glomerular filtration rate
GOT	Glutamate oxaloacetate transaminase
GPC	Glycerophosphocholine
GRAS	Generally recognized as safe
HEPES	N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]
HDL	High density lipoprotein
IOP	International Olympic Committee
KOH	Potassium hydroxide
LDL	Low density lipoprotein
MOPS	3-[N-Morpholino]propanesulforic acid
MRI	Magnetic reonance imaging
NAD^+	Nicotine Adenine Dinucleotide
NEC	Nonesterified carnitine
% BF	Percentage body fat
PCA	Perchloric acid
PDR	Physicians' Desk Reference

LIST OF ABBREVIATIONS (continued)

PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
RQ	Respiratory quotient
SN	Supernatant
SAM	S-adenosylmethionine
S1	Supplement 1
S2	Supplement 2
TC	Total carnitine
TCA	Tricarboxylic acid
VLDL	Very low density lipoprotein
VO_2max	Maximal oxygen uptake/ maximal volume of oxygen consumed
WHR	Waist-hip ratio

CHAPTER I

INTRODUCTION

Carnitine, choline and caffeine are common constituents in our diets. These compounds are also popular ingredients of dietary supplements, because of their involvement in certain metabolic processes, including those of energy metabolism for weight loss and sport performance.

We have previously reported that choline supplementation results in a significant conservation of carnitine in normal healthy humans (Dodson and Sachan 1996) and guinea pigs (Daily and Sachan 1995). The choline supplementation promoted tissue carnitine accretion, particularly in skeletal muscle of guinea pigs. In addition, choline supplemented diet decreased percentage of carcass fat and increased percentage of protein, without significantly changing body weight or the respiratory quotient (RQ) in guinea pigs (Daily et al. 1998). Based on these observations we hypothesized that a combination of choline and carnitine may further increase carnitine accretion by tissues, and the energy substrate utilization may be altered, if fat was mobilized by caffeine, and energy needs were increased by exercise. Simultaneous availability of caffeine, carnitine and choline was proposed to induce mobilization, transport and delivery of fat as the energy substrate of choice, and thus promote fat loss.

In the first study, rats were chosen for ease of exercise training and the choline dosage was increased to compensate high choline oxidase activity in this species. Male Sprague-Dowley rats were assigned to nonsupplement and supplemented groups and one-

half of each group was exercised. The diet was fortified with carnitine, choline and caffeine (CCC). We determined the effects of CCC on body weight, fat pad mass, metabolic indices of fat oxidation, and serum leptin concentration. This study is presented in Chapter III.

We postulate that CCC supplementation will enhance fat mobilization and fatty acid oxidation. To support this hypothesis, we determined the RQ and several metabolic markers of fat oxidation in the rat tissue, especially skeletal muscle, where most fat is oxidized. The functional consequence of CCC supplementation on fat oxidation was assessed by VO_2max of the nonsupplemented and supplemented, exercised rats. The method and findings of these experiments are presented in Chapter IV.

We were also interested in knowing how the combination of CCC affects various tissue carnitine concentrations. The carnitine concentrations of various tissues (liver, heart, kidneys, brain and testes) are reported in Chapter V .

After the establishment of the animal models (guinea pigs and rats), we asked if this unique nutrient-nutrient interaction could be seen in humans as well. Healthy, untrained adults of 18-54 y of age were randomly assigned to a placebo or supplement groups. All subjects were asked to exercise in the last part of the experiment (1 wk mild exercise intervention). Measurements were made of body weight, body fat, serum lipid profiles, serum leptin, 24-hr urinary metabolites and metabolic markers of fat oxidation as determined in the rat model. Also, nutrient intake analyses for every wk were determined by using 3-day dietary diary. Effects of CCC supplementation on fat metabolism in healthy humans are presented in Chapter VI.

The results of these studies provide evidence supporting our research hypothesis, i.e. increased fatty acid oxidation by the combination of CCC supplements. We found that the combination of supplements alters partitioning, metabolism and excretion of fatty acid metabolites in rats and humans.

CHAPTER II

LITERATURE REVIEW

CARNITINE

Carnitine, β-hydroxy-γ-trimethylammonium butyrate, is a very hygroscopic compound with a molecular weight of 161.2 g/mol (inner salt) (**Figure 2-1**). Only the L-isomer is biologically active, and it is essential for the oxidation of long-chain fatty acids. Carnitine is present in serum, urine and tissues of mammalian as free carnitine and as acylcarnitine, which are metabolic products catalysed by carnitine acyltransferases.

Carnitine was first isolated from meat extracts in 1905 (Gulewitsch and Krimberg 1905) and demonstrated to be an essential growth factor for a meal worm, Tenebrio molitor (Carter et al. 1952). In 1955 Friedman and Fraenkel demonstrated that carnitine can be acetylated to form acetylcarnitine from acetyl-Coenzyme A (CoA) and carnitine (Friedman and Fraenkel 1955), and Fritz showed that carnitine stimulates fatty acid oxidation in liver homogenates (Fritz 1955). The best characterized role of carnitine in β-oxidation of long chain fatty acids was discovered in the early 1960s (Bremer 1962, Fritz and Yue 1963). These findings stimulated a great amount of research for understanding the physiological and metabolic functions of carnitine. Since then, several other roles of carnitine in mammalian metabolism have been identified (Hoppel 1992). In addition, many studies were undertaken to investigate the mechanisms of metabolic effects of carnitine (Peluso et al. 2000) as well as effects of pharmacologic administration of carnitine on various disease conditions (Brass et al. 2001, Di Donato et al. 1992).

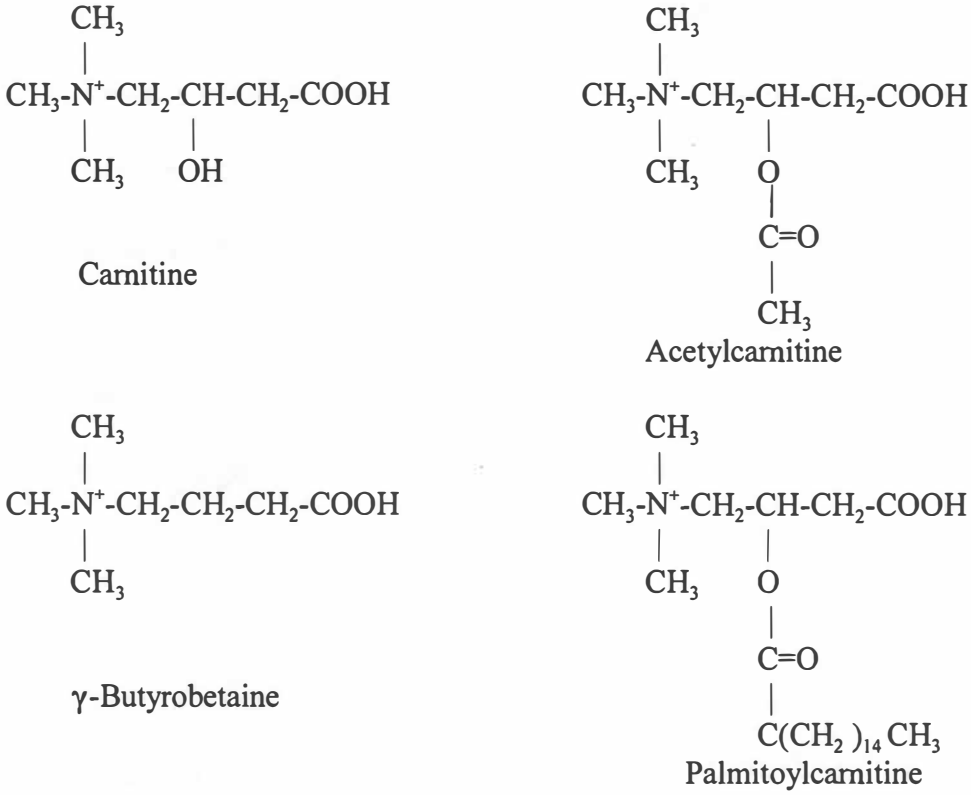


FIGURE 2-1 *Structure s of carnitine, its metabolic precursor gamma-butyrobetaine, and the acetyl and palmitoyl esters of carnitine.*

Carnitine is not a dietary essential nutrient for humans because of ample endogenous biosynthesis. However, there is nutritional and clinical evidence indicating that carnitine is a "conditionally essential" nutrient; that is, a dietary requirement of carnitine may become obligatory under certain pathophysiological conditions. An excellent example is supplementation of soy-derived infant formulas (containing little or no carnitine) with carnitine to obtain the carnitine concentration approximating that found in human milk. Newborn infants rapidly increase their reliance on endogenous and exogenous fat to meet energy needs (Wolf et al. 1974). According to rat study findings, immediately after birth an increase in the activity of carnitine palmitoyltransferase and in the capacity to oxidize fatty acids takes places in the liver (Augenfeld & Fritz 1969) and heart (Warshaw 1972). During this period, the availability of carnitine may be critical for optimal fatty acid oxidation in different tissues. Since 1985, all commercial soyprotein-based formulas manufactured in the United States are fortified with L-carnitine (Borum 1991). A substantial amount of research has been done to define a dietary requirement of carnitine for a special population that is physiologically and metabolically stressed, for examples, cardiac and renal patients.

Dietary Sources of Carnitine

In human, diets containing meat, poultry, fish and dairy products are the major sources of carnitine (Broquist 1994). **Table 2-1** lists the carnitine content of selected foods. Vegetables, fruits, grains and other plant-derived foods contain little or no carnitine. Normal non-vegetarian and strict vegetarian diets have been estimated to

TABLE 2-1

Carnitine content in common foods[1-2]

MEAT PRODUCTS		DAIRY PRODUCTS		VEGETABLES	
Beef steak	592	Whole milk	20	Asparagus (cooked)	1.21
Ground beef	582	Goat milk	15	Beets (cooked)	0.02
Pork	172	Sheep milk	100	Carrots	0.04
Bacon	146	Human milk	7	Green beans (cooked)	0.02
Fish (cod)	35	American cheese	23	Green peas (cooked)	0.04
Chicken breast	24	Ice cream	23	Potato (baked)	0.08
		Cottage cheese	7	Lettuce	0.00
BREAD AND CEREAL		**BEVERAGES**		**FRUITS**	
Whole-wheat bread	3	Coffee	0.01	Apples	0.01
White bread	0.9	Cola	ND[3]	Bananas	0.00
Rice	0.1	Grape juice	0.1	Peaches	0.01
Macaroni	0.8	Orange juice	0.01	Pineapple	0.01
Corn flakes	0.1	Tomato juice	0.03	Pears	0.01
MISCELLANEOUS				**INFANT FORMULAS**	
Eggs	0.08			Milk based formulas	7-23
Peanut butter	0.05			Premature formulas	5-30
Soy Milk	ND				

[1]Values are μmol/100 g (solid foods) or μmol/100 ml (liquids) of total carnitine.
1 μmol = 0.161 mg of carnitine.
[2]Modified from Rebouche and Engel 1984, Penn et al. 1987.
[3]ND, not detectable.

provide approximately 2-12 μmol and 0.1-0.2 μmol of carnitine/kg body weight/d, respectively (Rebouche 1992).

Carnitine Homeostasis

Dietary carnitine intake and nutritional status affect plasma and tissue carnitine concentrations. After feeding a carnitine-free diet over 7 months, plasma and tissue carnitine concentrations of rats were decreased 50% (Heinonen & Takala 1994), which is comparable to those in secondary carnitine-deficient patients (Tein et al. 1993). The carnitine free diet contained lysine, methionine, ascorbic acid and iron, which are essential for endogenous carnitine biosynthesis. Therefore, the study indicates that exogenous carnitine in a diet is necessary to maintain normal carnitine concentration.

Juvenile visceral steatosis (JVS) mice have been reported to have systemic carnitine deficiency (Kuwajima, et al. 1991). These mice are autosomal recessive mutants that exhibit severe fatty liver, hypoglycemia, and growth retardation (Koizumi et al. 1988). Their hepatic carnitine stores are below 5% of those normally observed in control mice. The JVS mice showed decreased tissue carnitine concentrations, decreased hepatic fatty acids metabolism, and decreased functions of other organs such as skeletal muscle and heart (Nakajima et al. 1997).

In neonatal rats, as in human newborns, carnitine concentrations were directly related to dietary supply, i.e. milk carnitine (Flores et al. 1996). Newborn rats were fed a rat milk substitute with or without 300 μmol/L carnitine, corresponding to the concentration of carnitine in rat milk, from birth to either 2 or 4 d. Carnitine supplementation resulted in significantly higher concentrations of carnitine in the heart,

skeletal muscle, liver and small intestine. The most pronounced differences in organ concentrations were found in the small intestine. Significant differences of carnitine concentration in the small intestine between supplemented and nonsupplemented pups were observed even after 2 d of milk feeding. The accumulation of carnitine was the greatest in the proximal intestine as has been previously reported in adult rats (Sachan & Ruark 1985). This indicated that the small intestine contributes substantial quantities of the body carnitine pool in rat pups. Other studies involving suckling animals demonstrated that milk-carnitine increased the concentrations of carnitine in the liver, heart and skeletal muscles in rabbits (Penn and Schmidt-Sommerfeld 1988) and piglets (Coffey et al. 1991). The increased carnitine in these organs was due to increased intake of exogenous carnitine, in addition to endogenous biosynthesis of carnitine. In suckling rats 45-59% of tissue carnitine is derived from endogenous sources (Davis 1989).

In healthy full-term infants fed carnitine-free soy formulas there was lower plasma carnitine concentration, reduction of ketogenesis, and accumulation of fatty acid in plasma, compared to the infants provided carnitine in either bovine milk-derived infant formula or in human milk (Novak et al. 1983). Similar observations have been made in preterm infants (Penn et al. 1980) and adult patients (Hahn et al. 1982, Bowyer et al. 1986) receiving total parenteral nutrition.

The carnitine status of individuals consuming self-selected diets low in carnitine were studied in lacto-ovo and strict vegetarians (Lombard et al. 1989, Sachan et al. 1997, Richter et al. 1999). Lombard et al. (1989) reported that in both adults and children, plasma carnitine concentrations and urinary carnitine excretion of both lacto-ovo and

strict vegetarians were significantly lower than those eating the mixed diet. The differences in plasma carnitine concentrations were greater in children than adults, indicating higher carnitine requirements for growth and tissue deposition in children. The relationship between plasma and urinary excretion of total carnitine suggests that low carnitine intake increased renal tubular carnitine transport (reabsorption) capacity to minimize renal loss of carnitine and thus maintain normal plasma carnitine concentration. Less efficient reabsorption of filtered carnitine by the kidney was observed when rats were administered diets high in carnitine (Rebouche and Mack 1984). These observations are in agreement with those of other investigators where lacto-ovo vegetarians were found to have lower circulating carnitine concentrations than omnivores (Richter et al. 1999).

Very little data exist concerning the carnitine status of elderly humans. Sachan et al. (1997) assessed dietary intake of nutrients essential for carnitine biosynthesis and urinary excretion of carnitine in elderly (mean age of 70.5 y) lacto-ovo vegetarian and non-vegetarian women. They found that dietary intake of protein and essential amino acids, lysine and methionine, were significantly lower in vegetarian than non-vegetarian women. Vegetarians showed 50% reduction in urinary excretion of carnitine that non-vegetarians. Mean (±SEM) urinary carnitine excretion for lacto-ovo vegetarians and non-vegetarians were 45.8 ± 6.7 and 92.5 ± 9.5 μmol/d, respectively. The reason for the significant reduction of urinary carnitine excretion in the lacto-ovo vegetarians was that these women consumed soy-milk, and did not consume cow's milk, which is a rich source of carnitine. From the dietary analysis and urinary carnitine excretion, it was concluded that elderly vegetarians do not meet a nutritional requirement for carnitine

(Sachan et al. 1997).

Dietary factors other than carnitine content in diets may influence carnitine status in humans. Cederblad (1987) determined the effect of dietary macronutrient content (carbohydrate and fat) on plasma carnitine concentration and urinary excretion of carnitine. Healthy male volunteers consumed a high-carbohydrate, low-fat diet (high-carbohydrate diet) and a low-carbohydrate, high-fat diet (high-fat diet). After baseline blood samples were drawn, each diet regimen was maintained for 4 d, followed by a 3 d break, and then other diet regimen for another 4 d. The two diet regimens contained same amount of energy and carnitine-rich foods. Total and free carnitine concentrations in plasma were higher in people on a high-fat diet than on a high-carbohydrate diet by d 3. Both diets increased plasma acylcarnitine level and acyl:free carnitine ratio compared to the initial levels. In urine of the high-fat diet, all carnitine fractions (i.e. total, free and acyl carnitine) were increased by d 2. Urinary excretion of the acylcarnitine increased significantly from an initial value of 283 ± 94 to 431 ± 119 μmol/24-h on d 5 with a high-fat diet. Increased urinary carnitine excretion observed in a high-fat diet may be due to an increased renal carnitine clearance. Renal carnitine clearance (C) was calculated as C (ml/min) = (U x V) /P where U and P are the carnitine concentrations in urine and plasma, respectively, and V is the urinary volume/unit of time. The study showed that composition of a diet with constant carnitine content affects plasma and urinary excretion of carnitine (Cederblad 1987).

Stadler et al. (1993) determined the mechanisms by which macronutrient (carbohydrate, fat and protein) content affect carnitine status by examining glomerular

filtration rate (GFR), excretion, reabsorption, and filtered load of carnitine. Healthy adults consumed five different diets: high-protein, low-protein, control, high-fat, high carbohydrate. The diets were isoenergetic and contained equal amount of carnitine, approximately 32 μmol/kJ/diet (equals to 133 μmol/kcal/diet). On d 6, plasma carnitine concentration was significantly lower after the high-carbohydrate diet than after either the high-fat or control diets. Urinary excretion of total carnitine was 4-fold higher after the high-fat diet compared to the high-carbohydrate diet, which confirmed the previous findings by Cederblad (1987). The rate of carnitine excretion was lower after the low-protein diet than after the high-protein diet because of lower GFR after the low-protein diet. An increased rate of carnitine excretion after a high-fat diet, compared with a high-carbohydrate diet, is due to a higher filtered load of carnitine. The results of this study may in part explain the decreased urinary carnitine excretion among individuals consuming vegetarian diets, which tend to be high in carbohydrate and low in fat and protein (Abdulla et al. 1981).

It is clear that dietary intake of carnitine and macronutrient affect plasma carnitine concentration and urinary carnitine excretion in humans. However, it is not known to what extent humans rely on dietary carnitine and what are the functional consequences of variations in dietary intake of carnitine. There are no known adverse effects attributed to the diet poor in carnitine. It must be noted that carnitine concentration in human muscle tissue is 4–5-fold higher than in rat, whereas plasma carnitine concentration are of the same order of magnitude (Cederblad and Lindstedt 1976). Total muscle carnitine in rats is ~1 μmol/g wet weight (Hongu & Sachan 2002), compared to ~4.2 μmol/g wet weight

(Lennon et al. 1983) in human. Therefore, humans have a large carnitine buffer in case of limited dietary intake or biosynthesis of carnitine. In summary, carnitine changes in urine and plasma involves dietary supply, biosynthesis, transport in and out of the tissue and excretion of carnitine, which together make up homeostasis of carnitine in the body.

Supplement Forms of Carnitine

Chemically synthesized carnitine (L-carnitine free base) became available on the market in the early 1980's (Zurbriggen 2000). Today, 4 main commercial forms of carnitine are available as nutritional supplements: L-carnitine free base, L-carnitine L-tartrate, L-carnitine magnesium citrate, and acetyl-L-carnitine hydrochloride. Supplementation studies have used L-carnitine with dosages ranging from 500 mg/d to 6 g/d. The typical dosage used in most studies were 1-2 g/d for 50-100 kg body weight, respectively. The dosages being even higher and reaching as much as 100-350 mg/kg body weight are mostly administered for medical treatment; e.g. removal of excessive organic acids as in variety of acidemia (Roe et al. 1984b, Itoh et al. 1996). It is important to note that a bioavailability of the 2 g dose of carnitine, if given orally, is reported ~15% (Harper et al. 1988).

It appears that L-carnitine supplementation is safe with typical dosages used in most studies and no adverse effects has been reported. Carnitine consumed in the large doses, about 5g/d by an adults, may cause diarrhea or fish odor syndrome in some people (Rebouche 1999). Importantly, myasthenia-like symptoms have been found in hemodialysis patients administered D,L-carnitine for 45 d (Bazzato et al. 1979). In rat study, intraperitoneal injection of D-carnitine (750mg/d) for 4 d produced an L-carnitine

deficiency in cardiac and skeletal muscle (Paulson and Shug 1981). Thus, the D,L-racemic mixtures, which are present in some commercial products, can result in harmful effects, therefore it should not be used in carnitine therapy. It should be also noted that carnitine supplement products may vary considerably in their purity (Borum 2000). Non-pharmaceutical grade carnitine products have been found to contain undetectable concentration of carnitine to 100 % pure carnitine. Bioavailability data were not available for these non-pharmaceutical grade products (Millington & Dubay 1993).

Carnitine Biosynthesis

In 1961, several investigators demonstrated that methionine provides the methyl groups of carnitine (Wolf and Berger 1961, Bremer 1961), and the immediate precursor of carnitine is γ-butyrobetaine, which is hydroxylated to form carnitine (Lindstedt and Lindstedt 1961, Bremer 1961). However, the source of the carbon chain of carnitine remained unknown. Later, Broquist and co-workers working with *Neurospora crassa* discovered that lysine provides the carbon-nitrogen backbone of carnitine (Horne et al. 1971, Tanphaichitr and Broquist 1973). It was clear then that carnitine is synthesized from two essential amino acids lysine and methionine (**Figure 2-2**).

The biosynthesis of carnitine begins with the enzymatic trimethylation of a peptide-bound lysine residue to form 6-N-trimethyllysine. Carnitine is formed from this 6-N-trimethyllysine after its release due to protein breakdown in the lysosomes (Dunn & Englard 1981). In the next step, the 6-N-trimethyllysine is hydroxylated to form 3-hydroxy-6-N-trimethyllysine. The reaction is catalyzed by 6-N-trimethyl-lysine hydroxylase (E.C. 1.14.11.8), which is found in mitochondria of kidney (Sachan and

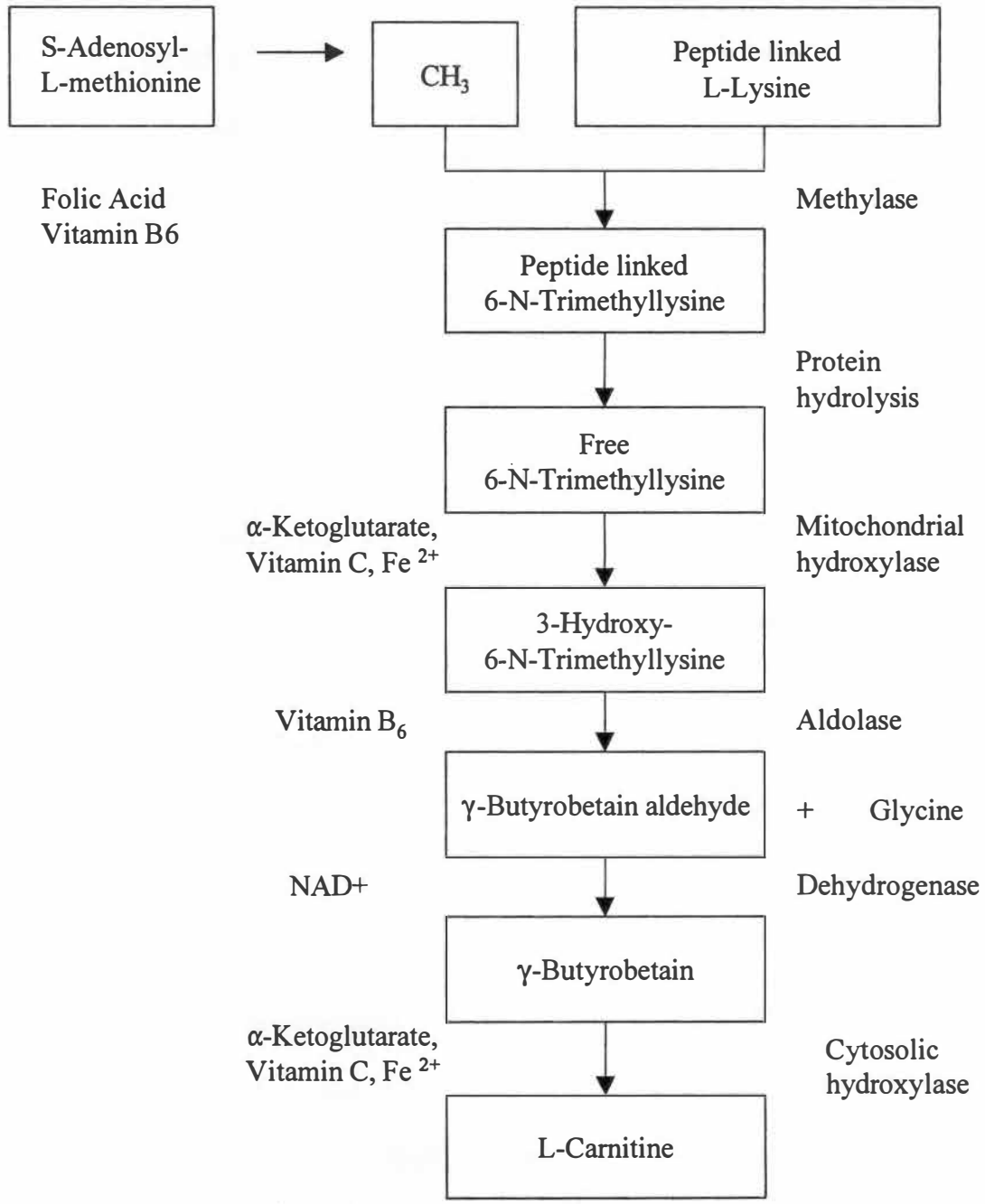


FIGURE 2-2 *Biosynthesis of L-carnitine.*

Hoppel 1980), liver, heart, skeletal muscle, and other tissues (Stein & Englard 1982). The enzyme requires α-ketoglutarate and oxygen as cosubstrates, and ferrous iron (Fe^{2+}) and ascorbic acid for optimal activity (Hulse et al. 1978). The 3-hydroxy-6-N-trimethyllysine undergoes aldol cleavage to glycine and γ-butyrobetaine aldehyde. Serine hydroxymethyltransferase (E.C. 2.1.2.1), a pyridoxal phosphate-requiring enzyme, catalyzes this reaction. The γ-butyrobetaine aldehyde is then oxidized to a precursor of L-carnitine, γ-butyrobetaine, by nicotine adenine dinucleotide (NAD^+) requiring aldehyde dehydrogenases (Hulse et al. 1978). The final step is a hydroxylation of γ-butyrobetaine to form L-carnitine. The enzyme catalyzing this reaction is γ-butyrobetaine hydroxylase (E.C. 1.14.11.1), which also requires an α-ketoglutarate and oxygen as cosubstrates, Fe^{2+} and Vitamin C as the reducing agent for its activity (Bremer 1983).

Most tissues can convert N-trimethyllysine to γ-butyrobetaine, but the last step of biosynthesis, the hydroxylation of butyrobetaine to carnitine, is limited to liver and testis in rats; liver, kidney and brain in humans (Bremer 1997). The rate of carnitine biosynthesis in humans is estimated to be about 1.2 μmol/ kg body weight/d (Lombard et al. 1989). Studies in rats have shown that the availability of 6-N-trimethllysine determines the normal rate of carnitine biosynthesis (Rebouche et al. 1986). However, regulation of carnitine biosynthesis in humans is not well understood. It is important to note that lysine and methionine are often the most limited amino acids in vegetable proteins consumed by vegetarians (Sachan et al. 1997, Siddiqui & Bamji 1980, Young and Pellet 1994). As was shown in Table 2-1, plant foods are also very low in carnitine content. Therefore, diets in certain populations may not adequately support the rate of

carnitine biosynthesis, when the demand for carnitine is increased under physiological and pathological conditions.

Absorption

Dietary carnitine is rapidly absorbed from the intestinal lumen by an active transport mechanism as well as by a passive diffusion process (Gross and Henderson 1984, Hamilton et al. 1986). Absorbed carnitine is taken up by the liver from the portal vein and then released into the systemic circulation (Gudjonsson et al. 1985). Live-animal studies, using male Sprague-Dawley rats, demonstrated 80% of L-carnitine was taken up by the intestinal tissue in 30 min after intraluminal injection of carnitine. After 5 h, 30% and 7% of L-carnitine were found in the skeletal muscle and urine, respectively (Gross and Henderson 1984).

It is estimated that about 70 to 80% of carnitine is absorbed under normal conditions in omnivores (Rebouche and Chenard 1991). The efficiency of absorption was reduced to 20% after 2 g daily supplementation of carnitine (Rebouche 1999). In mammals, carnitine is not catabolized except by microorganisms in the large intestine. Carnitine is mainly excreted in the urine as free and acylcarnitines (Brass and Hoppel 1978). Carnitine supplemented mice and rats have also shown increased urinary excretion of trimethylamine, trimethylamineoxide, γ-butyrobetaine, crotonobetaine (Bremer 1983). The previous study on the metabolism of L-[methyl-^{14}C]carnitine in rats showed conversion of carnitine to γ-butyrobetaine in feces and trimethylamineoxide in urine (Rebouche et al. 1984). It is also reported that nearly 100% radioactivity was recovered in rats receiving the isotope intravenously and in germ-free rats receiving the

isotope orally or intravenously. Results of this study concluded that L-carnitine is degraded in the intestine, and that intestinal microorganisms are responsible for these conversions. (Rebouche et al. 1984).

Transport and Tissue Uptake

Carnitine is present in most tissues and transported from the blood plasma. The concentration of total carnitine in blood in humans is 0.03 to 0.05 mM, and that is far lower than in tissues (Bieber 1988). Most of the carnitine is found in the skeletal muscles, which contains 95% of the total carnitine in the body (Rebouche and Engel 1984). Since carnitine concentration of tissue is 10-100-fold higher than that in blood plasma, a concentration gradient must exist to maintain carnitine homeostasis. Mechanisms of sodium-dependent, active transport systems have been suggested in the intestine (Shaw et al. 1983), liver (Christiansen and Bremer 1976), skeletal muscle (Rebouche 1977), and kidney (Rebouche and Mack 1984).

The carnitine transport system in the liver is important for the whole body carnitine homeostasis, because the liver is the major site of carnitine biosynthesis and the first site exposed to carnitine before the systemic carnitine distribution. The liver transport system has a high Km and the hepatic carnitine content is readily altered by exogenous carnitine (Brass and Hoppel 1980, Ruff et al. 1991), fasting (Brass and Hoppel 1978) and diabetes (McGarry et al. 1975). In the perfused rat liver Km for carnitine uptake was lower in fasted rats (2.59 mM) than fed rats (4.2 mM) with the unchanged maximal rate of transport (Kispal et al. 1987). Glucogan increased carnitine uptake in both fed and fasted rat liver, while insulin decreased carnitine transport in fasted rat liver.

The uptake and release of carnitine in liver are partially regulated by hormones (Bremer 1983) and by different membrane carriers (Hokland 1988).

The properties of transport systems in cardiac and skeletal muscles differ from those of the liver. The rate of maximal uptake in muscle is only 1/1000 of the rate of the maximal uptake in liver (Bremer 1983). This agrees with the turnover time of muscle carnitine, which is 100-150 times longer than liver carnitine in vivo (Brooks & McIntosh, 1975). Carnitine is slowly released from muscle cells, and exogenous carnitine stimulate this process. Normal serum carnitine concentration (26-69 μM) is positively correlated with skeletal muscles and heart carnitine concentration (Rebouche 1989).

A cardiac carnitine binding protein has been isolated from rat hearts which may serve as the carrier for the transport of carnitine across the heart cell membrane (Cantrell and Borum 1982). Maximum binding of carnitine was obtained after incubation for 1 h at 25°C. This relatively slow uptake of carnitine in the heart may be related to this slow binding. Another feature of this protein is that butyrobetaine is a relatively weak inhibitor of carnitine biding to this heart protein. In liver cells, butyrobetaine is taken up with a lower Km than is carnitine. This suggests that the cardiac carnitine binding protein is different from that of hepatocyte cell membrane (Cantrell and Borum 1982).

The kidney plays roles not only in carnitine biosynthesis, but also in carnitine reabsorption, acylation/deacylation, and excretion of carnitine. Carnitine, a small water-soluble molecule, is readily filtered by the renal glomerulus. Filtered carnitine is reabsorbed by the proximal tubule to prevent excess urinary loss (Rebouche and Engel 1984). A sodium-dependent transport system has been suggested in rat kidney cortical

slices (Huth and Shug 1980) and proximal tubular brush border membranes (Rebouche and Marck 1984). Guder and Wagner (1990) observed that the total carnitine content in isolated proximal tubule of rat kidney was 2.85 ± 0.15 μmol/g protein and about 50% of it was acetylated. When it was incubated at 37°C in vitro for 60 min, the acylcarnitine/carnitine ratio decreased without changing total carnitine, indicating deacylation of acylcarnitine. Subsequently, when 1 mmol/L of carnitine was added to the incubation medium, it showed 7-fold increase in acylcarnitine formation by the tubules. This observation suggested that proximal tubule cells actively take up carnitine and convert it to acyl derivatives (Guder and Wagner 1990).

Tissue uptake of carnitine occurs at widely varying rates (Bremer 1983). The turnover times for carnitine in rat tissues have been estimated to be 0.4 h in the kidney, 0.33 h in liver, 21 h in skeletal muscle, and 220 h in brain (Brooks and McIntosh 1975). In humans the turnover times of carnitine in skeletal muscle was estimated to be 191 h (Rebouche and Engel 1984). This suggests reutilization of carnitine among the various tissues of the body.

Excretion

When plasma carnitine concentration exceeds the renal reabsorption maximum, carnitine is eliminated in the urine. In rats 1-2 μmol of carnitine/100g body weight are excreted each day (Cederblad and Lindstedt 1976, Tsai et al. 1975). In fasting rats both the plasma concentration and the renal excretion of carnitine decreased at first, but increased above the initial values after fasting for 3-4 d (Brass and Hoppel 1978). Similar observations have been reported in humans. In man a filtered carnitine load is 4

μmol/min in contrast to the 125 μmol of daily urinary excretion (Brass 1992). Tubular reabsorption in the kidneys was estimated to be 96-99% (Frolich et al. 1978). When the plasma carnitine concentration is increased by supplementation or dietary intake of carnitine in vegetarians, the renal tubular reabsorption of carnitine declines, suggesting that plasma carnitine levels are regulated by renal clearance of carnitine (Engel et al. 1981, Lombard et al. 1989).

Other factors affecting urinary carnitine excretion have been reported. Adult men excreted more urinary carnitine than adult women (Cederblad and Lindstedt 1971, Maebashi et al. 1976), but adult male rats excrete less carnitine than adult female rats (Carter and Stratman 1982). Carnitine excretion varied widely in women during the menstrual cycle but reached maximum at the time of ovulation (Maebashi et al. 1976).

Functions

A. Mitochondrial fatty acid oxidation

Carnitine has a critical role in energy metabolism. Carnitine serves two important, well established functions in cellular metabolism; 1) mitochondrial fatty acid oxidation and 2) modulation of acyl-CoA/CoA ratio. Carnitine facilitates the entry of long-chain fatty acids into the mitochondrial matrix for oxidation (**Figure 2-3**). The mitochondrial inner membrane is impermeable to long-chain fatty acids and fatty acyl-CoA, which is formed on the outer surface of the mitochondrial outer membrane via a fatty acyl CoA synthetase (Lehninger et al. 1993). The long-chain fatty acyl-CoA between the outer and inner membranes is transesterified to carnitine to form long-chain fatty acylcarnitine by carnitine palmitoyltransferase I (CPT I), also designated as CPT-1,

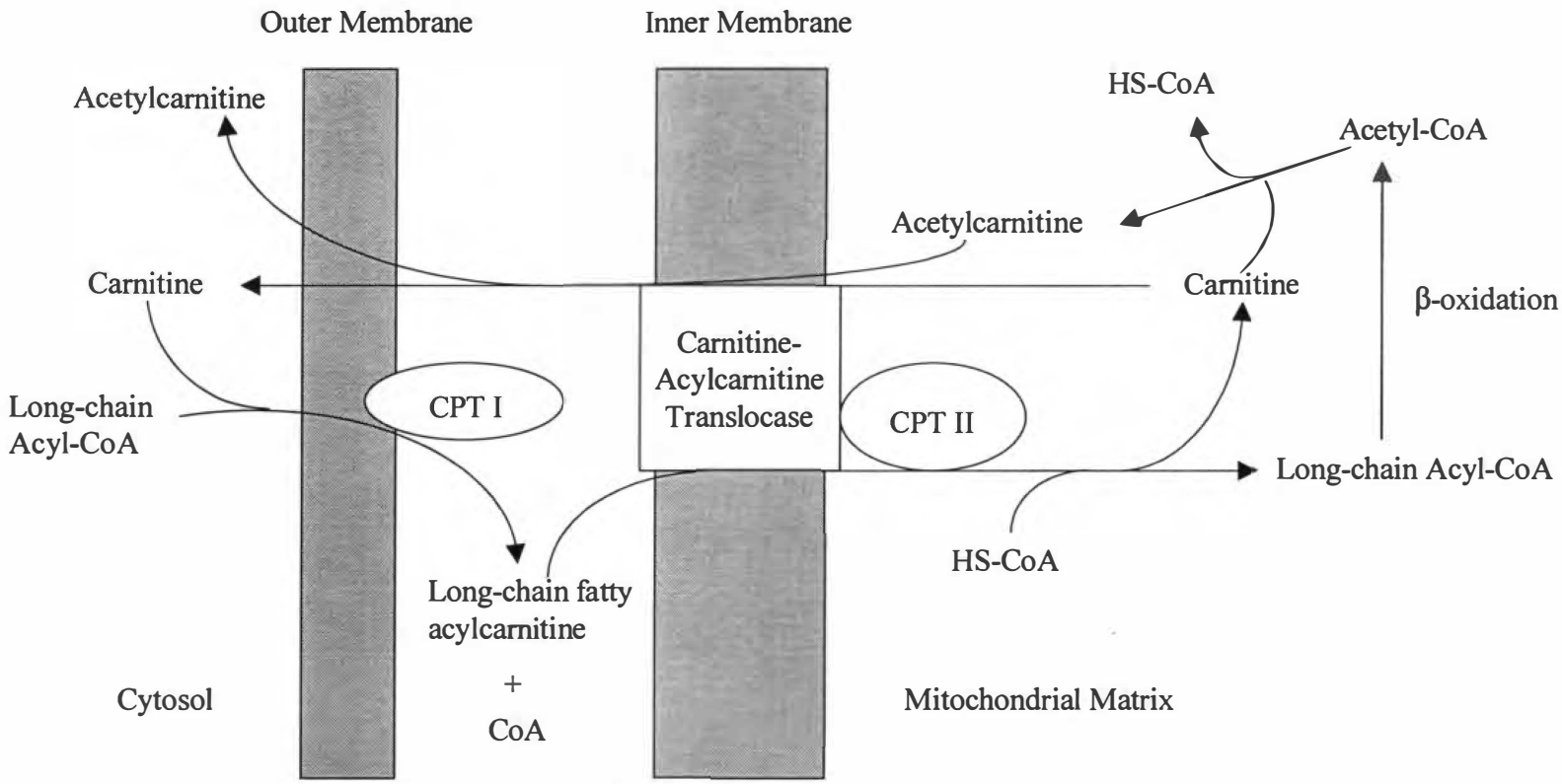


FIGURE 2-3 *Fatty acid entry into mitochondria via the acylcarnitine transport.* Long-chain fatty acid acyl-CoA in the cytoplasm is bound to carnitine in a reaction mediated by carnitine palmitoyltransferase I (CPT I). The acylcarnitine is transported across the mitochondrial inner-membrane via a carnitine-acylcarnitine translocase, where carnitine palmitoyltransferase II (CPT II) catalyzes the reaction between acylcarnitine and CoA. In this reaction, carnitine is regenerated and transported back to the inter-membrance space for further reactions and acyl-CoA is released and goes on to β-oxidation.

CPTo, CPT-A. The acylcarnitine is transported across the mitochondrial inner membrane via a carnitine acylcarnitine translocase. The second transesterification with CoA in the mitochondrial matrix is catalyzed by carnitine palmitoyltransferase II (CPT II), also designated as CPT-2, CPTi, CPT-B. This intramitochondrially regenerated fatty acyl-CoA enters the β-oxidation pathway to provide cellular energy. Free carnitine released in the matrix is translocated back to inter mitochondrial space by carnitine acylcarnitine translocase, where it can repeat the fatty acid transport process. In summary this transport process, the "carnitine transport shuttle", involves two transesterifications and three mitochondrial enzymes: CPT I, CPT II and carnitine translocase. The result of the concerted actions of these enzymes is that long-chain fatty acids are transported into the mitochondria by being esterified to carnitine (acylcarnitine).

Short-chain fatty acids, such as propionic acid and acetic acid, in all tissues can enter mitochondria without the carnitine translocator and be activated inside of the mitochondria to form the short-chain fatty acyl-CoA. Medium-chain fatty acids may be activated by the liver peroxisomal medium-chain acyl-CoA synthetase and trnsesterified to carnitine for entering into mitochondria (Bahl and Bressler 1987).

B. Modulation of acyl-CoA/CoA ratio

Under some metabolic conditions acetyl-CoA and other short CoA esters may accumulate in the mitochondria. For example, β-oxidation of fatty acid generates acetyl-CoA. If acetyl-CoA is not consumed by the tricarboxylic acid (TCA) cycle for energy production, the availability of free CoA is reduced. CoA is a required cofactors for many cellular oxidative and biosynthetic reactions. Therefore, if free CoA is not available, the

enzymes that required this cofactor (i.e. pyruvate dehydrogenase, fatty acyl CoA synthetase, etc.) will not function (Brass 1994). Carnitine facilitates the removal of excess acetyl residues generated by enhanced β-oxidation in mitochondria (Pearson and Tubbs 1967). In starved rat liver, the acetyl-CoA/CoA and the acetylcarnitine/carnitine ratios increase (Bøhmer 1967). In muscles these ratios increased with exercise. During 4 min intense exercise, 90% of the predicted VO_2max, there was an increase in skeletal muscle acetylcarnitine with almost equivalent decrease in free carnitine without changing total carnitine (Harris et al. 1987). When exercised for 30 min at a work load between the lactate threshold and maximal work capacity, the muscle short-chain acylcarnitne content increased 5.5-fold, while free and total carnitine content decreased, 66% and 19%, respectively. However, these changes in skeletal muscle carnitine were poorly reflected in the plasma and urinary carnitine concentrations (Hiatt et al. 1989). Similar results were observed in other studies in humans and rats (Sahlin 1990, Carter et al. 1981). These observations are consistent with the hypothesis that carnitine function in the regulation of the acetyl-CoA/CoA ratio by buffering excess accumulation of acetyl units.

This function of carnitine has important implication in normal and abnormal cellular metabolism associated with defects in organic and fatty acid metabolism. For example, the excretion of propionylcarnitine is elevated in propionyl-CoA carboxylase deficiency individuals (Roe and Bohan 1982). In this disorder there is a block in the oxidation of the propionyl-CoA, resulting in acyl-CoA accumulation and inhibition of oxidative phosphorylation. Propionic acidemia showed a significant clinical response to carnitine treatment (Roe et al. 1984a). The acyl moiety, propionate, becomes a carnitine

ester and is excreted in urine. Thus, it does not disturb the function of mitochondrial CoA dependent metabolic pathways. The excretion of specific acylcarnitine conjugates of two drugs that are metabolized to acyl-CoA in humans has been reported. Valprolylcarnitine and pivaloylcarnitine are excreted as a result of valproic acid (Vickers et al. 1985) and pivalic acid-therapy (Millington et al. 1985). These organic acidemias (a fall in blood pH caused by high levels of specific organic acids) are metabolic diseases that can be successfully treated by the pharmacological use of carnitine (Chapoy et al. 1980). Treatment generally consists of administration of 100 mg/kg/d of carnitine in 3 to 4 doses (Stumpf et al. 1985).

Numerous studies investigated the functions of carnitine and its derivatives beyond the well-defined roles of carnitine described above. These include the roles of carnitine in branched-chain amino acids metabolism (Solberg and Bremer 1970, Bieber and Choi 1977), ketone body utilization (McGarry and Foster 1980), peroxisomal β-oxidation (Bieber et al. 1981) and membrane trapnsport/permiability (Fix et al. 1986).

Nutrient-Nutrient Interactions

Carnitine status and metabolism are affected by several nutrients. Guinea pigs fed a vitamin C deficient diet decreased plasma carnitine levels and increased the rate of urinary carnitine excretion (Rebouche 1991), which corresponded to a 15% decrease in the rate of carnitine reabsorption in the vitamin C deficient guinea pigs (Rebouche 1995). Livers of iron deficient rat pups contained significantly less carnitine than livers of the control pups. Impaired ketogenesis and hyperlipidemia were observed in iron deficient pups (Bartholmey & Sherman 1985). The study of 70 children in Turkey suffering from

iron deficiency anemia aged 6 months to 2 years (mean age 14.5 ± 10.9 months) showed significantly lower serum free carnitine than the healthy controls (18.9 ± 0.43 nmol/ml and 45.9 ± 1.47 nmol/ml, respectively), where serum triglyceride, total cholesterol and very low density lipoprotein (VLDL) levels were significantly increased. The authors speculated that iron deficiency anemia may be linked to the endogenous carnitine synthesis which may be underlying causes of hyperlipidemia in pediatric age group (Tanzer et al. 2001).

Previous investigators reported that choline deficiency alters carnitine metabolism. Choline deficiency decreased carnitine concentration in liver, heart and skeletal muscle (Carter and Frenkel 1978, Corredor et al. 1967). Despite the decreased tissue carnitine concentrations, a significant increase in the serum carnitine in choline deficient rats was reported by Carter and Frenkel (1978) and Sheard and Krasin (1994) However, no explanation of this phenomena was given by these investigators.

The changes in carnitine concentration were observed in both choline deficient and choline supplemented rats after a 48 hr fast (Carter and Frenkel 1978). In the choline deficient rats, carnitine increased in the liver about 3-fold, but decreased slightly in the heart and muscles. In choline supplemented rats 48 hr fasting increased the carnitine concentration in the heart, skeletal muscles and the liver. There were no differences between choline deficient and supplemented rats in the concentration of plasma ketone bodies after a 48 hr fast. Subsequently, these choline deficient rats were injected with choline intraperitoneally to examine the changes in liver carnitine. Choline injection was able to raise the concentration of liver carnitine to normal values within 1.5 hr. In

addition to the choline injection, the other methyl-group donors, methionine, betaine and sarcosine were injected to determine their ability to increase the concentration of liver carnitine. None of these compounds were ineffective in increasing liver carnitine concentration within the time period of this experiment (Carter and Frenkel 1978). It seems that increased liver carnitine concentration, after choline injection and a 48 hr fast, were due to increased transport and uptake of carnitine into the liver.

In another study the decreased rate of palmitate oxidation was demonstrated in the heart and liver homogenates from choline deficient rats (Corredor et al. 1967). Addition of carnitine, but not of choline, to heart homogenates from both the choline deficient and control rats resulted in increased palmitate oxidation. When the rats were given intraperitoneal injection of choline or carnitine before being killed, the rate of palmitate oxidation was increased by liver and heart homogenate in both groups. Choline administration to choline deficient rats increased their tissue carnitine concentrations. The authors concluded that the decreased tissue carnitine in choline deficient rats are responsible for the decreased rates of long-chain fatty acid oxidation (Corredor et al. 1967).

Providing choline to choline-deficient rats resulted in significant increases in the carnitine concentrations of liver and muscle, and significantly lower urinary carnitine excretion (Carter and Frenkel 1978). Choline supplement also increased the total body pool of carnitine and increased carnitine turn over rate in rats (Tsai et al 1975). However, serum and urinary carnitine concentration were significantly lower in choline supplemented than choline deficient rats (Carter and Frenkel 1978, Sheard and Krasin

1994, Tsai et al. 1974). These studies suggested that the effects of choline on carnitine may extend the relationship beyond carnitine biosynthesis; that is choline, as a methyl-group donor for the methylation of lysine residues, choline may participate in modifying the transport of carnitine into all tissues where carnitine is needed.

Choline supplementation in choline sufficient humans and animals demonstrated the alteration of carnitine homeostasis. In 1996, Dodson and Sachan demonstrated for the first time, that choline supplementation resulted in conservation of carnitine altered renal clearance and urinary excretion of most fractions of carnitine in 23 to 52 y healthy humans of diverse ethnic background. Their urinary carnitine concentrations became similar to the ones reported in vegetarians (Lombard et al. 1989, Sachan et al. 1997). Serum total carnitine (TC), frequently used as the evaluation of carnitine status of humans, was not affected by the choline supplement. However, one case was reported to exhibit 50% reduction of serum carnitine concentration after 7 d of supplement of choline; and it would have been assessed as carnitine deficient (Dodson and Sachan 1996).

These observations in humans lead to a search for an animal model for studying choline-carnitine interaction, and rats were used first for this purpose (Daily and Sachan 1995). Male Sprague-Dawley rats were fed control and choline supplemented diets. There were no significant differences in the serum and urinary carnitine in the two groups. Other investigators were also unable to show the effects of choline on carnitine concentrations in rats (Sheard et al. 1992). It was postulated (Daily and Sachan 1995) that the lack of effects in rats might be due to their 60-fold higher hepatic choline oxidase

activity compared to humans showed by Sidransky and Farber (1960). The specie that is the closest to human in choline oxidase activity is guinea pig. Guinea pigs have hepatic choline oxidase activity about 3 times higher than that of humans (**Table 2-2**). Therefore, guinea pigs were used to determine the effects of choline on carnitine homeostasis (Daily and Sachan 1995).

Guinea pigs had free access to a nonpurified guinea pig diet (Purina Mills, Richmond, IN). The choline-supplemented diet was prepared by adding 3 g choline as choline chloride/kg diet to the control chow diet for 10 d. Plasma TC concentration was significantly higher in the choline supplemented than in nonsupplemented guinea pigs, which was different from results seen in humans study (Dodson and Sachan 1996).

TABLE 2-2

Hepatic choline oxidase in selected species[1-2]

Species	Choline oxidase activity	Comparison to humans
Rat	2408 ± 121	60.2
Chicken	1311 ± 8	32.8
Mouse	895 ± 72	22.4
Hamster	361 ± 63	9.0
Monkey	144 ± 21	3.6
Guinea Pig	136 ± 43	3.4
Human	40 ± 7	1.0

[1]Values are μL of O_2 uptake / hr / g wet liver ± SEM.
[2]Modified from Sidransky and Farber (1960)

Urinary excretion of carnitine was significantly lower in the choline supplemented guinea pigs. This conservation of carnitine led to the determination of carnitine distribution among various tissues in the guinea pigs. The concentration of carnitine were increased in all tissues, especially in skeletal muscle (Daily and Sachan 1995).

Subsequently, metabolic consequences of choline-induced carnitine accretion in guinea pigs were determined under normal, starvation and treadmill running exercise conditions (Daily et al. 1998). The respiratory quotient (RQ) of both groups decreased over the 8 hr starvation period. The increased hepatic CPT-I due to starvation was significantly increased in nonsupplemented, but not in the choline supplemented guinea pigs. There were no significant differences in RQ during exercise, but there was 30% increase in the running time of the choline supplemented guinea pigs. There was significant loss of body fat and gain of protein by the choline supplementation (Daily et al. 1998).

In summary, the human study clearly showed that choline supplementation results in carnitine conservation as indicated by the reduced urinary carnitine excretion. Plasma carnitine values were not altered except in few individuals (Dodson and Sachan 1996). These finding were confirmed in the guinea pig model (Daily and Sachan 1995). It demonstrated that reduced excretion of carnitine is not an indicator of choline-induced carnitine deficiency. Instead, there is an increased carnitine accretion in skeletal muscle. A consequence of this shift of carnitine is related to lower carcass fat in choline supplemented guinea pigs. However, there is no evidence of enhanced fat oxidation as determined by the change in RQ values in choline supplemented guinea pigs under

normal and exercised conditions (Daily et al. 1998).

Carnitine supplementation

A. Physical performance

It is clear that carnitine has important functions in cellular metabolism in muscles during exercise. Muscle carnitine homeostasis is perturbed during exercise. There is theoretical basis for carnitine supplementation to improve exercise performance in healthy humans. Brass (2000) summarized the potential mechanisms for a beneficial effect of carnitine supplementation on exercise performance in healthy humans. These include: 1) enhance muscle fatty acid oxidation, 2) preserve muscle glycogen during exercise, 3) enhance acylcarnitine production, 4) activate pyruvate dehydrogenase via lowering of acetyl-CoA content, 5) improve muscle fatigue resistance and 6) replace carnitine losses during training.

The obligatory role of carnitine is the transfer of long-chain fatty acids across the mitochondrial membrane. It has been hypothesized that carnitine supplementation might increase the availability and capacity to transport fatty acids into mitochondria as well as subsequent fatty acid oxidation, thus making more adnosine triphosphate (ATP) available for work. If carnitine supplementation increases muscle fatty acid oxidation, this would delay the depletion of muscle glycogen and thus delay fatigue development (Marconi et al. 1985). Carnitine supplementation showed increased concentration of carnitine in rat muscle (Simi et al. 1990), but the effects of carnitine supplementation on humans' skeletal muscle accretion are not unequivocal. Arenas and coworkers (1991) reported that carnitine supplementation, 2 g/d for 6 mo, increased muscle carnitine content by 6 %

in endurance athletes and sprinters. However, other studies with shorter periods of supplementation, 5 g/d for 5 d (Sumida et al. 1989), 6 g/d for 7-14 d (Vukovich et al. 1994), or 4 g/d for 14 d (Barnett et al. 1994) showed no effect on muscle carnitine concentration, despite increases in plasma carnitine concentrations (Vukovich et al. 1994, Barnett et al. 1994). The reasons for this discrepancies may well fit the pharmacokinetic considerations of carnitine. Carnitine is taken up by muscle against a concentration gradient, i.e. approximately 0.05 mM in plasma and 1 mM in skeletal muscle. This gradient is so large that even a substantial oral intake would not result in a measurable change (Hawley et al. 1998). Furthermore, after acute administration of large doses of carnitine, most of the carnitine is rapidly eliminated in the urine (Brass et al. 1994). Considering its gradient, renal loss, low bioavailability of oral carnitine supplement (~15%) and slow trunover rate in skeletal muscle (191 h), it seems that very large dosing for a long period of time would be necessary to significantly affect muscle carnitine in healthy people.

In agreement with the hypothesis of enhancing fatty acid oxidation by carnitine supplementation, Broderick and coworkers (1992) demonstrated increased oxidative metabolism of glucose, and improved function of isolated rat hearts perfused with exogenous carnitine. Shifting substrate used in muscle from fatty acid to glucose allows more ATP generation per O_2 consumption (Wasserman and Whipp 1975). This mechanism (carnitine induced enhanced glucose oxidation) may be associated with the activation of pyruvate dehydrogenase that is related to reductions of acetyl-CoA by acetylcarnitine formation (Uziel et al. 1988).

Carnitine concentration in skeletal muscle has been reported to decrease during high intensity exercise (Hiatt et al. 1989) and in the non-professional athletes during 4 mo period of training (Arenas et al. 1991). It is one of the hypothesized benefit of carnitine supplementation; replacement of carnitine loss during training would enhance exercise performance. However, it has not yet established that carnitine availability might become a rate-limiting factor of fatty acid oxidation. No experimental studies are available to assess the functional significance of changes in carnitine concentration in skeletal muscle in humans.

The idea of improving muscle fatigue resistance by carnitine supplementation was supported by the in vitro animal studies (Brass et al.1993, Dubelaar et al. 1991). High carnitine concentrations were shown to increase force generation and decrease muscle fatigue. This mechanism is applicable to human exercise, and needs to be further investigated.

Previous studies of carnitine supplement effects on exercise performance in healthy humans are summarized in **Table 2-3**. It should be noted that the experimental designs in these studies varied a great deal. Carnitine was supplemented in various doses, routes and durations of treatment. Some of these may or may not give pharmacological benefits of carnitine to individuals. In addition, these studies involved populations of groups diverse in age, gender and different physical-training experience. Endpoints of these studies were either performance (i.e. VO_2max, athletic performance, running time, etc.) or metabolic indexes of fatty acid oxidation (i.e. RQ, lactate accumulation, etc.).

Short-term administration (up to 2 wk) of carnitine failed to show an increase in

TABLE 2-3

Summary of results from studies of carnitine on exercise performance[1]

Study	Subject	Daily Carnitine Dose	Treatment Duration	Finding
Marconi et al. (1985)	6 competitive walkers	4 g	2 wk	↑ VO2max
Greig et al. (1987)	9 untrained	2 g	2 wk	No effect
Oyono-Enguelle et al. (1988)	10 untrained	2 g	2 wk	No effect
Soop et al. (1988)	7 trained	5 g	5 d	No effect
Gorostiaga et al. (1989)	10 trained	2 g	28 d	↓ RQ
Drågan et al. (1989)	110 trained	3 g	3 wk	↑ VO_2max, ↓FFA ↓ triglycerides
Siliprandi et al. (1990)	10 trained	2 g	1 dose 1 hr prior to test	↓ postexercise lactate & pyruvate
Vecchiet et al. (1990)	10 trained	2 g	1 dose 1 hr prior to test	↑ VO_2max, ↓ lactate
Wyss et al. (1990)	7 untrained	3 g	7 d	No effect
Natali et al. (1993)	12 untrained	3 g Intravenously	1 dose 40 min prior to test	↑ fatty acid oxidation during recovery
Trappe et al. (1994)	20 trained	4 g	7 d	No effect
Brass et al. (1994)	14 untrained	~1 g Intravenously	1 dose at start of test	No effect

[1] Abbreviation used: FFA, serum free fatty acids; RQ, respiratory quotient; VO_2max, maximal oxygen consumption.

TABLE 2-3 (continued)

Summary of results from studies of carnitine on exercise performance[1]

Study	Subject	Daily Carnitine Dose	Treatment Duration	Finding
Vukovich et al. (1994)	8 untrained	6 g	7-14d	No effect
Colombani et al. (1996)	7 untrained	4 g	Day of event	No effect
Zając et al. et al. (2001)	12 trained	2 g	8 wk	↑ VO2max, ↑ speed ↑ power, ↓ body fat
Volek et al. (2002)	10 trained	2 g	3 wk	↓ purine degradation, ↓ free radical ↓ tissue damage, and ↓ muscle soreness during recovery

[1] Abbreviation used: FFA, serum free fatty acids; RQ, respiratory quotient; VO_2max, maximal oxygen consumption.

exercise capacity (Colombani et al. 1996, Greig et al. 1987, Trappe et al. 1994, Soop et al. 1988), with few exceptions. As shown in rats (Berardi et al. 2000) and humans (Brass et al. 1994), administration of a single dose of carnitine may not be able to increase the carnitine skeletal muscle. On the other hand, if it were possible to increase the skeletal muscle carnitine content, an increase in physical performance could be expected.

Few studies have reported beneficial effects of short-term carnitine administration (up to 2 wk) on exercise performance (Marconi et al. 1985, Siliprandi et al. 1990, Vecchiet et al. 1990). Exercise-induced lactate accumulation was reduced by carnitine

supplementation (Siliprandi et al. 1991, Vecchiet et al. 1990). Siliprandi et al. (1990) found that plasma lactate and pyruvate concentrations were decreased after exercise in the carnitine-supplemented subjects. This decrease was accompanied by an increase in plasma acetylcarnitine (AC). The authors speculated that carnitine administration (2 g) 1 hr before high intensity exercise would stimulate pyruvate dehydrogenase, reducing lactate concentration and forming acetyl-CoA, as indicated increasing AC concentration. Vecchiet and coworkers (1990), using a similar experimental protocol, reported that supplementation (2 g/dose) significantly increased VO_2max and reduced lactate concentration. In another study, 3 g of intravenously injected carnitine lead to a significantly higher lipid oxidation rate during an exercise task, comprised of 40 min of aerobic exercise followed by 2 min of anaerobic exercise (Natali et al. 1993).

Long-term administration (4 wk or longer) of carnitine showed an increase in exercise capacity (Drågan et al. 1989, Gorostiaga et al. 1989, Marconi et al. 1985, Volek et al. 2001, Wyss et al. 1990, Zając et al. 2001). Gorostiaga and coworkers (1989) found that 28 d of carnitine supplementation, 2 g/d, resulted in a lower RQ during exercise at 66% VO_2max compared to a placebo group. Carnitine supplementation increased VO_2max following 2 to 3 wk of carnitine supplementation, 3 to 4 g/day in trained athletes (Drågan et al. 1987, Marconi et al. 1985). Work by Zając and coworkers (2001) provided evidence for positive effects of carnitine supplementation, 2 g/d for 8 wk in trained athletes. Speed and power were assessed by the 20 m sprint, using electronic photocells, and the vertical jump using the vertex device, respectively. Body fat content was significantly reduced in the supplemented athletes and showed improvement in speed

and power. In another study, carnitine supplementation (2 g/d for 3 wk) showed a favorable effect on assisting recovery from high-repetition squat exercise (Volek et al. 2002). There was significantly less accumulation of markers of purine degradation, free radical formation and tissue damage, and muscle soreness compared to placebo.

Studies that have used longer supplementation periods have reported no effects of carnitine supplementation (Greig et al. 1987, Oyono-Enguelle et al. 1988). Greig et al. (1987) reported no benefit from 2 g/d of carnitine for 4 wk. They found no changes in VO_2max, RQ, or plasma lactate and β-hydroxybutarate concentration. In another study, Oyono-Enguelle gave 2 g/d of carnitine for 4 wk, showed that plasma total and free carnitine concentration reached a plateau about 9-20 d after the supplementation. There was no change in VO_2max, and RQ in the untrained subjects. The elevated serum carnitine concentration returned to the baseline values at 6-8 wk after termination (Oyono-Enguelle et al. 1988).

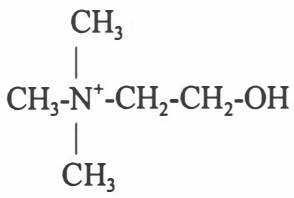
Given these diverse findings, it is difficult to draw definitive conclusions about the ability of carnitine supplementation in improving exercise performance in healthy humans. Although, there are insufficient data to support carnitine as an ergogenic aid, there are also insufficient data to ignore the effects of carnitine on exercise performance. It appears that additional comprehensive human studies are needed to evaluate the effects of carnitine supplementation on lipid metabolism during exercise, before general use of carnitine can be recommended for the stated promises.

B. Weight loss and management

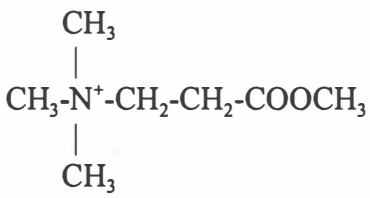
The relationship of the carnitine homeostasis to the critical metabolic processes of fatty acid oxidation has led to speculation about the potential benefits in obese persons undergoing weight reduction. The most frequently used strategy of weight loss is low calorie diets together with physical exercise. Both of these measures, diet and exercise, may induce reduction of carnitine in a body. Carnitine supplementation is known to provide a lipotropic effects. Carnitine increases the hepatocellular uptake of carnitine and fatty acids under the circumstance of starvation (Kispal et al. 1987) and alcohol induced fatty liver (Sachan et al. 1984). Low levels of carnitine may cause triglycerides to rise, weight to gain, and may lead to heart disease due to possible impairment of β-oxidation. These speculations may be justified on theoretical grounds, but they are not validated through in animal or human studies. In addition, comprehensive clinical trials in humans are lacking. At this time there is no experimental evidence of potential promotion of fat oxidation by carnitine in obese individuals.

CHOLINE

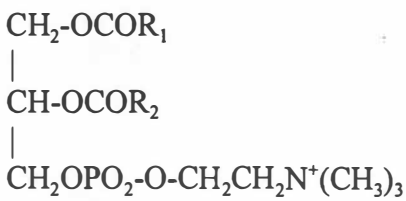
Choline, trimethyl-β-hydroxyethylammonium, is similar to carnitine in structure with a molecular weight of 121.2 g/mol (**Figure 2-4**). Choline is widely distributed in our foods and is essential for normal function of all cells (Zeisel 1981). Choline and its metabolites are important for providing structure of cell membranes, facilitating transmembrane signaling, manufacturing the neurotransmitter responsible for memory



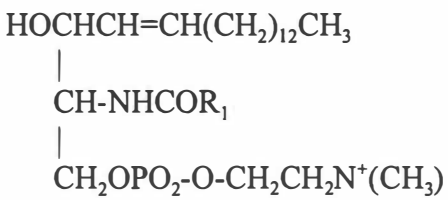
Choline



Acetylcholine



Phosphatidylcholine (Lecithin)



Sphingomyelin

FIGURE 2-4 *Structures of choline and related metabolites.* R_1 and R_2 represent fatty acyl groups.

and muscle contractions, and metabolizing fat and cholesterol. Choline is also known for a lipotropic compound, preventing deposition of fat in the liver (Best and Huntsman 1932).

Most choline is stored in the animal tissues such as brain, kidney and liver primarily as phosphatidylcholine (lecithin) and spingomyelin (Houtsmuller 1979). Other important metabolites of choline are listed in **Table 2-4**.

Choline was first discovered by Strecker in 1862, when it was isolated from the bile of a pig (Strecker 1862). The structure of choline was determined and synthesized in 1866 (Baeyer 1866). Choline was recognized as a component of phospholipids, but the pathway for its incorporation into phosphatidylcholine (lecithin) was not elucidated until 1956 (Kennedy and Weiss 1956). Early studies with insulin led to study of choline as an important nutrient. Metabolism of depancreatized dogs was demonstrated to prevent hepatic damage after the administration of raw pancreas, where the active component was pancreatic phosphatidylcholine (Allan et al. 1924, Best and Hershey 1932, Hershey 1930). The development of fatty livers in rats fed a low-choline, high-fat diet was prevented by choline supplementation (Best and Huntsman 1932). Subsequently, Best and co-workers showed that choline supplementation prevented the formation of fatty liver in chronic ethanol-fed rats (Best et al. 1949).

Dietary supplementation with soybean polyenylphosphatidylcholine, a purified extract comprising 94-96% of polyunsaturated phosphatidylcholines, prevented the development of alcohol-induced fibrosis and cirrhosis in nonhuman primates (Lieber et al. 1994). In another study the same authors showed attenuation of fatty liver and

TABLE 2-4

Choline-containing compounds and their physiologic importance [1]

Compound	Physiological function
Choline	Methyl group metabolism, constituent of various compounds (see below)
Betaine	Methyl group donor, renal osmolyte
Acetylcholine	Neurotansmitter used by cholinergic nerves
Phosphatidylcholine (Lecithin)	Necessary building block of biomembranes Component necessary for hepatic VLDL secretion Choline reserve; provides 1,2-*sn*-diacylglycerol (DAG), fatty acids, phosphorylcholine for cell signaling
Sphingomyelin	Necessary building block of biomembranes, Participate in cell signaling
Platelet-Activating Factor	Activation of platelets, inflammatory and immune response, egg implantation, induction of labor
Lysophosphatidylcholine	Putative second messenger modulating protein kinase C (PKC) activity
Lysosphingomyelin	Putative second messenger mediating growth-factor actions, mitogen
Glycerophosphocholine	Renal osmolyte
Phosphocholine	Intracellular storage pool of choline
Plasmalogen	Found in sarcolemma, the membrane of cardiac muscle; breakdown may contribute to sequelae of myocardial infarction

[1]Modified from Canty and Zeisel (1994), Zeisel and Blusztajn (1994).

hyperlipemia by the administration of polyenylphosphatidylcholine for 3 wk in rats (Navder et al. 1997). This finding suggested that the preventive effect of polyenylphosphatidylcholine was, at least in part, mediated by the correction of hepatic mitochondrial fatty acid oxidation.

There are exciting observations that greatly enhance our understanding of the metabolism, functions, physiologic effects and pharmacology of choline. In 1946, Copeland and Salmon first reported that prolonged choline deficiency developed liver cancer in rats (Copeland and Salmon 1946). Subsequently, numerous studies showed that a choline-deficient diet promotes liver carcinogenesis in animal models (Ghoshal and Farber 1984, Locker et al. 1986, Newberne and Rogers 1986). A number of possible mechanisms have been proposed for the development of cancer by choline deficiency. Some of these include increased cell proliferation with an associated rate of DNA synthesis (Rushmore et al. 1986), induced apoptosis in hepatocytes (Albright et al. 1996), decreased DNA methylation and repair (Locker et al. 1986), increased lipid peroxidation and free radical damage (Rushmore et al. 1984, Ghoshal and Farber 1993), and the formation of an excessive amount of DAG, which overstimulates PKC (da Costa et al. 1993, da Costa et al. 1995).

Since mid-1970, several researchers have reported that administration of choline accelerates the synthesis and release of acetylcholine by neurons (Cohen and Wurtman 1975, Haubrich et al. 1974, Rada et al. 1994, Wecker 1986 and 1990b). This relationship of choline and acetylcholine synthesis promoted an interest in dietary choline supplementation and brain function. Recent research has shown a beneficial effect of

prenatal choline supplementation in brain development in rats. Dietary choline supplementation during embryonic days 11 to 17, which is a period of high cell division and programmed cell death in fetal rat brain, leads to a life-long improvement of visuo-spatial memory performance in the offspring (Meck et al. 1989, Meck and Williams 1999). Possible mechanism of this memory facilitation induced by prenatal choline supplementation are; 1) alterations of acetylcholine synthesis and release (Blusztajn and Wurtman 1989, Cermak et al. 1998), 2) enhancement of hippocampal function (Jones et al. 1999, Montoya and Swartzwelder 2000, Pyapali et al. 1998) and 3) changes in choline containing phospholipids cell signaling event in the brain (Zeisel and Blusztajn 1994).

In 1998, choline was classified as an essential nutrient for humans by the Food and Nutrition Board of the Institute of Medicine of the National Academy of Science (Food and Nutrition Board 1998). This official recognition of the importance of choline was the result of many studies, that identified choline deficiency in humans under certain clinical conditions and disorders (Buchman et al. 1992, Sheard et al. 1986, Shronts 1997). When healthy humans consumed an experimental diet deficient in choline for 3 wk, they depleted stores of choline in tissues and developed signs of liver dysfunction (Zeisel et al. 1991). Thus, choline is an essential nutrient for humans.

Dietary Sources of Choline

Choline is widely distributed in many foods (**Table 2-5**), primarily in the form of lecithin (Engel 1943, Wurtman 1979). Lecithin is approximately 13% choline by weight. The richest food sources of choline are egg yolk and organ meats. Other good sources of choline include beef, nuts, leafy greens, soy beans, seed oils, and dairy products.

TABLE 2-5

Choline and choline ester content in common foods [1-2]

Food	Choline	Lecithin (mg/serving)	Sphingomyelin
Meat Products			
Beef liver (3.5 oz)	600	3363	135
Beef steak (3.5 oz)	80	466	69
Lamb chop (3.5 oz)	NR[3]	753	NR
Ham (3.5 oz)	120	800	NR
Trout (3.5 oz)	84	580	NR
Dairy Products			
Whole milk (1 cup)	3.0	28	15
Cheese (1 oz)	NR	14-28	NR
Margarine (1 oz)	0.08	1.4-2.8	0.04
Fruits and Vegetables			
Apple (1 medium)	0.4	4	0.2
Banana (1 medium)	4.4	0.7	0.4
Carrot (1 oz)	2-3	1.5-2.3	NR
Cabbage (1 oz)	13	NR	NR
Cauliflower (1 oz)	22	0.6	NR
Cucumber (1 oz)	0.63	0.22	0.08
Iceberg lettuce (1 oz)	8.5	3.0	9.0
Kale (1 oz)	25	0.6	NR
Orange (1 medium)	3.0	53	2.5
Potato (1 medium)	6.0	26.0	2.1
Spinach (1 oz)	1.1-4.9	1.7-4.0	NR
Tomato (1 oz)	1.3	0.2	0.1
Bread and Rice			
Oat meal (1 oz)	37	186	NR
Polished rice (1 oz)	NR	167	NR
Whole wheat bread (1 slice)	3.0	1.0	0.03
Beverages			
Coffee (6 oz)	19	2.0	3.0
Grape juice (6 oz)	9.0	2.1	0.7
Miscellaneous			
Egg (1 large)	0.2-0.3	256-350	82
Peanut butter (2 Tbsp)	13.0	97.4	0.2
Peanuts (1 oz)	13.2	107	1.6
Soybeans (1 oz)	68	423	NR

[1]Values are mg/serving. Serving size is indicated in the parentheses.
[2]Modified from Canty and Zeisel (1994), Wurtman (1979).
[3]NR; Data were not reported in the above referenced papers.

Choline, in the form of lecithin, is also added to many processed foods as an additive or emulsifier. Lecithin helps disperse water in oil, and it increases the wetting power of cocoa powder in chocolate bars (Wurtman 1979).

Human milk, bovine milk, and bovine milk-derived infant formulas contain approximately 1-2 mmol/L (121-242 mg/L) choline and choline containing compounds (Holmes-McNary et al. 1996, Zeisel et al. 1986). Human milk contains choline in the form of choline, phosphocholine, glycerophosphocholine, sphingomyelin, and phosphatidylcholine. Human milk has significantly higher phosphocholine than either bovine milk or infant formulas. Phosphatidylcholine and sphingomyelin concentrations are similar in both human and bovine milk. Soy-derived infant formulas have lower sphingomyelin concentration and higher phosphatidylcholine concentration than human milk or bovine milk-derived infant formula (Holmes-McNary et al. 1996).

It is estimated that average choline intake (free choline and choline esters) by human adults is in a range of 6-10 mmol (700-1200 mg) per day (Wurtman 1979, Zeisel 1999). Daily consumption of choline can be affected by many factors such as individual food habits, religion, ethnic background, economic status, geographic location, diet and health considerations, and personal food preferences (Wurtman 1979). Diets high in choline can be consumed by many normal healthy persons, if they include foods such as beef liver, egg and other processed foods in their diets (Shronts 1997).

Human infants consume a choline-rich diet. Assuming that an infant drinks 800 mL/d, the daily choline intake would be approximately 1.2 mmol (145 mg). This intake per kg body weight is 2-3 times more choline than average adults daily choline intake

(Zeisel 1999). Because human milk and colostrum are rich sources of choline, the Infant Formula Act of 1980 sets choline chloride at 7 mg/100 kcal as a requirement for all infant formulas in the United States (American Academy of Pediatrics 1985). Importantly, the Food and Nutrition board recognized that the growing fetus and neonate need increased amount of choline and issued separate choline intake guidelines for nonpregnant, pregnant and lactating women, which are 425, 450 and 550 mg/d, respectively (Food and Nutrition Board 1998).

Choline Homeostasis

Serum and tissue choline concentration depends on the dietary intake of choline. In human adults who are consuming common foods plasma choline concentrations fluctuate over approximately 2-3-fold range, 7-20 μmol/L (Zeisel 1992, Zeisel et al. 1980b). Plasma choline concentration was elevated 300-400% after a single dose of choline chloride in healthy humans (Hirsche et al, 1978, Hollister et al, 1978). Large oral doses of lecithin (0.3 g/kg body weight) increased serum choline concentration 400% and its significant elevation was sustained for 12 hours (Zeisel et al. 1980b). The prolong effects of lecithin may be due to it's resistance to degradation to trimethylamine within the gut (Zeisel 1981).

Lactating women consuming a low choline diet produced milk with lower choline than in those consuming more adequate diet high in choline (Zeisel et al 1982). When lactating rat dams were provided with either a control, choline-deficient, or choline-supplemented diet, their milk composition reflected the choline content of their diets. Milk phosphocholine concentrations were increased by 4-fold with the choline

supplemented diet in the lactating rats (Holmes-McNary et al. 1996). Thus, dietary intake of milk choline contributes to the maintenance of high serum choline concentration in the neonates, which have 4-7 times higher serum choline concentration than adult humans or rats (Zeisel et al. 1980a).

Choline Biosynthesis

Diet is not the only source of choline. De novo synthesis of choline molecules occurs by two pathways (**Figure 2-5**). In the first, phosphatidylethanolamine is sequentially methylated to form phosphatidylcholine, using S-adenosyl methionine (SAM) as methyl donor (Vance and Ridgway 1988). This is the only pathway for de novo synthesis of new choline moiety in adult mammals (Bremer and Greenberg 1961). It is catalyzed by the membrane bound enzyme phosphatidylethanolamine-N-methyltransferase (EC 2.1.1.17), which is active in liver, but it is also found in many other tissues, including brain (Blusztajn et al. 1979), and the mammary gland (Yang et al. 1988). In rats 15% of the daily requirement of choline, about 13 μmol/g of liver, is met by this methylation pathway (Bremer and Greenberg 1961, Schneider and Vance 1979). The activities of the methylation pathway are increased by choline deficiency and ethanol ingestion in rats (Fallon et al. 1961, Uthus et al. 1976).

Another pathway for phosphatidylcholine biosynthesis is the cytidine diphosphocholine (CDP) pathway. In this pathway choline is first phosphorylated, then converted to cytidine diphosphocholine (CDP-choline). This intermediate in combination with diacylglycerol, forms phosphatidylcholine. Within the liver most of the phosphatidylcholin is synthesized by the CDP pathway (Sundler and Akesson 1975).

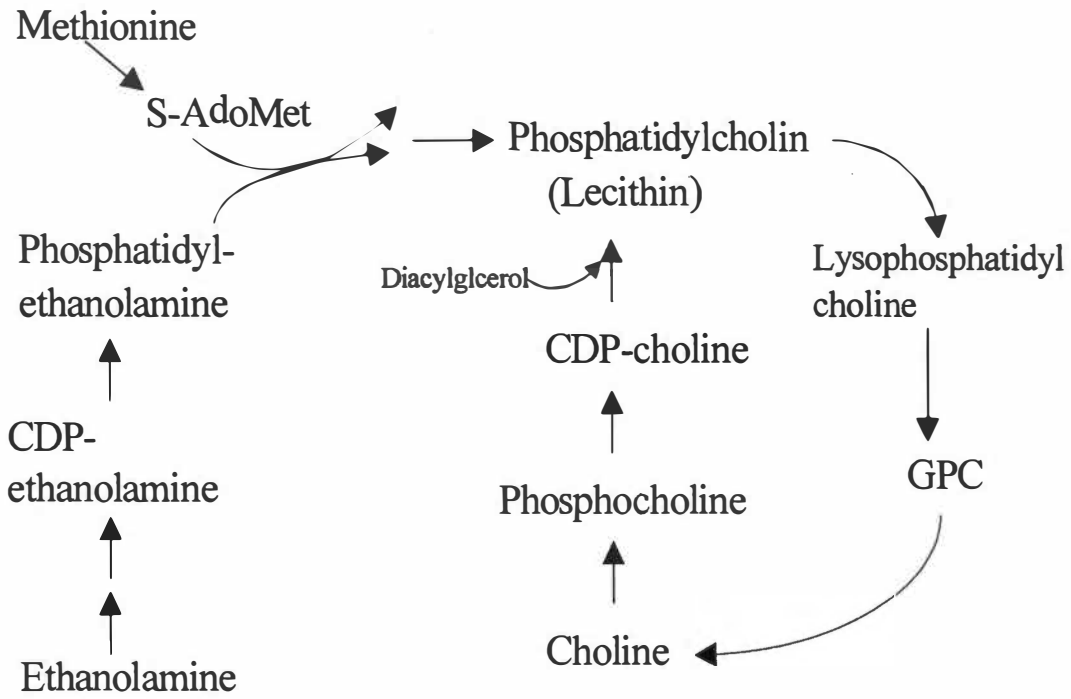


FIGURE 2-5 *Choline biosynthesis pathway.* CDP: cytidine diphosphocholine; GPC: glycerophosphocholine; S-AdoMet: S-adenosylmethionine (SAM)

The biosynthesis of phosphatidylcholine is regulated by the enzyme, phosphocholine cytidylyltransferase (EC 2.7.7.15). The content of phosphatidylcholine in the membrane also regulates the amount of enzyme bound to membranes. Binding of cytidylyltransferases to membranes increases when their phosphatidylcholine content decreases, whereas the reverse occurs when membrane phosphatidylcholine content increases (Watkins and Kent 1992, Jamil et al. 1993). These findings may explain why cytidylyltransferase activity increases in choline deficient hepatocytes (Yao et al. 1990).

Absorption

Choline is absorbed as free choline from the small intestine, mainly in the jejunum (Sanford and Smith 1971), and it enters the portal circulation. Unabsorbed choline is metabolized to betaine and trimethylamine by bacteria in the lower gut (De La Huerga and Popper 1951, Flower et al. 1972). As noted before, most dietary choline is in the form of phosphatidylcholine. Both pancreatic secretions and intestinal mucosal cells contain enzymes (namely phospholipase A_1, A_2, and B) that can hydrolyze phosphatidylcholine (Houtsmuller 1979, Parthasarathy et al. 1974). The other choline-containing phospholipid, sphingomyelin, is not degraded by intestinal microflora, but is taken up by the enterocyte, then extensively hydrolyzed in the mucosal cell. The enzyme sphyingomyelinase is found in the liver, which hydrolyzes sphyingomyeline and forms phosphorylcholine and ceramide (Heller and Shapiro 1966). In the brain, kidney, liver and spleen phospholipase C acts on syphingomyelin, forming phosphorylcholine. Then, they can be cleaved by phosphatase, producing free choline (Fujino 1952, Illingsworth and Portman 1973).

The ingestion of large amount of choline chloride causes the unpleasant smell, fish-odor syndrome, but this is not observed when lecithin is administered (Zhang et al. 1999). The explanation for this beneficial effect of lecithin is that lecithin is hydrolyzed by phospholipase A, found in pancreatic juice, to produce lysolecithin in the intestinal lumen. Further degradation does not occur in the upper gut and there is no release of choline available for the intestinal microflora (Houtsmuller, 1979).

Transport and Tissue Uptake

Most tissues take up choline by a combination of diffusion and carrier mediated transport systems (Haubrich et al. 1975). Choline is transported via an active uptake system in the liver where it is converted to betaine, phosphocholine and phosphatidylcholine (Zeisel 1990). Kidneys also oxidize choline to form betaine, which may serve as an important osmoprotectant within the kidney (Grossman and Hebert 1989). Mammary cells accumulate choline by both energy-dependent and passive transport mechanisms. Choline is delivered to the fetus across placenta, where it is stored in large amounts as acetylcholine. All of the acetylcholine synthesized within the placenta must be made by using exogenous choline, since the placenta lacks the ability to synthesize choline de novo. Acetylcholine within the placenta is not associated with neurons, and its function is not known (Welsch 1976).

Choline is used by brain cells for the synthesis of phophatidylcholine and other choline-containing lipids. The brain lacks the metabolic machinery to synthesize choline de novo. Therefore, the brain is dependent on circulating free choline and choline-phospholipid in plasma and the liver for its supply (Freeman and Jenden 1976). The choline-phospholipids in cholinergic neurons provide a precursor pool of free choline that can be used for the synthesis of the neurotransmitter, acetylcholine (Wecker 1990a). Free choline is transported across the blood brain barrier by a specific carrier (Cornford et al. 1978). Subsequently the choline is transported into brain cells via either a low-affinity or a high-affinity choline transport system (Yamamura and Snyder 1973).

Within cholinergic terminals, choline is coupled with glucose-derived acetyl-CoA to form the neurotransmitter acetylcholine.

Metabolism

Choline can be acetylated, oxidized and phosphorylated. A small fraction of dietary choline is acetylated to form acetylcholine by a reversible reaction (**Figure 2-6**).

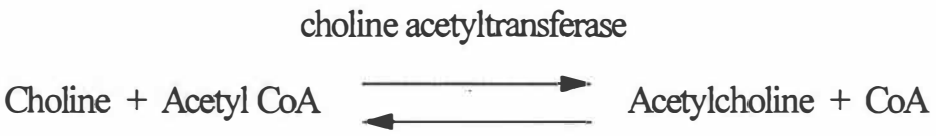


FIGURE 2-6 *Acetylcholine formation.*

The reaction is catalyzed by choline acetyltransferase (EC 2.3.1.6), a cytoplasmic enzyme that is highly concentrated in the terminals of cholinergic neurons (Haubrich et al. 1975). There is considerable interest in the role of choline as a precursor of acetylcholine. Exogenous choline administered by an acute injection or through dietary supplementation raises: 1) serum choline concentration, 2) brain choline and acetylcholine and 3) the acetylcholine at cholinergic neurons (Cohen and Wurtman 1975). Further studies are needed to elucidate the mechanisms that would account for increased synthesis of acetylcholine by choline supplementation. This information will allow the development of therapeutic strategies for the treatment of neuropsychiatric disorder, such as Alzheimer's disease (Wecker 1990a).

Oxidation of choline to betaine is a major pathway for choline catabolism and it is active in liver and kidney, but it does not seem to be present in brain and muscle. (Bernheim and Bernheim 1933). This process involves the following steps: 1) transport of choline from the cytosol to mitochondria, 2) oxidation of choline to betaine aldehyde by choline dehydrogenase (EC 1.1.9.91), 3) oxidation of betaine aldehyde to betaine by betaine aldehyde dehydrogenase (EC 1.2.1.8) and 4) release of betaine from the matrix into the cytosol.

Betaine, the end product of choline oxidation, cannot be reduced to form choline. However, it can donate a methyl group to homocysteine, forming dimethylglycine and methionine. The availability of choline as a methyl donor is the major factor that determines how quickly a choline-deficient diet leads to a pathologic condition (Zeisel 2001). Therefore, the metabolisms of methionine and folate is closely interrelated with choline in methyl group metabolism (**Figure 2-7**). Rats fed a diet deficient in choline develop diminished tissue concentration of methionine and SAM (Barak et al. 1982, Zeisel et al. 1989) and total hepatic folate (Selhub et al. 1991).

The phosphorylation of choline is catalyzed by choline kinase (EC 2.7.1.32) using adenosine triphosphate as the phosphate donor. It is a soluble, cytosolic enzyme, widely distributed in tissues including liver, brain, kidney and lung (Brophy et al. 1977). Phosphorylation of choline is the first step in the CDP-pathway for the synthesis of phosphatidylcholine (lecithin). In this pathway cytidine triphosphate and phosphocholine are combined and forms CDP-choline and inorganic phosphate. This is a rate limiting step for the pathway. The CDP-choline is subsequently combined with diacylglycerol

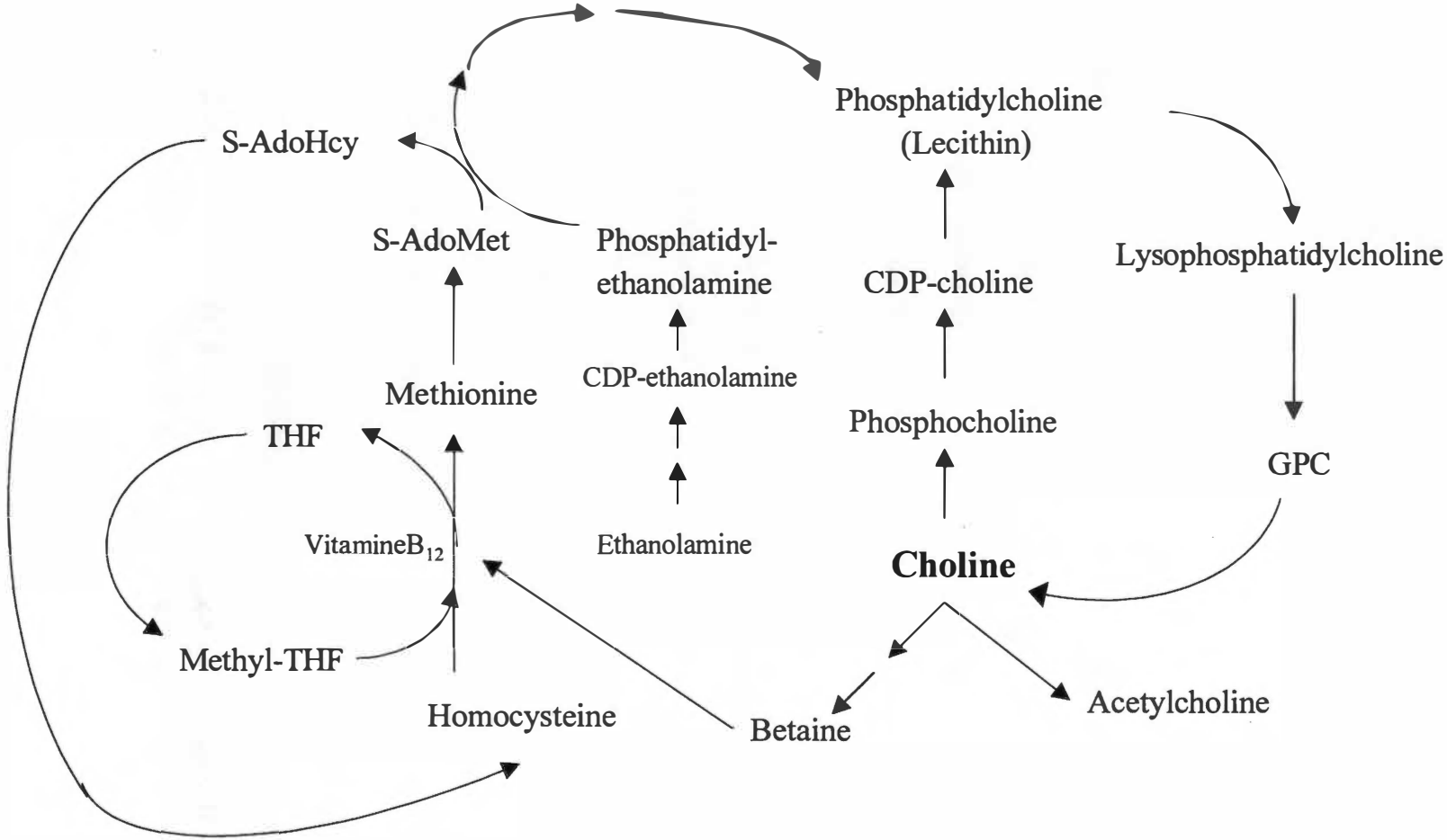


FIGURE 2-7 *Interrelationship of choline, methionine, folate and vitamin B_{12}.*[1] CDP: cytidine diphosphocholine, GPC: glycerophosphocholine, S-AdoHcy: S-adenosylhomocysteine, S-AdoMet: S-adenosylmethionine, THF: Tetrahydrofolate. [1] Modified from Zeisel and Blusztajn (1994).

and forms lecithin (Figure 2-5). The rate of phosphorylation of choline in forming phosphocholine is slower (1 μmol/hr/g) than the rate of oxidation of choline in forming betaine (9 μmol/hr/g) in rat liver (Weinhold and Sanders 1973).

Functions

Choline serves several biological functions in the body. Choline is a component of a number of important metabolites (Table 2-4). The formation of betaine from choline provides an important source of methyl groups for generating methionine (Figure 2-7). Betaine concentrations in livers of choline deficient rats are significantly reduced (Barak and Tuma 1983), as are total folate concentrations (Horne et al. 1989). A disturbance in choline metabolism results in changes in methionine and folate metabolism. Recently, oral administration of bataine for 1 y showed significant biochemical and histological improvement in patients with nonalcoholic steatohepatitis. There were significant improvements in the degree of steatosis, necroinflammatory grade, and stage of fibrosis. Authors suggested that increased SAM levels by betaine may play a role in marked improvement in these patients (Abdelmalek et al. 2001).

Another biochemical function of choline is the formation of phosphatidylcholine (lecithin). Due to the function of choline metabolites in membrane structures, a choline deficient diet results in a variety of phospholipid-related dysfunctions, such as fatty liver and impairment of lipoprotein metabolism. Choline acts as a lipotropic agent, and therefore, it decreases the rate of deposition and accelerates the rate of removal of fat from the liver (Best et al., 1950). During choline deficiency, large amounts of lipid, mainly the form of triglycerides can accumulate in the liver (Yao and Vance 1988). The

triglycerides produced by the liver are delivered to other tissues, mainly in the form of VLDL. Phosphatidylcholine is a required component of VLDL, and other phospholipid compounds similar to phospatidylcholine can not substitute for phosphatidylcholine (Yao and Vance 1989). Chronic ingestion of a diet deficient in choline diminishes synthesis of phosphatidylcholine, and that can result in intracellular accumulation of triglycerides, due to continuing triglycerides synthesis, regardless of choline status of rats. A soy phosphatidylcholine diet has been reported to be a potent hypocholesterolemic agent because it reduces the absorption of cholesterol and increase the excretion of sterols in rats (Omullane and Hawthorne 1982). Consistent hypocholesterolemic of phosphatidylcholine effects have been shown in hypercholesterolemic rabbits (Mastellone et al. 2000, Polichette et al. 2000) and in patients with hyperlipidemia after the dietary lecithin supplementation (Kesaniemi and Grundy 1986, Polichetti et al. 1998).

Recent studies show that choline phospholipids (i.e. phosphatidylcholine, sphingomyelin and their metabolites) play important roles in signal transduction (Zeisel 1993). Signal transduction begins with the binding of extracellular stimulus, such as a hormone, antigen and neurotransmitter, to a receptor on the cell membrane surface. This activates a guanosine triphosphate (GTP)-binding protein (G-protein), which then, activates phopholipase C (PLC), phospholipase D (PLD), or phospholipase A (PLA) within the plasma membrane. Phosphatidylcholine is broken dawn by lecithin-specific PLC, PLD, or PLA. PLC generates DAG and phosphocholine; PLD generates phosphatidic acid and choline; and PLA generates free acids (e.g. arachidonate) and lysophosphatidylcholine. All the hydrolysis products, except choline, may act as second

messengers (Besterman et al. 1986, Exton 1990). DAG, fatty acids, and lysophosphatidylcholine stimulates PKC. By catalyzing phosphorylation reactions, PKC can activate or inactivate a broad range of proteins including, membrane proteins, receptor proteins, contractile and cytoskeletal proteins, and microtubule-associated proteins (Nishizuka 1986).

In cell membranes, phosphatidylcholine exists in a variety forms due to fatty acyl chains. Therefore, hydrolysis of phosphatidylcholine can generate multiple forms of diradylglycerides (DRG) in addition to DAG. These subclasses of DRG may differ in their ability to activate PKC and may provide another mechanism for signal specificity (Cabot and Jaken 1984, Daniel et al. 1988).

It has been recognized only recently that choline sphingolipids are necessary for the survival and growth of cells (Hanada et al. 1992). Metabolites of sphingolipids also function as second messengers that inhibit the activity of PKC and terminate the signaling cascade (Merrill and Jones 1990). Sphingomyelin is hydrolyzed by sphingomyelinase, producing ceramide and phosphocholine. Ceramide is a potent inhibitor of cell growth as well as a mediator of apoptosis in response to extracellular agents and insults (Obeid et al. 1993). Sphingosine, metabolite of ceramide, is a potent inhibitor of PKC, cell growth, and it also exhibits antitumor properties (Hannun et al. 1991). Lysosphingomyelin, another product of sphingomyelinase hydrolysis, also inhibits PKC (Hannun and Bell 1989).

In summary, choline phospholipids have the unique functions in cellular regulation in addition to their essential roles as structural components of membranes and

lipoproteins. The new finding of the role of choline phospholipids in signal transduction may provide a molecular basis for the influence of choline in normal physiological and disease processes, such as cancer and Alzheimer's diseases. Additional research is required to determine how dietary and pharmacological intake of choline, phospatidylcholine or other phospholipids may favorably alter the phospholipid metabolism in humans.

Choline Supplementation

A. Nervous system and maintenance of normal lipid profile

Choline has been used to treat human diseases, primarily those of the nervous system and the liver. It has been shown that choline supplementation increases not only serum choline levels, but also brain choline and acetylcholine contents in rats (Cohen and Wurtman 1975, Haubrich and Pflueger 1979). Furthermore, the administration of choline as well as lecithin, has been demonstrated to be effective in increasing serum choline levels in humans (Hirsch et al. 1978, Zeisel 1980b). These findings and the fact that acetylcholine is a neurotransmitter in the central nervous system were used as rational for the choline loading of the patients who exhibited a deficiency of cholinergic neurotransmission. The therapeutic strategy has been to increase brain choline above normal level by large pharmacological doses of choline in order to stimulate acetylcholine synthesis within nerve terminals (Chan 1991).

Choline was first tried as therapy for a patient with tardive dyskinesia that is a movement disorder generally associated with the long-term use of neuroleptics (Davis et al. 1975). The pathophysiology of this syndrome was involved in inadequate

neurotransmission at striatal cholinergic interneurons (Davis and Berger 1978). All neuroleptics used in the United States have a potential for producing tardive dyskinesia and the estimate of prevalence was reported from 0.5% to 50% (Jus et al. 1976). Oral administration of choline or lecithin have shown considerable success in the treatment of tardive dyskinesia (Growdon et al. 1977). Choline and lecithin has been used in the treatment of other disorders or symptoms associated with inadequate cholinergic transmission including: Huntington's chorea, Alzheimer's disease, mania, Gilles de la Tourette, and ataxia. The efficacy of choline/lecithin supplementation in these disorder/symptoms has not been as convincing as it has been in tardive dyskinesia.

As discussed in a previous section, choline plays an important role in the maintenance of normal lipid transport. The beneficial effects of choline supplementation on serum lipids have been inconsistent. There are reports of a hypocholesterolemic effect of choline. It is hypothesized that choline lowers blood cholesterol by stimulating biliary cholesterol and lecithin excretion (Herrmann 1947, Robins and Armstrong 1976). Contrary to this effect of choline, there are reports that oral administration of choline increases plasma cholesterol, phospholipid, high density lipoprotein (HDL) and low density lipoprotein (LDL) in rats (Olson et al. 1958). If choline does increase blood cholesterol concentration, it may be due to mobilization of lipids from hepatic stores.

Lecithin has been marketed as a dietary supplement, with claims that it lowers blood cholesterol. Lecithin supplementation has been shown to lower serum cholesterol and the incidence of atherosclerosis in rabbits fed a high cholesterol diet (Kesten & Silbowitz 1942). In healthy humans the effects of lecithin on serum cholesterol have

been inconsistent (Grundy 1980, Hirsche et al. 1978, Steiner and Domanski, 1944). In patients with type IIA hyperlipidemia, lecithin administration did not lower serum cholesterol (Greten et al, 1980). In another study involving patients with type IIA hyperlipidemia there was a significant increase in plasma level of apo AI-HDL (the major contributor of the transport and exchange of cholesterol between tissues and plasma) after a daily dietary supplement of purified soybean lecithin for 6 wk (Polichetti, et al. 1998). There was no hypocholesterolemic effect; however, plasma concentration of unesterified cholesterol was increased, which resulted in a decrease the ratio of esterifed:total cholesterol. Authors suggested that soybean lecithin led increased unesterified cholesterol, which in turn stimulated the reverse cholesterol transport by enhanced AI-HDL system (Polichetti, et al. 1998).

B. Physical performance

Choline has been used as a dietary supplement or ergogenic aid as a means of enhancing acetylcholine synthesis and release at the neuromuscular junction (Kanter and Williams 1995). Choline supplementation has been demonstrated to increase plasma choline levels (Haubrich et al. 1974, von Allwörden et al. 1993) and increase acetylcholine release at the neuromuscular junction (Bierkamper and Goldberg 1980, Blusztajn and Wurtman 1983). When an electrically stimulated skeletal muscle was placed in a low choline medium, acetylcholine release decreased. However, when the nerve was not stimulated, changing the choline concentration in the medium did not change acetylcholine release (Bierkamper and Goldberg 1980). Thus, in theory, increased acetylcholine availability should result in increased muscular contraction and a delay in

muscular fatigue.

Physical exercise has been shown to reduce plasma choline concentrations in human subjects (Conlay et al. 1992, von Allwörden et al. 1993). There was about 40 % decline in mean plasma choline in subjects running the Boston Marathon (Conlay et al. 1992). Authors suggested that neuromuscular transmission may have been impaired by the end of the race in these runners. In another study (von Allwörden et al. 1993), triathletes performed a bicycle exercise at an average speed of 35km/hr for 2 hr showed about 17% decline (range: 3.6-54.7%) in plasma choline concentration. When 0.2 g lecithin/kg body weight was administered 60 min prior to exercise, mean plasma choline concentrations remained at the same level as the initial values. It is speculated that the decline of choline may be due to the enhanced use of choline in the synthesis of acetylcholine or as an intramuscular methyl donor during a long duration with a high intensity of exercise. However, there are no direct experimental data to support these mechanisms in exercising humans.

A few studies investigated the potential use of choline supplement as an independent factor for improving physical performance (Sandage et al. 1992, Spector et al. 1995, Warber et al. 2000). The preliminary report by Sandage et al. (1992) demonstrated that the mean run time of a 20-mile run was significantly shortened by ingestion of 2.8 g choline citrate 1 hr prior to, and again after, completing 10 miles of a 20-mile ran. The supplementation prevented the decline of plasma choline which was observed in placebo treated runners. Spector et al. (1995) reported that trained cyclist did not benefit from choline supplementation to delay fatigue nor improve work performance

under the conditions of 150% and 70% of VO_2max at cadence of 80-90 rpm. Warber et al. (2000) determined the plasma choline metabolites and physical performance measures through a 4 hr load carriage treadmill exercise (3% grade at 5.6 km/hr) carrying a total load of 34.1 kg in 14 male soldiers with or without 8.425 g of choline citrate. Following the treadmill test, a run to exhaustion test and leg strength test using a barbell squat were performed. Their mean (± SD) VO_2max was 60.3 ± 2.3 ml/kg/min, which is higher than that previously reported for a similarly aged Amy population (Sharp et al. 2001, Vogel et al. 1986). Results showed that plasma choline, glycerophosphocholine, and phosphatidylcholine concentrations did not change from baseline to post-exercise for either treatment. There was no significant effects of choline supplementation on any performance tests in this study. Researchers concluded that no merit at this time to support a requirement of choline supplementation to reduce muscle fatigue and increase physical performance in physically active humans (Warber et al. 2000).

CAFFEINE

Caffeine, 1,3,7-trimethylxanthine, is a white odorless powder with a molecular weight of 194.2 g/mol (**Figure 2-8**). It is one of a family of purine derivative methylated xanthines, which are often referred to as methylxanthines or merely xanthines. While the class of methylxanthines is large, caffeine is the most common methylxanthine in nature (James 1991). Figure 2-8 shows the structure of caffeine, and its dimethylxanthine isomers: paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine).

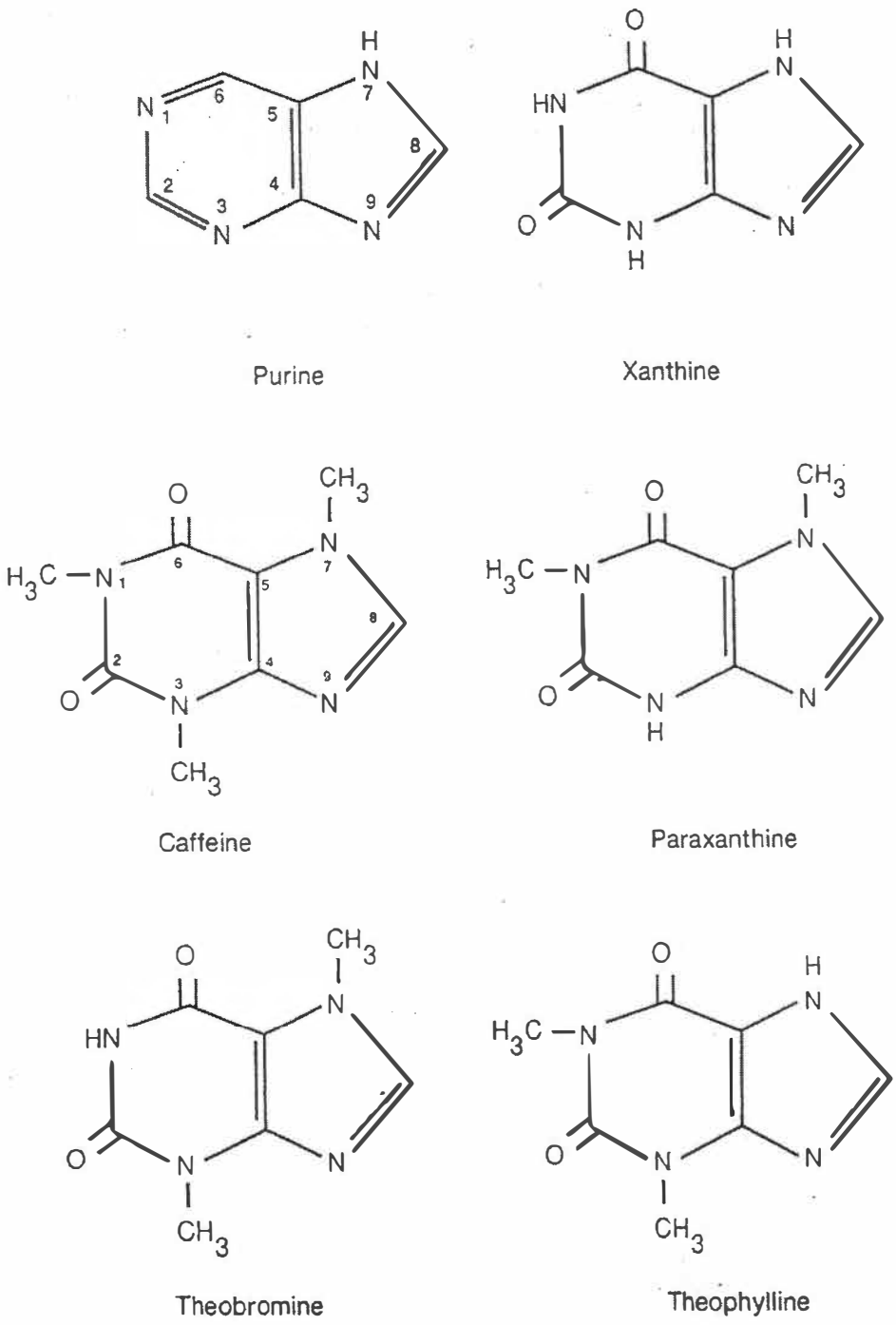


FIGURE 2-8 *Structures of purine, xanthine, caffeine (1,3,7-trimethylxanthine) and its dimethylxanthine isomers: paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine).*

Caffeine was first isolated from green coffee beans in Germany by Runge (1820) and confirmed by von Giese the same year. Theobromine, an immediate precursor in caffeine biosynthesis, was discovered in cocoa beans by Woskresensky in 1842 (Woskresensky 1842). In 1861, Strecker converted theobromine to caffeine, leading to the conclusion that theobromine was a dimethylxanthine, and caffeine was a trimethylxanthine (Strecker 1861). In 1888 Kossel isolated theophylline from tea leaves and identified it as a dimethylxanthine (Kossel 1888). The third dimethylxanthine, paraxanthine, was recently isolated from plants by Chou and Waller (1980). However, Salomon who isolated purine alkaloids from human urine, discovered paraxanthine for the first time in 1883 (Salomon 1883).

Caffeine is the most widely consumed psychophysiologically active substance in the world (Gilbert 1984). Today, more than five million tons of green coffee beans are produced annually in approximately 50 coffee-growing countries. The U. S. is the largest importing country and 1.1 million tons were imported in 1991 (National Coffee Association, 1991). That equals 3 billion dollars worth of green coffee beans (Lundsberg 1998). It is estimated that four out of five Americans consume caffeine knowingly or unknowingly every day (Harland 2000).

Although humans have consumed caffeine-containing plant products over the centuries, interest in caffeine among the general public and the scientific community was low before the late 1970s. Intensive investigations of caffeine started after the re-evaluaiton of the risk of caffeine for human health by a special committee on generally recognized as safe (GRAS) substances by the Food and Drug Administration (FDA) of

the U.S. Public Health Service in 1978 (Dews 1984). Since then, significant numbers of studies have been done to determine, if caffeine is potentially harmful to health. There have been many contradictory results, thus, no conclusive recommendation yet exists for the general population concerning how much caffeine can be consumed safely on a regular basis (James 1991).

The therapeutic use of caffeine has a long list due to its complex effects on different organ systems, i.e. cardiovascular, respiratory, gastrointestinal, neuroendocrine, and reproductive systems. General therapeutic applications, including effects and rationales for the use of caffeine, are listed in **Table 2-6**. Most of the applications listed in Table 2-6, except a few exceptions, have not been researched in sufficient detail to permit firm conclusions about the effects of caffeine. In addition to the clinical applications of caffeine listed in Table 2-6, it has been used as an ergogenic aid by athletes of all ages to get an edge on their competitors (Lamarine 1998). Caffeine is a common substance in the diets of most athletes, and it is also consumed in many new products, including energy drinks, energy bars, caffeinated waters, alcoholic beverages, diet aids and supplement pills (Graham 2001). In the last decade the popularity of caffeine in athletes has increased, probably because of the more convincing research on the ergogenic nature of caffeine (Graham et al. 1994, Jacobson & Kulling 1989). Recently, several investigators looked at the additive effects of caffeine and another drug (ephedrine) and nutrients (carnitine, choline, creatine) on enhancement of physical performance (Bell et al. 1998, Bell et al. 2002, Sachan and Hongu 2000, Vanakoski, et al. 1998). These studies are still in their early stage of the final conclusion. There have been

TABLE 2-6

Therapeutic use of caffeine [1]

Application	Effects of caffeine (references)
Neonatal apnoea	Successful treatment (Martin et al. 1986), used in preference to theophylline (Bairam et al.1987, Nahata et al. 1989)
Sperm motility	Increased by direct exposure to caffeine (Bunge 1973)
Cancer	Potentiating effect on the action of certain antineoplastic agent (Tomita & Tsuchiya 1989, Yunis et al. 1987)
Pain	Analgesic adjuvant (Beaver 1984), vasoconstrictor action on cerebral blood vessels (Mathew & Wilson 1985
Nutrition	Dietary sources of minerals: manganese, potassium, copper (Gillies & Birkbeck 1983)
Obesity	Thermogeinic drug (Acheson et al. 1980, Dulloo et al. 1989, Grollman 1930, Haldi, et al. 1941, Jung et al., 1981, Means et al., 1917), used in combination with other substances, i.e. ephedrine (Astrup et al. 1992), chromium (Pasman et al. 1997, Hoeger et al. 2000), carnitine (Décombaz et al. 1987), choline and carnitine (Hongu et al. 2000), inhibit adipocite proliferation (Bukowiecke et al. 1983)
Respiratory dysfunction	Relaxant effects on bronchial smooth muscle (Lima et al. 1989)
Postprandial hypotension	Pressor effects (Onrot et al. 1985)
Allergic reaction	Successful in the treatment of atopic dermatitis (Kaplan et al. 1978) and allergic rhinitis (Shapiro 1982)

[1]Modified from James 1991.

only a few studies to confirm the findings on these additive effects of caffeine and other drugs and nutrients. Thus, there has not been a final conclusions on the efficacy of the combination of these substances as an aid to athletic performance. The interest in caffeine's potential as an ergogenic aid will continue, and its use will not decline because it is an inexpensive, relatively safe and readily available legal drug.

Dietary Sources and Consumption of Caffeine

The primary dietary sources of caffeine are coffee and tea, with varying amounts in soft drinks, chocolate products, and medicine (Lundsberg 1998). The caffeine content of beverages, foods and medicines are listed in **Table 2-7**. As shown in the Table 2-7, caffeine content of coffee, tea and other products can vary substantially. Caffeine content in 5 oz of coffee is as high as 156 mg (Starbucks coffee) and 74 mg for percolated coffee. Instant coffee contains significantly less caffeine at 66 mg, and negligible caffeine is found in decaffeinated coffee. Thus, caffeine concentrations in coffee depends on the product (e.g. ground roasted vs instant), the method of brewing, the amount of coffee used, and the brewing time. In addition to these conditions, the caffeine content of the natural product (e.g. coffee beans, tea leaves) can significantly differ due to the plant variety and growing conditions of the plants (Kaplan et al. 1974). Recognizing that there are significant variability in caffeine content from a given sources, the FDA has selected the following standard range of caffeine per 5 oz cup of coffee and bagged tea: 75-155 mg and 28-44 mg, respectively (FDA 1980).

Americans consume 75% of caffeine from coffee, and the remaining 25% of caffeine from both tea and cocoa products (Barone and Roberts 1996). Caffeine content

TABLE 2-7

Caffeine content of beverages, foods and medicine[1]

Product	size	Caffeine (mg)	Product	size	Caffeine (mg)
Coffee			**Tea**		
Roasted and ground, percolated	5 oz	74	Average blend	5 oz	27
Roasted and ground, drip	5 oz	112	Green Tea	5 oz	19
Roasted and ground, decaffeinated	5 oz	2	Leaf Tea	5 oz	30-48
Instant	5 oz	66	Bagged Tea	5 oz	28-44
Instant, decaffeinated	5 oz	3	Tea, decaffeinated	5 oz	0
General foods			Instant Tea	5 oz	9
International swiss mocha	8 oz	55	Lipton Iced Tea, assorted	16 oz	35
International Viennese chocolate	8 oz	26	Lipton Natural Brew Iced Tea, unsweetened	8 oz	25
Maxwell house regular, drip	5 oz	69	Lipton Natural Brew Iced Tea, sweetened	8 oz	15
Cappuccino, amaretto	8 oz	25	Lipton Natural Brew Iced Tea, diet	8 oz	10
Cappuccino, French vanilla, or Irish cream	8 oz	45	Lipton Natural Brew Iced Tea, decaffeinated	8 oz	<5
Cappuccino, decaffeinated	8 oz	3	Nestea pure sweetened Iced Tea	8 oz	17
Starbucks coffee, short	8 oz	250	Nestea pure lemon, Sweetened Iced Tea	8 oz	11
Starbucks coffee, tall	12 oz	375	Arizona Iced Tea, assorted varieties	16 oz	15
Starbucks coffee, grande	16 oz	550			
Starbucks caffe, Americana	8 oz	35			

[1]Values are mg/serving size. Modified from Barone and Roberts 1996, FDA 1980, Gilbert 1981, Nutrition action health letter 1996, Physicians' Desk Reference (PDR) 2000.

TABLE 2-7

Caffeine content of beverages, foods and medicine (Continued)

Product	size	Caffeine (mg)	Product	size	Caffeine (mg)
Coffee			**Tea**		
Starbucks caffe, mocha, short	8 oz	250	Snapple Iced Tea, all	16 oz	48
Starbucks caffe, latte, short	8 oz	35	**Chocolate beverages,**	8 oz	5
Starbucks espresso	1 oz	35	1 envelope hot-cocoa mix		
Starbucks espresso, decaffeinated	1 oz	5	**Chocolate cake, cookies, candy**		
Soft drinks			Chocolate cake	2.5 oz	6
Canada Dry cola	12 oz	30	Chocolate frosting	2.5 oz	7
Canada Dry cola, diet	12 oz	1	Chocolate chip cookies	0.7 oz	4
Coca Cola	12 oz	46	Chocolate brownies	1.5 oz	10
Coca Cola, diet	12 oz	46	Fudge	0.8 oz	11
Dr. Pepper	12 oz	10	Chocolate bar, Hersheys	1.5 oz	10
Mellow Yellow	12 oz	53	**Caffeinated water**		
Mountain Dew	12 oz	54	Aqua Blast, 0.5L	12 oz	64
Pepsi	12 oz	38	Java Water, 0.5L	12 oz	90
7-Eleven Big Gulp Cola	64 oz	190	**Medicines**		
Ice cream, Yogurt			Anacin	1 tablet	33
Häagen-Dazs, coffee ice cream	8 oz	58	Excederin	1 tablet	65
			Dexatrim (weight loss)	1 dose	200
			Dristan (decongestant)	1 tablet	16
Ben & Jerry's coffee fudge	8 oz	85	No Doz, (stimulant)	1 tablet	10

[1]Values are mg/serving size. Modified from Barone and Roberts 1996, FDA 1980, Gilbert 1981, Nutrition action health letter 1996, Physicians' Desk Reference (PDR) 2000.

in coffee is 50-70% higher than it is in tea. Alternative sources of caffeine include soft drinks, which are increasing in popularity in all industrialized nations of the world (Gilbert 1984). Other dietary sources of caffeine are foods that contain cocoa or chocolate, such as candies and sweets. Substantial quantities of caffeine are present in prescription and non-prescription drugs. It was estimated in 1980 that there was more than 1000 prescription and 2000 non-prescription drugs containing caffeine in the U.S. (Barone & Roberts 1996). The primary indications for these caffeine-containing drugs are pain relief, cough or cold, diet or weight loss, the need for alertness and diuresis (PDR 2000, Roberts & Barone 1983).

From various caffeine-consumption survey and product usage data bases, the mean daily intake of caffeine was estimated to be 3-4 mg/kg body weight for all adults (Barone and Roberts (1984). For the heaviest caffeine consumers (90^{th} - 100^{th} percentiles) the mean intake was 7 mg/kg/d. For children under 18 y, the mean intake was 1 mg/kg, and for the 90^{th} - 100^{th} percentiles it was 3-5 mg/kg (Barone and Roberts 1984). Many children consume caffeine from soft drink, iced tea, chocolate-containing foods and candy, resulting in significant cumulative caffeine intake (Lundsberg 1998). The daily caffeine intake by pregnant women was 2 mg/kg/d and the 90^{th} - 95^{th} percentiles were 5.0 - 6.5 mg/kg/d, respectively (Barone and Roberts 1984). It is important to recognize that 1 can of cola (about 50 mg of caffeine) may not produce the same biological and physiological effects of caffeine in children as it does in grown adults (Skinner et al. 2000).

The trends in consumption of caffeinated beverages in the U.S.A. over the 1970-

1992 is shown in **Table 2-8**. There was a reduction in coffee consumption, a slight increase in tea consumption, and a dramatic increases in soft drink consumption (Lundsberg 1998, USDA 1995). The declines in coffee consumption may be explained by increasing consumption of other caffeinated beverages, particularly soft drinks and iced teas, that attract children and adolescents. The general public may have been cutting back coffee consumption, due to the information of the epidemiological studies and scientific finding on caffeine's negative influence on health. However, the overall exposure to caffeine has increased approximately 20% over these last 20 years.

TABLE 2-8

Per capita consumption of various beverages in the USA [1]

Year	1970	1980	1985	1990	1992
Coffee	35.7	27.2	26.8	26.4	26.1
Tea	5.2	7.8	7.3	7.0	6.8
Soft drinks	22.7	34.2	40.8	47.7	48.0

[1]Values are gallons. Data from USDA 1995.

Absorption

Caffeine is absorbed through the gastrointestinal tract into bloodstream (Blanchard & Sawers 1983a). Peak plasma concentration of caffeine is typically reached within 30 to 120 min after ingestion. This variability in peak time appears to be due to several factors. The absorption rate for caffeine contained in soft drinks has been found to be slower than that for coffee and tea (Marks & Kelly 1973). Coffee and soft drinks have a pH which varies from 5.0-6.0 and 2.0-4.0, respectively (Bettelheim & March 1991). Several investigators have reported that increased doses of caffeine consumption may raise the absorption rate (Bonati et al. 1982, Garattini et al. 1980, Passmore et al. 1987). The findings suggest that the characteristics of the medium in which caffeine is dissolved (volume, pH, chemical components) influence its absorption rate. Caffeine absorption after intraperitoneal administration was similar to the one after oral ingestion (Wang & Lau 1998). However, the absorption rate was lower after intramusculer than oral ingestion (Sant'Ambrogio et al. 1964). This indicates that solubility of caffeine at the site of administration affects the absorption rates.

Transport and Tissue Uptake

Following absorption, caffeine is readily distributed into all tissues and total body fluids by a simple diffusion (Rall 1985). Concentration and half-life (approximately 3-5 hr) of caffeine in saliva significantly correlated with serum concentration (Cook et al. 1976, Zylber-Katz et al. 1984). Caffeine is also present in semen in concentrations that are equivalent to serum levels (Beach et al. 1984). Caffeine crosses the placenta and

enters into fetus and amniotic fluid. It also enters into breast milk in lactating mothers (Bonati & Garattini 1984).

Excretion

Caffeine is biotransformed to several metabolites in the liver. Less than 3% of the administered caffeine is unmetabolized and eliminated in urine (Arnaud 1987). The excretion rates of caffeine may vary among individuals, and are influenced by a variety of factors (**Table 2-9**). Gender differences were observed by Callahan et al. (1983) when catabolism of caffeine was determined in males, females, and females on oral contraceptives during 24 hr. Females showed a 55.9% higher rate of catabolism (17.3 vs. 11.1 μg/min/kg) than men in the first hour after ingesting 5 mg/kg caffeine. The half-life of caffeine in females was 20-30 % shorter than in males. However, more caffeine was left in the body of females on contraceptives than those not on contraceptive. The difference was especially noticeable in the first 12-24 hr after ingestion of caffeine. Also, 25% of total ingested caffeine was still in the bodies of oral contraceptive users, compared to the complete elimination of caffeine in men during the same span of time (Callahan et al. 1983).

Metabolism

Caffeine is metabolized by the liver through a series of sequential reactions (**Figure 2-9**). The dimethylxanthines (paraxanthine, theophylline and theobromine) are first metabolized to dimethyluric acid or monomethylxanthines, and then they are converted to monomethyluric acids (Yesair et al. 1984). The major pathway of caffeine metabolism begins with the demethylation of caffeine catalyzed by cytochrome P450

TABLE 2-9

Factors influence half-life of caffeine

Factors	Effects	References
Gender	Female ↓	Callahan et al. (1983)
	on oral contraceptives ↑	
Age	Older men ↓	Blanchard & Sawers (1983)
	with vitamin C ↔	Trang et al. (1982)
Pregnancy	↑ (2-fold/trimester)	Aldridge et al. (1981)
Newborns	↑	Aranda et al. (1976), Parsons et al. (1976)
Cigarette smoking	↓ (2-fold)	Parsons & Neimes (1978)
Hepatic disease	↑	Desmond et al. (1980)
Exercise	↓	Collomp et al. (1996)

Arrows indicate: ↑ , increases half-life (caffeine remains in a body for a longer time); ↓ , decrease half-life (caffeine remains in a body for a shorter time); ↔ , no effects on half-life of caffeine.

1A2. The predominant (84%) metabolite of caffeine is paraxanthine. Theophylline and theobromine are produced in much less quantities (Kalow and Tang 1993).

Various factors affect the metabolism of caffeine. Chronic use of caffeine leads to its increased metabolism due to up-regulated cytochrome P450 1A2 with a higher affinity for caffeine (Goasduff et al. 1996). Furthermore, it has been reported that exercise increases the expression of cytochrome P450 1A2, decreases peak plasma levels of caffeine, and decreases half-life of caffeine (Collomp et al. 1996).

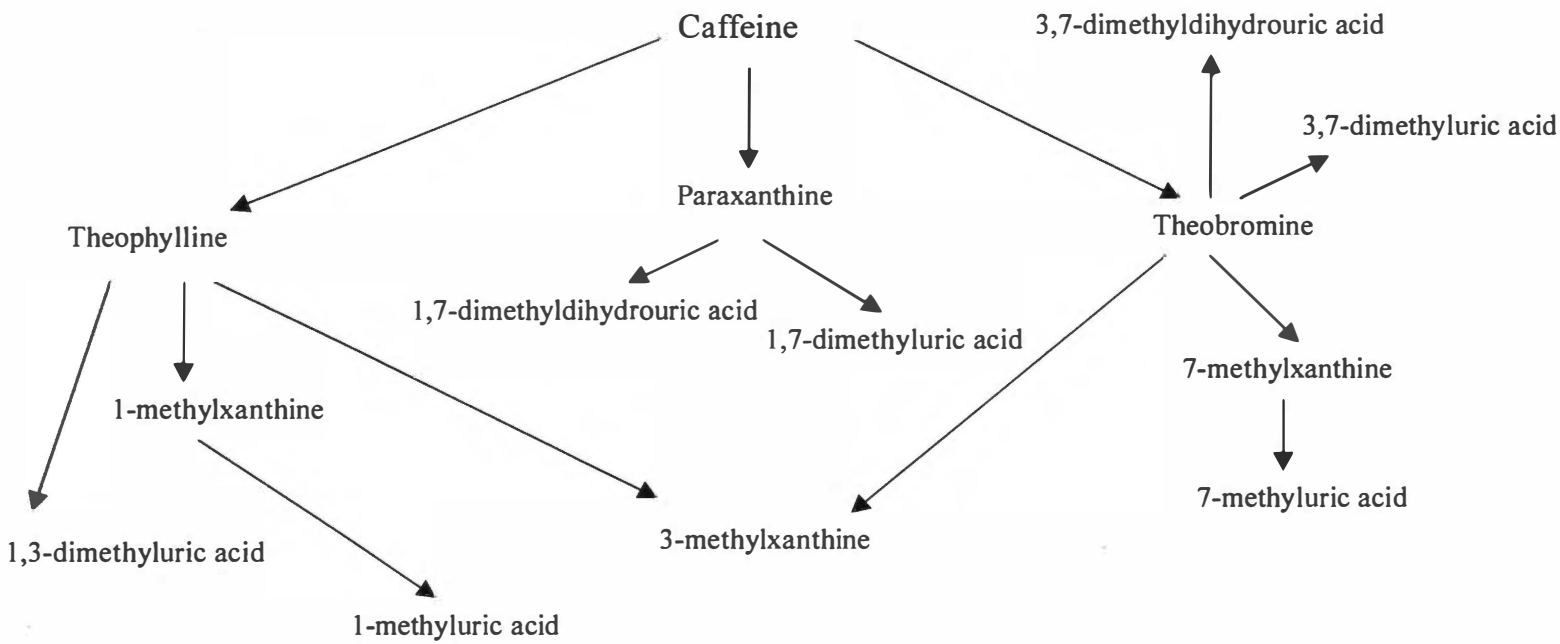


FIGURE 2-9 *Metabolic pathways of caffeine*[1] .
[1] Modified from Tarka (1982).

Mechanism of Action

Extensive research has been conducted to determine the mechanisms by which caffeine produces its pharmacological effects on major organ systems. Three hypotheses to explain the diverse actions of caffeine in humans have received the most attention. These are A) translocation of intracellular calcium, B) inhibition of phosphodiesterases, and C) the competitive antagonism of adenosine receptors.

A. Translocation of intracellular calcium

Studies of calcium translocation have been concerned mainly with the effects of caffeine on skeletal muscles (Bättig 1985). It is speculated that caffeine increases the release of calcium from the stores in the sarcoplasmic reticulum (Rall 1985). Experiments in vitro have demonstrated that caffeine lowers the excitability threshold and extends the length of muscular contractions via calcium release (Blinks et al. 1972). Alternatively, caffeine may inhibit calcium reuptake by the sarcoplasmic reticulum for continuous availability of calcium for muscle work (Endo 1977, Fabiato & Fabiato 1977). Furthermore, caffeine may increase levels of calcium indirectly by increasing the levels of fatty acyl-CoA (Spriet et al. 1992), epinephrine and cortisol (Lovallo et al. 1996). As a result of these actions, caffeine elevates intracellular calcium in skeletal muscles. However, the mechanism appears to be of limited importance in explaining the effects of caffeine in humans. The concentration of caffeine required to increase the levels of intracellular calcium would be a toxic, even lethal, dose in humans (Nehling et al. 1992).

B. Phosphodiesterase inhibition

Caffeine was found to indirectly facilitate the accumulation of cyclic adenosine

3',5'-monophosphate (cAMP), which is known to mediate the actions of many neurotransmitters and hormones in the nervous system (Sutherland et al. 1968). Butcher and Sutherland (1962) suggested that caffeine stimulates brain norepinephrine by inhibiting the enzyme, cAMP phosphodiesterase, hence blocking the breakdown of cAMP. As a result, the concentration of cAMP rises, which leads to an increase in fatty acid metabolism and subsequent ATP production (Sutherland & Butcher 1962).

It has been estimated that plasma concentrations required for calcium translocation and phosphodiesterase inhibition are 300 μg/ml and 100 μg/ml, respectively (Fredholm 1985). The concentration of caffeine in plasma after ingestion of 200-300 mg of caffeine is approximately 10 μg/ml or less in humans.

C. Adenosine receptor antagonism

Cumulative evidence indicates that many of the actions of caffeine are mediated by antagonism to cell surface adenosine receptors (von Borstel & Wurtman 1984). Unlike other mechanism describe above, the majority of the pharmacological effects of adenosine on the central nervous system can be inhibited by dosages well within physiological norms. For an example, when adenosine binds to its adenosine receptors, it inhibits lipolysis by suppressing adenylate cyclase activity. In the opposite case, chronic caffeine ingestion has been shown to stimulate adenylase cyclase activity in platelet membranes, which has been suggested to increase lipolysis (Zhang and Wells 1990). There are 4 adenosine receptors subtypes that have been identified and characterized: A_1, A_{2a}, A_{2b}, and A_3 receptors (Olah and Stiles 1995). The activation of these receptors by endogenous adenosine regulates biological processes throughout the body including, the

cardiovascular and nervous systems. Caffeine and adenosine purportedly have antagonistic effects on blood pressure, central nervous system activity, lipolysis, catecholamine release, respiration, renin release, urine output and intestinal peristalsis (James 1991). In general, caffeine has a stimulatory effect and adnosine decreases the activity of each of the functions listed above.

Functions

A. Central nervous system

Caffeine is classified as a drug with pharmacological effects, especially as a central nervous system (CNS) stimulant (Rall 1985). The widespread use of caffeine is certainly related to its ability to stimulate the nervous system and increase alertness (Sinclair and Geiger 2000). The mechanism of caffeine increasing neuronal excitability is not completely understood, however, it may involve neurotransmitter-mediated mechanisms. The best known effect of caffeine on a neurotransmitter/neuromodulatory system is its ability to block the inhibitory action of adenosine in the CNS (described in this previous section). Caffeine can increase neuronal excitability via increasing the release of excitatory neurotransmitters through blockage of adenosine receptors (Meeusen and Meirleir 1995).

Caffeine is also closely linked to the other neurotransmitter, including epinephrine, norepinephrin, dopamine, serotonin, gamma-aminobutyric acid (GABA) and acetylcholine (Spindel and Wurtman 1984). Several studies have found that caffeine ingestion increases concentrations of epinephrine and norepinephrine in blood and urine (Bellet et al. 1969, Robertson et al. 1978). Caffeine has been reported to increase brain

levels of serotonin in rats (Schlosberg et al. 1981). However, there are some studies that report no effect of caffeine on urinary and serum catecholamine in humans (Jung et al. 1981, Spindel 1984). The mechanisms responsible for these effects are unknown, but it is speculated that the effect is mediated via putative adenosine receptors (Fernstrom and Fernstrom 1984).

B. Renal Function

Another well-known function of caffeine is its diuretic effect. This effect seems to be due in part to an increase in renal blood flow and increased glomerular filtration rate (Fredholm 1984). Adenosine regulates renal function and caffeine is an antagonist of endogenous adenosine (Spielman and Thompson 1982).

C. Cardiovascular system

Caffeine may have direct effects on the contractility of the heart and blood vessels, as well as indirect peripheral actions mediated by catecholamines and renin-angiotensin system (Fredholm 1984). Other possible effects include reduced cerebral blood flow (Mathew et al. 1983). There is evidence that caffeine withdrawal after habitual caffeine intake produces severe headache, which is relieved by consuming caffeine (Roller 1981). It seems that cerebral blood vessels dilate during caffeine withdrawal, but the process is reversed by caffeine ingestion. These effects are related to caffeine's antagonism of endogenous adenosine which is a potent dilator of cerebral blood vessels (Fredholm 1984).

D. Respiratory system

In general, caffeine has little effect on respiration. An exception is the patients

with a suppressed respiratory system (Bellville 1964). It appears that caffeine increases blood flow throw the lungs by relaxing arterial smooth muscle, which increases the supply of air to the lungs by relaxing bronchiolar and alveolar smooth muscle (Dorfman & Jarvik 1970, Gilbert 1976).

E. Gastrointestinal system

The most frequently reported gastrointestinal symptom after the ingestion of coffee is heartburn (Cohen 1980). Boekema et al. (1999) suggested that coffee promotes a gastro-oesophageal reflex and is associated with this common disorder. Coffee induces cholecystokinin release and gallbladder contraction (Douglas et al 1990). There is evidence that coffee increases rectosigmoid motor activity (Brown 1990). Its effects on the colon were found to be comparable to those of a 1000 kcal meal (Rao et al. 1998). The mechanisms responsible for those effects have not yet been elucidated, and caffeine alone can not account for these gastrointestinal effects.

Caffeine Supplementation

A. Physical performance

Because of caffeine's availability and social acceptance, it has become the most widely used ergogenic aid by athletes at all levels of competition, including the elite, Olympic level athletes and recreational athletes (Applegate 1999). Young athletes start using caffeine to enhance performance. The Center for Drug-Free Sport found that 35% of 16,000 Canadian youths, aged from 11 to 18 y believe caffeine could enhance performance and 27% reported having used caffeine to enhance performance (Graham et al. 1994).

The ergogenic potential of caffeine has been recognized, and currently the International Olympic Committee (IOC) and National Collegiate Athletic Association regard caffeine as a restricted substance with and allowance limited to 12 μg caffeine/ml of urine (Sinclair & Geiger 2000). It was estimated that about 6-8 cups of coffee (95-150 mg caffeine/250 ml) must be ingested in a 1-2 hr period to reach 12 μg/ml in urine (Birkett & Miners 1991). Thus, it seems that the legal limit allows athletes to consume caffeine and manipulate their metabolism without fear of disqualification from a competition.

Studies on ergogenic effects of caffeine have produced confusing and inconsistent results. The lack of standardized experimental protocols and small number of subjects may contribute to some of the contradictory findings. Methodological problems include: differences in work performance (types, intensity, and duration), caffeine dosages, time of ingestion of caffeine, test environments, and physiological and somatic differences in subjects.

a. Endurance performance

From 1978 to 1980 Costill and co-workers demonstrated that caffeine ingestion increased time to exhaustion during submaximal (80% VO_2max) cycle ergometry (Costill et al. 1979) and increased total work output during prolonged (2 hr) cycle ergometry (Ivy et al. 1979). It was proposed that caffeine ingestion was associated with metabolic substrate shifts, implying that caffeine enhanced fat oxidation and reduced carbohydrate oxidation. This may effectively spare glycogen and help delay fatigue during prolonged exercise (Essig et al. 1980). Subsequently, many other studies produced positive results

about caffeine's ergogenicity (**Table 2-10**).

McNaughton et al. (1987) examined effects of caffeine on high intensity, short duration exercise (approximately 7 min) using caffeine naive subjects who received a high dose (10-15 mg/kg body weight) of caffeine 1 hr before testing. This study showed significant enhancement of work performance and increased free fatty acid (FFA) production (McNaughton et al. 1987).

Flinn et al. (1990) conducted a study to determine the effects of high doses of caffeine (10 mg/kg body weight) on endurance performance in recreational athletes who were caffeine native. The subjects ingested caffeine 3 hr prior to exercise allowing FFA to reach at peak. These investigators suggested that their positive findings are due to: 1) the use of caffeine naive subjects, 2) use of recreational athletes, 3) high doses of caffeine and 4) timing of the caffeine ingestion (Flinn et al. 1990).

In another well-designed study, Graham and Spriet (1991) examined the effect of caffeine on the endurance of 7 trained athletes (mean VO_2max, ≈73 ml/kg/min). The subjects ingested caffeine (9 mg/kg body weight) 1 hr prior to be tested with 2 types of exercise (running and cycling) at high intensity, ≈85% VO_2max. The study confirmed previous findings, demonstrating longer performing time in both running and cycling (Graham and Spriet 1991).

In 1992 Spriet and his associates examined the glycogen sparing effects of caffeine using muscle biopsy techniques. They took muscle biopsies from the subjects at rest, 15 min after exercise and at exhaustion. The glycogen conservation by caffeine was observed only in the initial phase of exercise, indicating only transient sparing of

TABLE 2-10

Summary of key results from studies of caffeine and endurance exercise[1]

Study	Test condition	# of subjects	Caffeine dose	Finding
Costill et al. 1978	80% VO_2max to exhaustion	9	330 mg	↑ time to exhaustion
Ivy et al. 1979	69% VO_2max	9	250 mg	↑ [FFA], ↑ fat oxidation ↑ endurance
Essig et al. 1980	65-75% VO_2max 30 min	7	5 mg/kg bw	↑ [FFA], ↑ m. TG use ↑ endurance
Power et al. 1983	80% VO_2max	7	5 mg/kg bw	↑ [FFA], No improvement
Butts & Crowell 1985	75% VO_2max to exhaustion	28	5mg/kg bw	No improvement
Bond et al. 1987	75% VO_2max to exhaustion	6	5 mg/kg bw	No improvement
McNaughton 1987	Incremental exercise ≈ 7 min	10	10 or 15 mg/ kg bw	↑ [FFA], ↓ lactate ↑ time to exhaustion
Tarnopolsky et al. 1989	80% VO_2max 90 min	6	6 mg/kg bw	No improvement
Flinn et al. 1990	Incremental exercise	9	10 mg/kg bw	↑ time to exhaustion ↑ [FFA]
Dodd et al. 1991	80% VO_2max to exhaustion	17	3 or 5 mg/ kg bw	No improvement
Graham & Spriet 1991	85% VO_2max to exhaustion	7	9 mg/kg bw	↑ time to exhaustion

[1] Abbreviation used: bw, body wight; CHO, carbohydrate; FFA, serum free fatty acids; m., muscle; TG, triglycerides

TABLE 2-10

Summary of key results from studies of caffeine and endurance exercise[1] (Continued)

Study	Test condition	# of subjects	Caffeine dose	Finding
Spriet et al. 1992	80% VO_2max to exhaustion	8	9 mg/kg bw	↑ time to exhaustion, ↓ m. glycogen ↑ m. acetyl CoA/CoA
Pasman et al. 1995	80% VO_2max to exhausiton	9	0, 5, 9, 13 mg /kg bw	↑ time to exhaustion, ↑ [FFA]
Graham & Spriet 1995	80% VO_2max to exhaustion	8	0, 3, 6, 9 mg /kg bw	↑ time at 3 & 6 mg, but not at 9 mg/kg bw
Kovacs et al. 1998	1 hr time trial cycling	15	2, 2.3, 3.2 mg kg/ bw + CHO	↑ endurance, additive effect
Anderson et al. 2000	2000 m rowing < 8 min	8 (all ♀)	6 or 9 mg/ kg bw	↑ endurance
Bell et al. 2002	10 km run ≈ 46 min	12	4 mg/kg bw + ephedrine 0.8 mg /kg	ephedrine ↓ run time

[1] Abbreviation used: bw, body wight; CHO, carbohydrate; CoA, Coenzyme A; FFA, serum free fatty acids; m., muscle; TG, triglycerides. ♀: Female subjects

glycogen. However, following ingestion of caffeine (9 mg/kg body weight) the subjects were found to increase their endurance. The investigators also determined the intramuscular concentrations of several other metabolites that could explain the metabolic shifts between fat and carbohydrate oxidation. At rest, 1 hr following caffeine ingestion, muscle citrate and the acetyl-CoA/CoA ratios were elevated; however, throughout the exercise, there were no differences between placebo and caffeine groups in these muscle metabolites (Spriet et al. 1992).

The following year van Soeren et al. (1993) examined the metabolic and catecholamine responses to caffeine in coffee users and nonusers after acute ingestion of caffeine (5 mg/kg body weight) during 1 hr of steady state exercise (50 %VO_2max). The nonusers responded to caffeine with a large rise in plasma epinephrine during 1 hr of moderate exercise. However, there was no shift in RQ or plasma FFA concentration. In contrast to the nonusers, the users did not exhibit any increased epinephrine after caffeine ingestion, but the epinephrine response to exercise was 2-fold greater than that of the nonusers. The RQ and plasma FFA were not significant differences between the groups. Thus, these findings suggest that the epinephrine response to caffeine is influenced by the subject's caffeine habits and it is not directly related to metabolic shifts after caffeine ingestion or exercise (van Soeren et al. 1993).

Several studies examined dose-response of caffeine. Pasman and associates examined the effect of different dosages of caffeine (0, 5, 9, 13 mg/kg body weight) on endurance performance. Trained athletes ingested caffeine capsules and cycled until exhaustion. All subjects were caffeine users in varying degrees (100-250 mg/d).

Significant increases in both endurance and plasma FFA production were found for all caffeine groups compared to placebo group. They also found that there was no dose-response effects on endurance performances. The stimulating effect of caffeine was observed even at the lowest dose (5 mg/kg body weight) of caffeine given (Pasman et al. 1995).

In another study, similar results were obtained when various dosages (0, 3, 6, 9 mg/ kg body weight) of caffeine were used. Trained athletes were asked to run to exhaustion and they showed significant improvement after 3 and 9 mg doses, but not after the 9 mg dose. The investigators speculated that the failure to find the significance at 9 mg/kg body weight was due to the large numbers of caffeine naive subjects who may have experienced adverse effects at this high dose (Graham and Spriet 1995)

Most of the previous studies on ergogenic effects of caffeine determined performance (the time taken to reach exhaustion). Kovacs and coworkers (1998) examined the effects of caffeine on 1 hr time trial cycling performance using a caffeinated CHO-electrolyte solution mixture. Subjects ingested the mixture with different doses of caffeine during a warm-up and at one-third and two-thirds of a 1-hr time trial. Mean caffeine contents of the mixtures were 2, 3.2, and 4.5 mg/ kg body weight. The study demonstrated that the subjects completed the time trial significantly faster after ingestion of the mixtures. An ergogenic effect of caffeine was observed at the lowest dosage (2 mg/kg body weight), and the highest dose did not result in further improvements (Kovacs et al. 1998).

There have been many studies that have evaluated an ergogenic effect of caffeine

on performance and metabolism in trained and untrained male subjects. Anderson and associates examined the effect of caffeine ingestion on short-term (< 10 min), 2000-m rowing performance, in trained (mean VO_2max , 48 ml/kg/min) female athletes. The subjects ingested 6 or 9 mg/kg body weight caffeine 1 hr before the 2000-m time trials on a rowing ergometer. The study demonstrated caffeine induced a dose-dependent performance time improvement: 0.7 % with 6 mg/kg caffeine and 1.3 % with 9 mg/kg caffeine. To date, this is the first study showing that caffeine produced an enhancement of performance by improving the first 500-m of a 2000-m row in female athletes (Anderson et al. 2000).

Bell and associates published a series of papers on the additive effects of caffeine and ephedrine on both aerobic and anaerobic exercise performance (Bell et al. 2001, Bell et al. 2002). In an anaerobic exercise study they demonstrated a significant improvement by caffeine (5 mg/kg body weight) and ephedrine (1 mg/kg body weight) in untrained healthy men. These results were contradictory to their previously published work (Bell et al. 1998), where the additive effects of caffeine and ephedrine were not observed. The authors speculated that differences were due to the type of exercise, and the improvement on anaerobic exercise performance was produced by caffeine and ephedrine independently, which means that ephedrine may be influencing CNS drive and caffeine may be enhancing muscle metabolism (Bell et al. 2000). Subsequently, Bell and associates examined the effects of the combination of caffeine (4 mg/kg body weight) and ephedrine (0.8 mg/kg body weight) on 10-km run performance in recreational runners, where the half of the subjects were coffee users (1 cup/d ≈ 150 mg/d). The study showed

run times for the ephedrine and the combination of ephedrine and caffeine significantly reduced run times compared with placebo and caffeine alone. Caffeine increased exercise-associated ephedrine response. The additive effects of caffeine and ephedrine, as seen in the previous anaerobic study (Bell et al. 2000) was not evident in this study (Bell et al. 2002).

Despite the impressive numbers of caffeine studies suggesting an ergogenic effect of caffeine, there are equally as many studies that have failed to show enhancement in endurance exercise by caffeine. Bond et al. (1987) and Powers et al. (1982), both using 5 mg/kg body weight caffeine, failed to demonstrate that caffeine increases exercise time to exhaustion. Butt and Crowell (1985) recruited untrained subjects to test on a cycle ergometry at submaximal exercise (75%VO_2max) after ingesting 300 mg caffeine. No significant effect was detected in the time to exhaustion.

Tarnopolsy and colleagues (1989) studied the effect of caffeine using a 90-min treadmill run with trained runners. All subjects were coffee users (mean intake 200 mg/d) and they took 6 mg/kg caffeine 1 hr prior to testing. No significant difference in metabolic effects were found, except that plasma FFA were significantly elevated following administration of caffeine.

In another study, Dodd et al. (1991) determined the VO_2max and a time-to-exhaustion on a bicycle ergometer in trained men after administration of 3 or 5 mg/kg body weight caffeine. There was no significant caffeine effect on exercise performance in both caffeine naive (<25 mg/d) and tolerant (> 310 mg/d) subjects.

In summary, there have been convincing replicated findings that could permit the

conclusion concerning the effects of caffeine as an ergogenic aid for endurance performance. Since there are also a large number of studies indicating no effects, there may be some variables that have not been clearly elucidated. Different exercise protocols (intensity, duration and mode of exercise) and doses of caffeine have been tested to resolve the confounding results. Individual differences including nutritional differences, muscle fiber differences, nutrient-nutrient interactions, genetic differences, mood and motivation of athletes may be good candidates to be consider as modulating variables in human performances.

There has been no well-designed study that could test the psychostimulant effect of caffeine during exercise. Caffeine increases alertness and enhances mood. When the improvements in performance were indirectly due to the caffeine's effects on mood, we are not able to distinguish, if that is simply the placebo effect or caffeine induced placebo-like effect.

b. Muscle strength

In vitro studies have indicated that caffeine may enhance the production of muscular force by altering rate of substrate utilization, neuromuscular transmission (Butcher and Sutherland 1962), and calcium manipulation (Kavaler et al. 1978). Human studies have consistently failed to demonstrate the significant effects of caffeine on muscular strength (Jacobson and Edwards 1991, Lopes et al. 1983, Powers and Dodd 1985, Williams et al. 1988) with a very few exceptions (Kruk et al. 1999).

Lopes and co-workers (1983) investigated the effects of 500 mg caffeine on voluntary isometric and electrically stimulated contractions. The subjects performed 3

maximal voluntary contractions followed by electrical stimulation of the ulnar nerve at 10, 20, 30, 50 and 100 Hz. The results of a curve force showed that caffeine produced an increase in tension for all stimulation frequencies, except at 100 Hz. However, there was no difference between placebo and experimental groups in the maximal voluntary contraction (Lopes et al. 1983).

In another study, the effect of caffeine (5 mg/kg body weight) on voluntary dynamic contraction was investigated by Bond et al. (1986). The subjects were tested for isokinetic knee flexion and extension at selected velocities at 30, 150 and 300°/s. The authors concluded that caffeine has no effect on skeletal muscle contraction at different velocities (Bond et al. 1986). For the present, research findings do not offer much support for caffeine's ergogenic effects on muscle strength.

B. Weight loss and management

Caffeine is one of the most popular ingredients of weight loss supplements (Egger et al. 1999). The rational behind this popularity is that caffeine has the lipolytic potential (Essig et al. 1980, McNaughton 1986) and thermogenic property (Dulloo & Miller 1986, Hollands et al. 1981). Caffeine stimulates catecholamine secretion by acting as an adenosine receptor antagonism and by other unknown mechanisms. This in turn stimulates adipose tissue lipolysis and elevates serum FFA concentration. Active skeletal muscles take up FFA, and this causes a greater fat oxidation. Some studies demonstrated the lipolytic effects of caffeine, for example, increasing FFA concentration and altering metabolic rate after ingestion of caffeine in humans (Astrup et al. 1990, Bracco et a. 1995, Horton and Geissler 1996).

Furthermore, the effects of caffeine was investigated to determine whether it continues to elevate oxygen consumption after exercise (55% VO_2max). Donelly and McNaughton (1992) have determined the effects of caffeine ingestion (5 and 10 mg/kg body weight) on post-exercise oxygen consumption in 6 untrained women. During exercise and 1-hr-post-exercise, oxygen consumption was found to be significantly higher in the caffeine groups compared to the placebo group. During the entire period of experiments, FFA concentration was significantly higher, and RQ values were significantly lower in the caffeine groups than in the placebo group. The study demonstrated that caffeine increased metabolic rate above normal levels in untrained women during as well as after exercising at 55% VO_2max (Donelly and McNaughton 1992).

Well-designed studies have examined the effects of caffeine in combination with ephedrine on weight loss in humans. Astrup and associates (1992) determined whether the combination of ephedrine and caffeine produces an additive effect on weight loss compared with ephedrine and caffeine alone. In their randomized, placebo controlled, double blind study, 180 obese patients were treated with low calorie diet (1000 kcal/d) and either an ephedrine/caffeine combination (20 mg/200 mg), ephedrine (20 mg), caffeine (200 mg) or placebo for 24 wk. They found that when administered as an adjuvant to a low calorie diet, the combination of caffeine and ephedrine significantly reduced body weight, 1.5 kg after 12 wk, 3.4 kg after 24 wk, more than placebo; however, neither ephedrine nor caffeine alone induced a significantly greater weight reduction than placebo (Astrup et al. 1992).

Several other studies examined the effectiveness of weight loss using caffeine in combination with fiber or chromium in humans. However, the findings of these studies are confusing and farther studies are needed. In general, there is a theoretical basis for using caffeine in weight control, and good evidence for its lipolysic effects. However, there is no controlled study on fat loss in over weight individuals using caffeine without an energy-restricted diet. No studies have reported on the effects of caffeine in individual with high or low habitual caffeine uses.

OVERWEIGHT AND OBESITY

In adults, overweight is defined as a Body Mass Index (BMI) of 25 kg/m^2 or more. And obesity is defined as a BMI of 30 kg/m^2 or more. Currently, the National Institutes of Health (1998), Healthy People 2010 (2000), the 2000 Dietary Guidelines for Americans (2000) and the WHO (1998) all use similar cut-off points of BMI for defining overweight and obesity. BMI is calculated as an individual's weight in kilograms divided by the square of his or her height in meters (Bray 1994). BMI units are used in classification of overweight and obesity (**Table 2-11**). Allowing for the ease of measuring height and weight in a field setting, the BMI is an accepted indicator of the risk of overweight in adults. However, the BMI has limitations; it does not incorporate any measure of body composition. There is evidence that the relationship between BMI and percentage body fat (%BF) may be different in different ethnic groups (Dudeja et al. 2001, Norgan 1994).

The assessment of body fat may help achieve a more accurate definition of

overweight and obesity. The assessment of body fat is also important due to the established association between high body fat and chronic disease conditions, such as type 2 diabetes mellitus, hypertension and hyperlipidaemia. The upper limits of body fat for defining obesity have been set as 25% and 30% for males and females, respectively (Hortobagyi et al. 1994).

TABLE 2-11

Classification of underweight, normal range, overweight and obesity in adults according to BMI[1]

Classification	BMI (kg/m^2)
Underweight	<18.5
Normal range	18.5-24.9
Overweight	25.0-29.9
Obese	≥ 30.0
Class I	30.0-34.9
Class II	35.0-39.9
Class III	≥ 40.0

[1]BMI = Body Mass Index

There are various methods available for measuring body fat (**Table 2-12**). Hydrodensitometry, or hydrostatic weighing (underwater weighing), is considered to be the gold standard of the densitometric methods. Hydrostatic weighing uses the Archimedes principle to determine body volume by measuring the difference between a subject's weight in water and that in air and thus determining whole-body density (Bubb 1992). This technique requires the subject to be completely submerged underwater while exhaling maximally for yielding residual lung volume and minimize the effect of buoyancy from lung air (Behnke et al. 1942). The procedure provides a two-compartment model of fat free mass and body fat. The limitations associated with this method include the requirement of specialized technicians and equipment, time consumption, subject discomfort and inaccessibility for many special populations such as elderly, infants, or disabled.

Skinfold thickness is often used in clinical studies. However, it has been reported to have large inter-observer and inter-individual variances (Pollock and Jackson 1984). In some obese individuals the skinfold caliper, the instrument used for the skinfold thickness measurement, will not allow a satisfactory measurement of skinfold in some areas. Furthermore, the equations for converting skinfold thickness into body fat of an individual are often population specific and have been reported an overestimate or underestimate of body fat in some population (Pollock and Jackson 1984).

Dual-energy X-ray absorptiometry (DEXA) is becoming increasingly popular for body composition assessment. DEXA provides a three-compartment model, which

TABLE 2-12

Methods of measuring body fat

Method	Advantage	Disadvantage
Density Hydrostatic weighing	Accuracy	Time consuming, Subject discomfort, Impractical in certain populations, Operator training, Specialized equipment, Can not measure regional fat
Air displacement Plethysmograph	Quick, Simple operation, Accessible in special population,	Expensive equipment, Can not measure regional fat
Skinfold thickness	Easy, Low cost, Measure regional fat	High inter-individual variances
Dual energy X-ray absorptiometry (DEXA)	Easy, Accuracy	Expensive equipment
Bioelectric impedance (BIA)	Easy, Accuracy	Can not measure regional fat
Computed tomography (CT)	Accuracy, Measure regional fat	Expensive equipment, Radiation exposure
Magnetic resonance imaging (MRI)	Accuracy, Measure regional fat	Expensive equipment, Radiation exposure
Circumferences Waist circumference Waist-hip ratio (WHR)	Easy, Accuracy	Population specific

separates the fat-free mass into bone mineral mass and lean tissue mass (Mazess et al. 1990). It appears to offer a precise and simple way of measuring total and regional body fat and lean masses with a very low radiation exposure (Kohrt 1998). Svendsen et al. (1993) found that abdominal fat measured by computed tomography (CT) in postmenopausal women was better correlated to central abdominal fat estimated by DEXA than either waist circumference or waist-hip ratio (WHR).

Bioelectric impedance analysis (BIA) has been used to measure body fat, which estimates body resistance (impedance) from a voltage drop in a small current passed between electrodes. The level of impedance is used to estimate lean tissue content and body-water volume, then assuming a hydration fraction of lean tissue, farther estimate is made of body lean and fat mass (Kuczmarski 1996). The method provides an easy and fast means of estimating body fats, with very little inter-observer variation (Heitmann 1990a).

An alternative method for measuring body fat is air displacement plethysmorgraphy, which determines body volume by measuring the reduction in chamber volume caused by introduction of a subject into a chamber with a fixed air volume. Similar to hydrostatic weighing, whole body density is determined and body composition is estimated by using previously validated equations (Dempster and Aitkens 1995, McCrory et al. 1995). The device, BOD POD® (Life Measurement Instruments, Concord, CA), has been developed based on the plethysmorgraphic measurement of body volume for the purpose of estimating human body composition (Dempster and Aitkens 1995). The entire test process requires about 5 min, the device has an easy testing

protocol and it is suitable for a wide variety of subjects, including the obese and elderly.

It has been recognized that measurements of body fat distribution are of great value in assessing the degree of overweight (Kissebah et al. 1983, Vague 1956). Increased abdominal, particularly visceral adipose tissue, substantially increases the risk of diabetes, high blood pressure and heart disease compared with lower body fat distribution (Kissebah and Krakower 1994). The recognition of visceral fat accumulation as a potential risk factor led to the development of techniques to measure the amount of intra-abdominal fat directly by computed tomography (CT) and magnetic resonance imaging (MRI). They made a substantial contribution to the advancement of knowledge in body composition research. However, because of the expense and radiation exposure of these techniques, there are clear limitations to their use. Instead, simple anthropometric measures, waist circumference and the ratio of the waist circumference divided by the hip circumference (WHR), have been the most widely used to measure fat distribution. There are several cut-off points for abdominal obesity based on waist circumference and WHR. Some of the recommendations frequently cited in various studies are listed in **Table 2-13**.

In this section, the definitions and how we measure overweight and obesity were examined. In the United States 63% of men and 55% of women aged 25 years or older are overweight or obese defined by a BMI of higher than 25 (Must et al. 1999). There have been some concerns that the conventional cut-off points of the BMI underestimates of overweight and obesity, when %BF is used as the standard to define overweight (Dudeja et al. 2001). Based on the health-related risk factors and comorbidities, the

TABLE 2-13

Cut-off points for abdominal obesity

	Cut-off points	References
Waist circumference	102 cm for men 88 cm for women	Lean et al. (1995), American Heart Association (2000)
	100 cm for ≤ 40 y 90 cm for ≥ 40 y (Not gender specific)	Lemieux et al. (1996)
	102 cm for men 88 cm for women (For BMI ≤ 30 kg/m^2)	Bray (1998)
WHR	1.00 for men 0.80 for women	Björntorp (1985)
	1.00 for men 0.85 for women (For BMI ≤ 30 kg/m^2)	Bray (1998)
	0.95 for men 0.80 for women	Dietary guidelines for Americans (1990)
	0.94 for men 0.88 for women	Lemieux et al. (1996)

BMI = body mass index; WHR = waist-hip ratio.

WHO Western Pacific Region (2000) recommended different ranges for classifying overweight and obesity for populations within the Asian-Pacific region. The lower cut-off points for Asians, BMI≥23.0 for overweight and BMI≥25.0 for obesity, and the higher cut-off points for Pacific Islanders, BMI≥26.0 for overweight and BMI≥32.0 for obesity.

There is a variety of methods for estimating body fat and its distribution with various advantages and disadvantages (Table 2-12). There is a consensus about the cut-off points for abdominal obesity (central fat) based on WHR and waist circumference (Table 2-13). However; it is important to note that these cut-off points lack a systemic evaluation, taking into account possible difference between the genders, age categories, ethnic groups, and their rates of different diseases and mortality. More comprehensive assessment in large population studies are required for the broader evaluation of possible cut-off points for abdominal obesity.

RESEARCH OBJECTIVES

The overall objective of this study was to understand the interactive effects of carnitine, choline and caffeine (CCC) on fat metabolism under the conditions of mild exercise regimen in humans and animals. The specific objectives were:

1) To determine if CCC will alter the serum, urinary and tissue (liver, skeletal muscles, heart, kidneys, brain and testes) carnitine profiles before and after exercise.
2) To determine if CCC will reduce body weight and fat pad mass (% BF).

3) To determine if CCC will promote fatty acid oxidation with and without exercise.

4) To determine if CCC will affect a biochemical marker of body fat mass (leptin), and physiological markers of exercise performance (RQ, VO_2max and running time to exhaustion).

CHAPTER III

CAFFEINE, CARNITINE AND CHOLINE SUPPLEMENTATION OF RATS DECREASES BODY FAT AND SERUM LEPTIN CONCENTRATION AS DOES EXERCISE

This chapter is a slightly revised version of a manuscript published with co-author Dileep S. Sachan in the Journal of Nutrition in 2000.

Nobuko Hongu and Dileep S. Sachan (2000) Caffeine, carnitine and choline supplementation of rats decreases body fat and serum leptin concentration as does exercise. J. Nutr. 130: 152-157.

ABSTRACT

The effect of a combination of caffeine, carnitine and choline with or without exercise on changes in body weight, fat pad mass, serum leptin concentration and metabolic indices was determined in 20 male, 7-wk-old Sprague-Dawley rats. They were given free access to a nonpurified diet without or with caffeine, carnitine, and choline at concentrations of 0.1, 5, and 11.5 g/kg diet, respectively. In a 2x2 factorial design, one-half of each dietary group was exercised, and the other half was sedentary. Body weight and food intake of all rats were measured every day for 28 d. Rats were killed and blood and tissue samples were collected and analyzed for biochemical markers. Food intake of the groups was not different, but the body weight was significantly reduced by exercise in both dietary groups. Fat pad weights and total lipids of epididymal, inguinal and perirenal regions were significantly reduced by the supplements as well as by exercise.

Regardless of exercise, supplements significantly lowered triglycerides in serum but increased levels in the skeletal muscle. Serum leptin concentrations were equally lowered by supplements and exercise. Serum leptin was correlated with body weight ($r = 0.55$, $P < 0.01$), fat pad weight ($r = 0.82$, $P < 0.001$) and glucose ($r = 0.51$, $P < 0.05$). We conclude that the indices of body fat loss due to dietary supplements were similar to those due to mild exercise, and there were no interactive effects of the two variables.

INTRODUCTION

Carnitine is a facilitator of fatty acid oxidation by virtue of its role in inter-organelle translocation of fatty acids (Bremer 1997). Clinical studies have shown that carnitine supplementation improves muscle function in patients with carnitine deficiency (Brass and Hiatt 1994). Several studies suggest an increased carnitine utilization during endurance exercise in humans (Decombaz et al. 1992, Lennon et al. 1983). However, there is no clear agreement on the benefits of carnitine supplementation in normal healthy individuals (Neumann 1996). Choline is a lipotropic agent (Best and Huntsman 1935, Leiber et al. 1994), and its supplementation has been shown to enhance synthesis and release of acetylcholine at the neuromuscular junction (Buyukuysal et al. 1995, Zeisel and Blusztajn 1994). It has been observed that there is a decline in plasma choline concentration in athletes after running a marathon (Conlay et al. 1992). Therefore, choline supplementation may improve acetylcholine balance and prevent decrement in physical performance. However, there are no definitive studies on the effects of choline supplementation in normal healthy people. Caffeine is used widely as a general stimulant

and a fatty acids mobilizer from adipose tissues (Bellet, et al. 1968, Costill, et al. 1978, Denadai 1994). It has been hypothesized that fat-mobilizing effects of caffeine and other methylxanthines elevate blood free fatty acid concentration and spare carbohydrate stores, prolonging endurance exercise. However, this hypothesis remains controversial, because of a lack of standardized experimental procedures: the type, intensity and duration of training, the dose of caffeine used and habitual use of caffeine by participants (Nehlig and Debry 1994).

Recently, we reported interactive effects of choline and carnitine in normal healthy humans and animals. Choline supplementation resulted in significant conservation of carnitine in humans and guinea pigs (Daily and Sachan 1995, Dodson and Sachan 1996); however, this effect of choline was not seen in the adult rats given choline dosage similar to that given to humans and guinea pigs (Daily and Sachan 1995, Rein et al. 1997). The choline supplementation promoted tissue carnitine accretion, particularly in skeletal muscle of guinea pigs (Daily and Sachan 1995) and livers of rats (Rein et al. 1997). In addition, a choline supplemented diet decreased percentage of body fat and increased percentage of protein without significantly changing body weight or the respiratory quotient (RQ) in guinea pigs (Daily et al. 1998). Guinea pigs would have been a preferred animal model for their likeness to humans with regard to choline carnitine interactions, but guinea pigs are not easily made to exercise on a treadmill. So, we settled on a rat as a model with modification of dietary treatment in two ways. First, we increased the supplementary level of choline to about 5-fold used in guinea pigs or humans. Second, we included caffeine as one of the supplements for its fat-mobilizing

property. We rationalized that besides increasing energy demand by exercising muscle, simultaneous availability of caffeine, carnitine and choline may induce mobilization, transport and delivery of fat as the energy substrate of choice. This theoretically sound rationale required experimental evidence which is presented in this chapter.

Leptin, a 16-kDa protein released from adipose tissue as a product of the obese (*ob*) gene (Zhang et al. 1994), is thought to play a role in the regulation of body weight, energy expenditure and food intake (Campfield et al. 1995, Halaas et al. 1995, Pelleymounter et al. 1995). Studies have shown that leptin circulates in proportion to body fat mass in humans (Considine et al. 1996) and rodents (Ahren et al. 1997) and reflects body lipid content in mice (Frederich et al. 1995). The data on effects of nutritional perturbations on serum leptin are beginning to accumulate. For example, serum leptin concentration falls after fasting (Boden et al. 1996) and energy restriction (Wadden et al. 1998) with relatively small decreases in body weight and fat mass. In addition, serum leptin concentrations fell following ultramarathon running in men (Landt et al. 1997) and 12 wk of aerobic exercise in women without changes in adiposity (Hickey et al. 1997). The list of nutritional factors that influence circulating leptin concentrations is small, and there are no studies on the effect of dietary supplements plus exercise on circulating serum leptin concentrations. Thus the objective of this study was to determine changes in body weight, fat pad weights, serum leptin concentrations and metabolic indices in rats fed a diet supplemented with caffeine, carnitine and choline with and without exercise.

MATERIALS AND METHODS

Animals and treatment

The protocol of this study was approved by the University of Tennessee Institutional Review Board. Twenty, 7-wk-old male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were housed in a room controlled for temperature (20-22°C), relative humidity (50%), and light (12-h light:dark cycle). Their initial mean body weight was 218 g. Rats had free access to a ground nonpurified diet, Harlan Teklad 22/5: protein 22%, fat 5% and fiber 3.83%, (Harlan Teklad, Madison, WI) and water. The diet was fortified with caffeine, carnitine, and choline at concentrations of 0.1, 5, and 11.5 g/kg nonpurified diet, respectively, as the supplemented diet.

The endogenous concentrations of choline and carnitine in this commercial diet were 2.1 g and 30 mg/kg diet, respectively. All rats were weighed daily and their food intake was determined daily by the difference in the weight of food containers. Daily food intakes throughout the 4-wk experimental period were used to calculate average food intake. After arrival (1 wk), the rats were randomly divided into two groups (n = 10) and assigned to the nonsupplemented or supplemented diet. Each dietary group was subdivided into exercise and non-exercise groups (n = 5).

One-half of each dietary group was exercised on a rodent treadmill (Columbus Instruments International, Inc., Columbus, OH). All rats in exercise groups were made to run on the treadmill for 10 min at 15% grade starting at wk 3. The running speed and duration were continuously increased during the course of exercise to maximize at 18 m/min for 25 min/d. The 2 x 2 factorial design was scheduled as follows: wk 1, all rats

consumed the nonsupplemented diet; wk 2, one-half of the rats consumed the nonsupplemented diet and one-half did the supplemented diet; wk 3, one-half of the nonsupplemented and supplemented rats were made to exercise on a treadmill 5 d/wk; wk 4, the diet and exercise regimen continued; and wk 5, the same treatments as in wk 4.

Sample collection and assays

At the end of the 5-wk experimental period, rats were anesthetized with methoxyflurane (Pitman-Moore, Mundelein, IL) and killed by exsanguination after cardiocentesis using 10-mL syringes fitted with 23-gauge, 1.9 cm disposable needles. The blood samples were immediately centrifuged at 2000 X g for 10 min at 4°C. Serum was removed and stored at -80°C until used for determination of glucose, triglycerides, free fatty acids and leptin concentrations. Following blood collection, regional fat pads of epididymal, perirenal and inguinal fat were quickly excised from the carcasses, weighed, rinsed with saline, blotted dry, frozen in liquid nitrogen and stored at -80°C until used for triglycerides and total lipid determination. Glucose was determined by glucose oxidase method using Sigma kit no. 510 (Sigma, St Louis, MO). Triglycerides, total lipid, nonesterified fatty acids and lactate were determined by the methods of Giegel et al. (1975), Ellefson and Caraway (1976) Novak (1965) and Gutmann and Wahlefeld (1974), respectively. Serum leptin concentrations were determined using a commercial radioimmunoassay kit (Linco Research, St Louis, MO).

Statistical analysis

All results are presented as group means ± SEM. Data were analyzed using two-way ANOVA to test the effects of exercise, supplementation and their interaction using

SAS (1997). The main effects of diet and exercise were tested using specific linear contrasts, as was the interaction. Pearson correlation coefficients were calculated using data from all 20 rats. Statistical significance level was set at $P < 0.05$.

RESULTS

Nonsupplemented rats consumed no caffeine, but consumed about 45 mg choline and 0.66 mg carnitine/d, because the nonpurified diet, Teklad 22/5, contains 2.1 g choline and 30 mg carnitine/kg of diet (**Table 3-1**). Supplemented rats consumed in their diets ~2.1, 105 and 240 mg/d caffeine, carnitine and choline, respectively. The final body weight and weight gain of the exercised rats were significantly lower ($P = 0.001$) than those of nonexercised rats with or without supplement (Table 3-1). There were no significant differences in food intakes of the groups.

Fat pad weights generally were affected by exercise as well as by the supplement (**Table 3-2**). The weights of epididymal fat pad of supplemented and nonsupplemented rats with exercise were significantly lower than their nonexercised counterparts. The weight of inguinal fat pads of exercised rats with and without supplementation were significantly lower than their paired nonexercised rats. The weights of perirenal fat pads of exercised rats were significantly lower than their nonexercised counterparts. The perirenal fat pad weights of the supplemented, nonexercised rats were lower than those of the nonexercised, nonsupplemented rats. Total fat pad weight (sum of the three) showed a pattern similar to that of the other three fat pads, particularly the inguinal fat pad weight. Although the duration and intensity of exercise were the same in the two exercised

TABLE 3-1

Body weight and food; caffeine, carnitine and choline intakes in exercised or nonexercised male rats with and without caffeine, carnitine and choline supplementation[1]

	Nonsupplement		Supplement		Statistical significance[2]		
	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[3]
Initial body weight, *g*	218 ± 2.7	220 ± 7.8	216 ± 7.3	218 ± 5.6	NS[4]	NS	NS
Final body weight, *g*	377 ± 5.9[a]	349 ± 8.8	365 ± 9.2[a]	332 ± 6.5	NS	0.0012	NS
Total weight gain, *g*	158 ± 5.1[a]	129 ± 8.9	149 ± 11[a]	114 ± 7.2	NS	0.0013	NS
Food intake, *g/d*	21.8 ± 0.4	21.1 ± 0.4	22.2 ± 0.5	20.7 ± 0.4	NS	NS	NS
Caffeine intake, *mg/d*	-----	-----	2.11 ± 0.04	2.07 ± 0.04	-----	-----	-----
Carnitine intake, *mg/d*	0.65 ± 0.01	0.67 ± 0.01	105 ± 2.0	104 ± 1.8	0.0001	NS	NS
Choline intake, *mg/d*	45 ± 0.9[b]	44 ± 0.8[c]	242 ± 4.6	238 ± 4.1	0.0001	NS	NS

[1] Values are means ± SEM, $n = 5$. The diets were supplemented with caffeine, carnitine, and choline at 0.1, 5, 11.5 g/kg diet. The nonsupplemented diet contained 2.1 g choline and 30 mg carnitine per kg of diet. The energy distribution of diet: 22% protein, 5% fat, 73% carbohydrate. The diet provides 16.7 kJ/g (3.99 kcal/g) and 3.8% fiber.

[2] *P* values from 2-way ANOVA, using the factors supplementation and exercise.

[3] S x E, supplement and exercise interaction.

[4] NS, not significant, $P < 0.05$.

[a] Significant differences between nonexercised and exercised rats of a dietary group, tested by specific linear contrasts.

[b] Significant differences between nonexercised rats with and without supplement, tested by specific linear contrasts.

[c] Significant differences between exercised rats with and without supplement, tested by specific linear contrasts.

TABLE 3-2

Three regional fat pads and total fat pad weights in exercised or nonexercised male rats with and without caffeine, carnitine, and choline supplementation[1]

	Nonsupplement		Supplement		Statistical significance[2]		
	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[3]
Epididymal fat, g	4.2 ± 0.2	3.7 ± 0.4[a]	3.7 ± 0.3[b]	2.9 ± 0.4	0.0350	0.0169	NS[4]
Inguinal fat, g	4.6 ± 0.3[b]	3.1 ± 0.5	3.7 ± 0.2[b]	2.2 ± 0.3	0.0141	0.0003	NS
Perirenal fat, g	3.7 ± 0.3[b,c]	2.5 ± 0.4	2.6 ± 0.3	1.9 ± 0.3	0.0245	0.0141	NS
Total fat pad[5], g	12.4 ± 1.6[b]	9.3 ± 2.6	10.1 ± 1.8[b]	7.0 ± 1.4	0.0140	0.0019	NS

[1] Values are means ± SEM, $n = 5$. The diets were supplemented with caffeine, carnitine, and choline at 0.1, 5, 11.5 g/kg diet. The nonsupplemented diet contained 2.1 g choline and 30 mg carnitine per kg of diet. The energy distribution of diet: 22% protein, 5% fat, 73% carbohydrate. The diet provides 16.7 kJ/g (3.99 kcal/g) and 3.8% fiber.

[2] P values from 2-way ANOVA, using the factors supplementation and exercise.

[3] S x E, supplement and exercise interaction.

[4] NS, not significant, $P < 0.05$.

[a] Significant differences between nonexercised and exercised rats of a dietary group, tested by specific linear contrasts.

[b] Significant differences between nonexercised rats with and without supplement, tested by specific linear contrasts.

[c] Significant differences between exercised rats with and without supplement, tested by specific linear contrasts.

groups, total fat pad reductions due to exercise were 25% in nonsupplemented rats and 44% in supplemented rats compared to the nonsupplemented, nonexercised rats. However, there was not a significant interaction between diet and exercise.

Dietary supplement and exercise independently affected total lipids of the regional fat pads (**Table 3-3**). The total lipid of epididymal and perirenal fat pad was significantly lower in exercised, supplemented rats, reflecting the difference in the corresponding fat pad weight (Table 3-2). The effects of both supplement and exercise on total lipid were remarkable in the inguinal fat pad as there was 62% less of total lipid in the supplemented, exercised rats compared to the nonsupplemented, nonexercised rats. However, the interactive effects of supplement and exercise were not significant. The triglycerides of epididymal fat pads were affected by exercise, however, those of the inguinal and perirenal fat pads were not significantly affected by either supplementation or exercise. The total lipids made up ~ 80-90% of the fat pad weights and 70-80 % of the total lipids were triglycerides.

Differences in serum and muscle triglycerides were produced by the supplement in exercised and nonexercised rats. Serum triglycerides were significantly lower in the supplemented rats, but their muscle triglycerides were significantly higher than in the nonsupplemented groups (**Table 3-4**). Serum leptin concentration was significantly lower in the supplemented, exercised rats compared to any other group. Supplementation and exercise did not significantly affect serum glucose, lactate or free fatty acids.

There were significant correlation between body weight and fat pad weights as

TABLE 3-3

Total lipid and triglycerides of three regional fat pads in exercised and nonexercised male rats with and without caffeine, carnitine, and choline supplementation[1]

	Nonsupplement		Supplement		Statistical significance[2]		
	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[3]
Total lipid, *g*							
Epididymal fat pads	3.5 ± 0.1	3.2 ± 0.3[a]	3.5 ± 0.3[b]	2.5 ± 0.2	NS	0.0165	NS[4]
Inguinal fat pads	2.6 ± 0.2 [b, c]	1.8 ± 0.2[a]	1.8 ± 0.2[b]	1.0 ± 0.1	0.0001	0.0001	NS
Perirenal fat pads	2.5 ± 0.2 [b, c]	1.6 ± 0.2	1.7 ± 0.2	1.3 ± 0.2	0.0173	0.0082	NS
Triglycerides, *mmol/g fat*							
Epididymal fat pads	7.89 ± 0.3	8.63 ± 0.3	7.94 ± 0.3[b]	9.22 ± 0.3	NS	0.0039	NS
Inguinal fat pads	7.73 ± 0.2	7.55 ± 0.5	6.93 ± 0.4	6.43 ± 0.5	NS	NS	NS
Perirenal fat pads	9.25 ± 0.4	8.66 ± 0.1	8.67 ± 0.1	8.79 ± 0.2	NS	NS	NS

[1] Values are means ± SEM, $n = 5$. The diets were supplemented with caffeine, carnitine, and choline at 0.1, 5, 11.5 g/kg diet. The nonsupplemented diet contained 2.1 g choline and 30 mg carnitine per kg of diet. The energy distribution of diet: 22% protein, 5% fat, 73% carbohydrate. The diet provides 16.7 kJ/g (3.99 kcal/g) and 3.8% fiber.

[2] *P* values from 2-way ANOVA, using the factors supplementation and exercise.

[3] S x E, supplement and exercise interaction.

[4] NS, not significant, $P < 0.05$.

[a] Significant differences between nonexercised and exercised rats of a dietary group, tested by specific linear contrasts.

[b] Significant differences between nonexercised rats with and without supplement, tested by specific linear contrasts.

[c] Significant differences between exercised rats with and without supplement, tested by specific linear contrasts.

TABLE 3-4

Effect of supplement and exercise on blood metabolites and muscle triglycerides in male rats fed the nonsupplemented diet or supplemented diet with caffeine, carnitine and choline[1]

	Nonsupplement		Supplement		Statistical significance[2]		
	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[3]
Serum glucose, *mmol/L*	9.26 ± 0.57	9.10 ± 0.19	9.18 ± 0.36	7.94 ± 0.28	NS	NS	NS[4]
Serum lactate, *mmol/L*	1.42 ± 0.25	2.67 ± 0.65	1.36 ± 0.11	1.89 ± 0.24	NS	NS	NS
Serum triglycerides, *mmol/L*	0.76 ± 0.04[a]	0.69 ± 0.09[b]	0.41 ± 0.06	0.46 ± 0.04	0.0003	NS	NS
Muscle triglycerides, *μmol/g of wet muscle*	32.1 ± 2.7[a]	34.1 ± 4.7[b]	61.2 ± 5.2	62.2 ± 6.2	0.0001	NS	NS
Serum free fatty acids, *μmol/L*	282 ± 30.9	290 ± 38.9	284 ± 29.4	195 ± 24.4	NS	NS	NS
Serum leptin, *μg/L*	2.24 ± 0.17[a,c]	1.73 ± 0.15[b]	1.69 ± 0.11[c]	1.19 ± 0.15	0.0018	0.0032	NS

[1] Values are means ± SEM, $n = 5$. The diets were supplemented with caffeine, carnitine, and choline at 0.1, 5, 11.5 g/kg diet. The nonsupplemented diet contained 2.1 g choline and 30 mg carnitine per kg of diet. The energy distribution of diet: 22% protein, 5% fat, 73% carbohydrate. The diet provides 16.7 kJ/g (3.99 kcal/g) and 3.8% fiber.

[2] *P* values from 2-way ANOVA, using the factors supplementation and exercise.

[3] S x E, supplement and exercise interaction.

[4] NS, not significant, $P < 0.05$.

[a] Significant differences between nonexercised and exercised rats of a dietary group, tested by specific linear contrasts.

[b] Significant differences between nonexercised rats with and without supplement, tested by specific linear contrasts.

[c] Significant differences between exercised rats with and without supplement, tested by specific linear contrasts.

well as between body weight and concentrations of serum leptin and free fatty acids (**Table 3-5**). Serum leptin concentrations were correlated with serum glucose but not with food intake.

DISCUSSION

Since choline and carnitine have been used as lipotropic agents in number of studies, we hypothesized that their combined effect on fat metabolism may be greater than each alone, and may even be enhanced by exercise where energy demand is increased. Our earlier studies had shown that choline supplementation promoted carnitine conservation in both humans and guinea pigs but not in rats (Daily and Sachan 1995, Dodson and Sachan 1996). This conservation of carnitine in guinea pigs resulted in accretion of carnitine in all tissues, especially skeletal muscle, where the differences were significant (Daily and Sachan 1995). The exercise regimen is difficult to impose upon guinea pigs because they do not run very well on a treadmill. Rats, on the other hand, will run on a treadmill which enhances adipose tissue turnover rate (Askew et al. 1977). However, rats do not conserve carnitine as do humans or guinea pigs at equivalent dosage of choline (Daily and Sachan 1995). This was believed to be due to very high activity of choline oxidase in rat liver compared to that in guinea pigs or humans (Sidransky and Farber 1960). We speculated that high choline oxidase activity may be counterbalanced by high levels of choline supplementation in the rat diet. Therefore, supplementary choline level in the nonpurified diet was about 5-fold endogenous levels and that used in earlier rat studies by us (Daily and Sachan 1995) and others (Rein et al. 1997). After

TABLE 3-5

Correlations between body weight, three regional and total fat pad weights, food intake, blood metabolites, and muscle triglycerides in exercised and nonexercised male rats with and without choline, caffeine and carnitine supplementation[1,2]

Variable	BW	EF	IF	PF	TF	FI	LP	GLU	LA	STG	FFA
EF	0.76#										
IF	0.71**	0.86#									
PF	0.64**	0.86#	0.84#								
TF	0.74#	0.95#	0.96#	0.64**							
FI	0.77#	0.54*	0.51*	0.40	0.50*						
LP	0.55**	0.78#	0.80#	0.75#	0.82#	0.29					
GLU	0.24	0.34	0.51	0.43	0.46*	0.21	0.51*				
LA	-0.33	-0.03	-0.15	0.07	-0.05	-0.49*	-0.06				
STG	0.21	0.17	0.31	0.29	0.28	-0.31	0.32	0.22	0.19		
FFA	0.48*	0.40	0.49*	0.25	0.41	0.37	0.13	0.23	0.19	0.26	
MTG	-0.31	-0.34	-0.39	-0.41	-0.40	0.06	-0.38	-0.36	-0.21	-0.41	-0.41

[1] Values are correlation coefficients, n = 20.

[2] Abbreviation used: BW, body weight; EF, epididymal fat; IF, inguinal fat; PF, perirenal fat; TF, total fat; FI, food intake; LP, leptin; GLU, nonfasting serum glucose; LA, lactic acid; STG, serum triglycerides; FFA; serum free fatty acids; MTG, skeletal muscle triglycerides.

* $P < 0.05$, ** $P < 0.01$, # $P < 0.001$.

taking into account the daily food consumption by the rats (Table 3-1) it is estimated that caffeine, carnitine and choline intakes were 6, 300 and 800 mg/kg of body weight. The pharmacological doses of caffeine, carnitine and choline in humans are 7-14, 40-45 and 150-200 mg/kg body weight, respectively. Thus choline and carnitine were 4-6 fold higher than usual dosage but far below toxic dosage (LD_{50} of choline in rat is 3.4-6.7 g/kg body weight and none established for carnitine). It was anticipated that this amount of choline may be more than sufficient to compensate for the high activity of rat choline oxidase and therefore, may induce significant carnitine conservation. Choline supplemented at 10 g/kg diet (seven- to tenfold higher than normal) and fed for 1- 42 d increased urinary excretion of choline 19-fold in rats just after 1 d of feeding the diet (Rein et al. 1997). In guinea pigs, choline supplementation (3 g choline/kg diet) over and above the normal dietary choline concentration (1.8 mg/kg), resulted in eight- to ninefold higher choline excretion in the urine. These observations indicate that supplementary choline must get into the systemic circulation before it can be filtered into urine at these high concentrations. We can only assume that enough choline escapes choline oxidase to bring about anticipated conservation of carnitine.

The combination of choline, carnitine and caffeine superimposed with mild exercise was hypothesized to enhance oxidation of fat by skeletal muscle and as a consequence reduce the amount of body fat. Mild exercise preferentially promotes fat oxidation for meeting energy needs over and above the basal energy requirements (Romijn et al. 1993). The results of this study support this hypothesis as there was significant loss of adipose fat mass in the supplemented as well as exercised rats in 4 wk

(Table 3-2). This is important because carnitine alone had no significant effect on perirenal fat pad weight of rats fed 0.5% carnitine-supplemented diet and trained on treadmill for 5 wk (Askew et al. 1977). Diets supplemented with carnitine alone have produced variable responses regarding substrate utilization and muscle fatigue in exercise (Brass and Hiatt 1994, Decombaz et al. 1992, Lennon et al. 1983, Neumann 1996). Similarly, choline alone is not known to alter energy substrate utilization (Spector et al. 1995) or reduce body weight (Tsai et al. 1974). Likewise, caffeine alone has been shown to increase plasma free fatty acid concentration without altering substrate preference for oxidation in running exercise (Casal and Leon 1985). Caffeine by itself at a dosage of 200 mg, three times a day for 24 wk had no effect on body weight in obese patients. However, when combined with 20 mg of ephedrine, it caused a significant decrease in body weight (Astrup et al. 1992).

The reduction in body fat is supported by reduction in the endogenous marker of adiposity, leptin, which was significantly lower in the supplemented as well as exercised rats (Table 3-4). The leptin concentrations were positively correlated with body fat ($r = 0.82$, $P < 0.001$) and body weight ($r = 0.55$, $P < 0.01$) as shown in Table 3-5 and has been seen elsewhere (Ahren, et al. 1997, Frederich, et al. 1995). Factors other than adipose tissue mass influence leptin secretion (Havel 1998). For example, the decrease in circulating leptin concentration during energy restriction in human is closely related to the decrease in plasma glucose (Dubuc, et al. 1998). However, in the rats of our study, feed-intake was not affected by any of the treatments (Table 3-1), and serum concentration of leptin was positively correlated with serum glucose ($r = 0.51$, $P < 0.05$).

The differences in the concentrations of the metabolites presented in Table 3-4 suggest that supplementation favored partitioning of triglycerides from serum to skeletal muscle because there was 33-46% lower concentration in serum and 82-90% greater level in the muscle. Increased esterification and increased permeability of muscle membranes to fatty acids are possible (Marconi et al. 1985, Neumann, 1996). Lower concentrations of serum glucose (13%) and free fatty acids (31%) in the supplemented, exercised group also suggests a promotion of energy substrate utilization by supplementation under the conditions of mild exercise. Mild exercise has been shown to promote preferential use of fat for energy needs over and above the basal requirements (Romijn et al. 1993). The rise in serum lactate concentration due to exercise (88%) was buffered by the supplements where it rose less than half (39%), which may be due to decreased glycogen utilization or increased renal loss of lactate.

We thought it was relevant to characterize the chemical nature of the adipose fat mass which was found to be 80-90 % total lipids of which 70-80% were triglycerides (Table 3-3). These values are close to the upper end of the range of lipid contents of adipose tissue. Adipose tissue is, on the average, 80% fat, 18% water and 2% protein (Heymsfield et al. 1999). The protein contents of the adipose tissues were not different among the groups (data not shown).

From these data, clearly, the combination of choline, carnitine and caffeine with or without mild exercise reduces body fat, as indicated by decrease in fat pad weights and total lipids as well as serum leptin in rats. We conclude that the dietary supplement used in this study promotes fat loss as much as does the exercise, and there is no significant

interactive effect. This does not preclude reassessment of individual effects of the components of this supplement. These results may or may not be applicable to humans, and further research is necessary to determine whether similar effects would result in other species.

CHAPTER IV

INCREASES IN VO_2max AND METABOLIC MARKERS OF FAT OXIDATION BY CAFFEINE, CARNITINE AND CHOLINE SUPPLEMENTATION IN RATS

This chapter is a slightly revised version of a manuscript published with co-author Dileep S. in the Journal of Nutritional Biochemistry in 2000.

Dileep S. Sachan and Nobuko Hongu (2000) Increases in VO_2max and metabolic markers of fat oxidation by caffeine, carnitine and choline supplementation in rats. J. Nutr. Biochem. 11: 521-526, 2000.

ABSTRACT

We have previously shown that the combination of caffeine, carnitine and choline supplementation decreased body fat and serum leptin concentration in rats and this was attributed to increased fat utilization for energy. As a result, it was hypothesized that the supplements may augment exercise performance including physiological and biochemical indices. Twenty, 7-wk-old male Sprague-Dawley rats were given free access to a nonpurified diet with or without supplementation of caffeine, carnitine and choline at concentrations of 0.1, 5 and 11.5 g/kg diet, respectively. One-half of each dietary group was exercised on a motor-driven treadmill for 3 wk and maximal aerobic power (VO_2max) was determined on 18th day of exercise. Rats were killed 24-h postexercise, and blood, regional fat pads and skeletal muscle were collected. The VO_2max was increased ($P < 0.05$) in the supplemented, exercised group; however, the respiratory quotient (RQ) was not affected. Postexercised concentrations of serum triglycerides were

decreased but β-hydroxybutyrate, acylcarnitine and acetylcarnitine were increased in the supplemented rats. The changes in serum metabolites were complemented by the changes in the muscle and urinary metabolites. The magnitude of increase in urinary acylcarnitines (34–45-fold) is a unique effect of this combination of supplements. Cumulative evidence indicates enhanced β-oxidation of fatty acids without a change in the RQ because acetyl units were excreted in urine as acetylcarnitine and not oxidized to carbon dioxide. For this phenomenon, we propose the term "fatty acid dumping". We conclude that supplementation with caffeine, carnitine and choline augments exercise performance and promotes fatty acid oxidation as well as disposal in urine.

INTRODUCTION

Professional and nonprofessional athletes seek nutritional supplements that will enhance exercise performance. These substances theoretically improve exercise capacity by enhancing lipid oxidation and slowing rates of muscle glycogen depletion (Essig, et al. 1980, Hawley et al. 1998), therefore reducing fatigue (Green 1991). Substantial research has been done on the use of a single substance (or nutrient) to enhance endurance performance, but only a few studies have been carried out to determine favorable effects of a combination of ergogenic substances on metabolic processes (Decombaz et al. 1987). It is well known that athletes supplement their diets not only with single but multiple nutritional factors (Clarkson 1996, Short and Marquart 1993). Therefore, a study using a combination of three popular dietary supplements (caffeine, carnitine and choline) was envisioned, where the parameters of energy substrate utilization and exercise could be

determined.

It has been suggested that caffeine enhances lipolysis and fat oxidation and reduces glycogen breakdown (Costill et al. 1978, Essig, et al. 1980, Pasman et al. 1978) However, the results of studies on the ergogenic effects of caffeine are equivocal. Carnitine is essential for translocation of long-chain fatty acids into mitochondrial matrix as well as for the shuttling of acyl groups out of the mitochondria (Bremer 1997). Clinical studies have shown that carnitine supplementation improves muscle function and exercise capability in peripheral arterial disease and hemodialysis patients (Brass and Hiatt 1998). Yet, it is not clear whether carnitine supplementation benefits normal healthy individuals. Choline supplementation has been shown to enhance acetylcholine synthesis (Zeisel and Blusztajn 1994). A decline in plasma and urinary choline concentrations has been observed in athletes after running a marathon (Buchman et al. 1999, Conlay et al. 1992). This has lead to the hypothesis that choline supplementation may prevent decrement in physical performance. However, the mechanism by which choline could affect exercise performance has not been clearly established.

Recently, nutrient-nutrient interactions between choline and carnitine have been reported in healthy humans and animals (Daily and Sachan 1995, Daily et al. 1998, Dodson and Sachan 1996, Hongu and Sachan 2000, Rein et al. 1997). Choline supplementation promotes tissue carnitine conservation, especially in skeletal muscle, which may have positive functional consequences in terms of exercise performance (Daily and Sachan 1995, Daily et al. 1998). However, choline-supplemented exercised guinea pigs showed no change in RQ even though their carcass fat was significantly

reduced (Daily et al. 1998). Adult rats did not produce carnitine conservation, when choline dosage similar to that given to humans and guinea pigs were administered (Daily and Sachan 1995, Rein et al. 1997). In the previous study we found that relatively higher doses of choline in combination with caffeine plus carnitine given to rats for 4 wk significantly reduced their body fat and serum leptin concentration (Hongu and Sachan 2000). We report now the effects of the combination of caffeine, carnitine, and choline supplementation on RQ, VO_2max and relevant metabolic and biochemical markers of fatty acid oxidation.

MATERIALS AND METHODS

Animals and treatment

The experimental protocol was approved by the University of Tennessee Institutional Review Board. Twenty, 7-wk-old male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN), weighing 210-230 g, were individually housed in wire mesh cages in a room with controlled temperature (20-22°C), relative humidity (50%), and light cycle (12-h light/dark cycle). The rats were randomly divided into two dietary groups to receive either a nonsupplemented or supplemented diet with caffeine, carnitine and choline at concentrations of 0.1, 5 and 11.5 g/kg nonpurified diet, respectively. Food intake and body weight were determined daily throughout the 5-wk experimental period.

After 1 wk of dietary treatment, one-half of each dietary group (n = 5) was put to exercise on the rodent treadmill (Columbus Instruments International, Inc., Columbus,

OH) for 10 min at 15% grade, 6 d/wk. The running speed and duration were gradually increased during the experiment to maximize at 18 m/min for 25 min/day. Expired gasses were continuously analyzed using an Ametek S-3A oxygen analyzer and CD-3A carbon dioxide analyzer (Ametek, Inc., Paoli, PA). The expired gases were monitored to calculate RQ. Resting RQ of each rat was measured on the first day of exercise period (wk 3), after a rat was sitting quietly in the metabolic chamber for 20 min. On the 18th day of exercise, the VO_2max was determined according to the method described by Bedford et al. (1979). The exhaustion RQ was measured at the time a rat stopped running, and the running time was recorded as the exhaustion time. A tail-vein blood sample was taken at exhaustion for lactate determination.

Sample collection and assays

At the end of the experimental period, rats were anesthetized with methoxyflurane (Pitman-Moore, Mundelein, IL) and killed by exsanguination after cardiocentesis. In the exercised rats, blood (cardiac) and tissue collections were done 24 hr after the last bout of exercise. The blood samples were immediately centrifuged and serum was stored at -80°C. Following blood collection, regional fat pads and skeletal muscle (gastrocnemius) samples were collected and frozen in liquid nitrogen, and stored at -80°C. The samples were assayed for the concentrations of lactate (Gutmann and Wahlefeld 1974), triglycerides (Giegel et al. 1975), free fatty acids (Novak 1965) and carnitine fractions (Cedarblad and Lindstedt 1972, Sachan et al. 1984) according to the established procedures. Glucose and β-hydroxybutyrate were determined by using Sigma kits no. 510 and 310, respectively (Sigma, St.Louis, MO).

Statistical analysis

Data are reported as means ± SEM (n = 5). The differences between exercise groups at exhaustion were tested with Student's *t* test. Two-way ANOVA was used to determine the effects of exercise, supplementation and their interaction on serum and skeletal muscle metabolites and urinary carnitine excretion. Statistics were performed using SAS (1997). A value of $P < 0.05$ was considered to be significant.

RESULTS

There were no significant differences in food intakes of the groups (Table 3-1). Final body weight was significantly affected by exercise (Table 3-1), but total fat pad weight was affected by exercise as well as by the supplement (Table 3-2). The mean total fat pad weight of supplemented, exercised rats was 25% lower than nonsupplemented, exercise rats (Table 3-2).

No significant differences were observed in the mean RQ values of the groups at rest and at exhaustion between the two exercised groups (**Table 4-1**). The VO_2max of the supplemented rats was significantly higher than that of the nonsupplemented rats, which is indicative of the enhanced exercise capacity of the supplemented rats. Although exhaustion time and blood lactate were not statistically significant, the 70% longer time to exhaustion and 29% lower blood lactate concentration in the supplemented, exercised rats are physiological changes too large to be easily ignored (Table 4-1).

TABLE 4-1

Effects of caffeine, carnitine and choline supplementation on RQ at rest and exhaustion, VO_2max, exhaustion time, and blood lactate concentrations at exhaustion in exercised rats[1]

	Nonsupplement		Supplement	
Parameters	Nonexercise	Exercise	Nonexercise	Exercise
RQ				
At rest	0.879 ± 0.02	0.875 ± 0.02	0.866 ± 0.02	0.847 ± 0.01
At exhaustion	----------	0.920 ± 0.02	----------	0.906 ± 0.03
VO_2max (ml/kg/min)	----------	47.80 ± 1.12	----------	52.13 ± 1.28*
Exhaustion time (min)	----------	32.26 ± 9.3	----------	54.72 ± 15.8
Lactate at exhaustion (mmol/L)	----------	2.66 ± 1.46	----------	1.89 ± 0.53

[1]Values are mean ± SEM, $n = 5$.
Rats were fed diet without supplemented or supplemented with caffeine, carnitine, and choline at concentrations of 0.1, 5, and 11.5 g/kg diet, respectively. The energy distribution of diet: 22% protein, 5% fat, and 73% carbohydrate. The diet provides 16.7 kJ/g (3.99 kcal/g) and 3.8% fiber.
* $P < 0.05$.

One day after the last bout of exercise, cardiac blood and tissues were collected. Triglycerides were significantly lower in serum (**Table 4-2**) but significantly higher in skeletal muscle (**Table 4-3**) of both supplemented groups. There were no significant differences in serum glucose and free fatty acids. Serum β-hydroxybutyrate was affected by exercise, even though it was determined in the blood samples collected 24 hr after exercise periods (Table 4-2). All fractions of serum carnitine were significantly higher in the supplement rats with or without exercise (Table 4-2). Serum carnitine fractions of the nonsupplemented, exercised rats were 20-29% lower compared with the nonsupplemented, nonexercised rats. There was significant interactive effect of supplement and exercise on total carnitine (TC), nonesterified carnitine (NEC) and acid soluble acylcarnitine (ASAC) in serum.

The supplementation resulted in significantly higher concentration of triglycerides, TC, NEC and ASAC in skeletal muscle (Table 4-3). There was 18% less TC in the nonsupplemented, exercised rats compared to nonsupplemented, nonexercise rats; however, TC concentration remained the same in the two supplemented groups with or without exercise. The muscle free fatty acids and total lipid were significantly higher in the exercised rats with or without supplements (Table 4-3).

Urinary excretion of TC and all fractions of carnitine were significantly higher in the supplemented groups (**Table 4-4**). There was 34–45-fold increase in ASAC and 56–80-fold increase in acid-insoluble acylcarnitine (AIAC) in the urine of the supplemented rats. There were no differences between the nonexercised and exercised

TABLE 4-2

Effects of supplement and exercise on serum metabolites at rest in male rats fed the nonsupplemented diet or supplemented diet with caffeine, carnitine and choline[1-2]

	Nonsupplement		Supplement		Statistical significance[3]		
Metabolites	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[4]
Glucose, *mmol/L*	9.26 ± 0.57	9.10 ± 0.19	9.18 ± 0.36	7.94 ± 0.28	NS[5]	NS	NS
Triglycerides, *mmol/L*	0.76 ± 0.04	0.69 ± 0.09	0.41 ± 0.06	0.46 ± 0.04	0.0003	NS	NS
β-Hydroxybutyrate *mg/dL*	0.61 ± 0.15	1.57 ± 0.32	1.31 ± 0.38	2.20 ± 0.44	NS	0.0153	NS
Free fatty acids, *μmol/L*	282 ± 30.9	290 ± 38.9	284 ± 29.4	195 ± 24.4	NS	NS	NS
TC, *μmol/L*	82.1 ± 1.6	66.5 ± 0.9	106.9 ± 0.9	117.6 ± 4.9	0.0001	NS	0.0003
NEC, *μmol/L*	64.8 ± 2.3	53.7 ± 1.4	85.6 ± 2.0	95.0 ± 3.5	0.0001	NS	0.0007
ASAC, *μmol/L*	14.4 ± 1.7	10.2 ± 1.7	16.1 ± 1.1	18.3 ± 1.2	0.0035	NS	0.0380
AIAC, *μmol/L*	3.0 ± 0.2	2.4 ± 0.3	5.2 ± 0.3	4.3 ± 0.5	0.0001	NS	NS
AC, *nmol/L*	42.7 ± 1.1	34.3 ± 0.7	71.9 ± 5.68	73.7 ± 4.66	0.0001	NS	NS

[1]Values are means ± SEM, $n = 5$. Rats were fed diet without supplemented or supplemented with caffeine, carnitine, and choline at concentrations of 0.1, 5 and 11.5 g/kg diet, respectively. The energy distribution of diet: 22% protein, 5% fat, and 73% carbohydrate. The diet provides 16.7 kJ/g (3.99 kcal/g) and 3.8% fiber.

[2]Abbreviation used: AC, acetylcarnitine; AIAC, acid-insoluble acylcarnitine; ASAC, acid-soluble acylcarnitine; NEC, nonesterified carnitine; TC, total carnitine.

[3]*P* values from two-way ANOVA, using the factors, supplementation and exercise.

[4]S X E; supplement and exercise interaction.

[5]NS: not significant, $P > 0.05$.

TABLE 4-3

Effects of supplement and exercise on skeletal muscle metabolites at rest in male rats fed the nonsupplemented diet or supplemented diet with caffeine, carnitine and choline[1-2]

Metabolites (units/g wet tissue)	Nonsupplement		Supplement		Statistical significance[2]		
	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[3]
Triglycerides, *μmol*	32.1 ± 2.7	34.1 ± 4.7	61.2 ± 5.2	62.2 ± 6.2	0.0001	NS	NS
Free fatty acids, *μmol*	2.1 ± 0.1	2.8 ± 0.3	2.3 ± 0.1	2.8 ± 0.1	NS	0.0050	NS
Total lipid, *mg*	13.6 ± 0.8	20.2 ± 1.2	11.2 ± 1.2	19.1 ± 2.1	NS	0.0001	NS
TC, *nmol*	1025 ± 35.4	966 ± 59.6	1270 ± 26.2	1282 ± 56.5	0.0001	NS	0.0002
NEC, *nmol*	576 ± 32.2	542 ± 33.7	637 ± 31.4	727 ± 49.8	0.0001	NS	NS
ASAC, *nmol*	270 ± 29.8	295 ± 24.5	533 ± 30.6	419.2 ± 24.0	0.0001	NS	0.0218
AIAC, *nmol*	164 ± 16.5	138 ± 6.3	109.8 ± 8.7	147.8 ± 5.6	NS	NS	0.0165
AC, *nmol*	245 ± 14.9	204 ± 19.9	276 ± 15.6	254.3 ± 22.1	NS	NS	NS

[1]Values are means ± SEM, $n = 5$. Rats were fed diet without supplemented or supplemented with caffeine, carnitine, and choline at concentrations of 0.1, 5 and 11.5 g/kg diet, respectively. The energy distribution of diet: 22% protein, 5% fat, and 73% carbohydrate. The diet provides 16.7 kJ/g (3.99 kcal/g) and 3.8% fiber.
[2]Abbreviation used: AC, acetylcarnitine; AIAC, acid-insoluble acylcarnitine; ASAC, acid-soluble acylcarnitine; NEC, nonesterified carnitine; TC, total carnitine.
[3]*P* values from two-way ANOVA, using the factors, supplementation and exercise.
[4]S X E; supplement and exercise interaction. [5]NS: not significant, $P > 0.05$.

TABLE 4-4

Effects of supplement and exercise on urinary carnitine excretion in male rats fed the nonsupplemented diet or supplemented diet with caffeine, carnitine and choline[1-2]

Carnitine *μmol/day*	Nonsupplement		Supplement		Statistical significance[2]		
	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[3]
TC	0.99 ± 0.26	0.87 ± 0.08	100.4 ± 7.9	105.6 ± 8.7	0.0001	NS	NS
NEC	0.57 ± 0.2	0.48 ± 0.04	85.2 ± 7.2	87.5 ± 9.2	0.0001	NS	NS
ASAC	0.4 ± 0.06	0.36 ± 0.04	13.6 ± 3.2	16.4 ± 2.2	0.0001	NS	NS
AIAC	0.02 ± 0.00	0.03 ± 0.01	1.6 ± 0.1	1.7 ± 0.1	0.0001	NS	NS
AC	0.66 ± 0.16	0.56 ± 0.06	13.4 ± 2.5	14.4 ± 2.8	0.0001	NS	NS

[1]Values are means ± SEM, $n = 5$. Rats were fed diet without supplemented or supplemented with caffeine, carnitine, and choline at concentrations of 0.1, 5 and 11.5 g/kg diet, respectively. The energy distribution of diet: 22% protein, 5% fat, and 73% carbohydrate. The diet provides 16.7 kJ/g (3.99 kcal/g) and 3.8% fiber.
[2]Abbreviation used: AC, acetylcarnitine; AIAC, acid-insoluble acylcarnitine; ASAC, acid-soluble acylcarnitine; NEC, nonesterified carnitine; TC, total carnitine.
[3]*P* values from two-way ANOVA, using the factors, supplementation and exercise.
[4]S X E; supplement and exercise interaction.
[5]NS: not significant, $P > 0.05$.

rats. The majority (88-98%) of the urinary ASAC was acetylcarnitine (AC) in the supplemented groups.

DISCUSSION

The known biochemical and physiological effects of caffeine, carnitine and choline formed the basis of our hypothesis that coadministration of these agents should augment exercise endurance and provide energy from fat oxidation in muscle. The conceptual model was that caffeine will promote fatty acid release from adipose tissue, choline will readily allow entry of carnitine into skeletal muscle cells, and carnitine will enhance translocation of fatty acids (as acylcarnitine) into the mitochondrial matrix of muscle. As a result, there should be a shift in the indices of exercise and fat oxidation. Measurements of VO_2max, RQ, enzyme activities and substrate or metabolite concentrations are often chosen as endpoints in exercise studies to assess aspects of physical performance. VO_2max, provides an assessment of maximal exercise capacity (Wasserman and Whipp 1975) and is an accepted index of the functional limit of the cardiovascular system (Rowell 1974). Also, it has been used to evaluate the effect of the ergogenic aids including nutritional supplements (Marconi et al. 1985, Spector et al. 1995). RQ is a convenient and general guide to the relative rates of glucose and fat oxidation during steady state exercise below the lactate threshold (Jansson 1982).

The results of this study show that the combination of supplements augmented exercise performance as indicated by 8% increased VO_2max, 70% longer time to exhaustion, and 29% lower blood lactate concentration (Table 4-1). These results are

consistent with those reported by Marconi et al.(1985) who found a significant increased in the VO_2max after carnitine loading (4 g/d for 2 wk) of the competitive athletes engaged in training programs. However, the results of other studies in humans supplemented with carnitine are contradictory (Greig et al. 1987, Wyss et al. 1990). Some of the contradiction may be related to variations in carnitine loading. For example, we gave carnitine at the rate of 105 mg/d for 4 wk, which was 150-fold higher than that of the nonsupplemented rats and 5-10-fold greater than the dosage used in humans (2-4 g/d). Further, the polypharmacy nature of the supplement (combination of choline, carnitine and caffeine) may lend a unique advantage to the system not present in other studies.

The RQ range (0.84-0.87) of the rats (Table 4-1) suggests that energy substrates were both fat and carbohydrates (50:50), according to the published reports (McArdle et al. 1986). The RQ was not altered by the supplement in our study; similar results have been reported by Marconi et al. (1985). This means that fat was not the major energy substrate under these conditions, which is difficult to reconcile with the significant loss of body fat in supplemented, as well as in exercised rats (Table 3-2) (Hongu and Sachan 2000). We have argued that the loss of body fat was due to fat being used as the energy substrate, as feed composition and intakes were not different among the groups (Table 3-1). Therefore, markers of fatty acid oxidation other than RQ needed closer examination.

The lower serum lactate concentration in the supplemented, exercised rats compared to nonsupplemented, exercised rats (Table 4-1) is suggestive of lesser glucose catabolism and more fatty acid oxidation to provide energy for exercise (Sugden and Holness 1994). A more direct evidence of fatty acid oxidation is seen in the higher β-

hydroxybutyric acid (> 40%), lower triglycerides (40%) and free fatty acids (31%) in the serum of the supplemented, exercised rats compared to the nonsupplemented, exercised rats (Table 4-2). Additional evidence is presented by the changes in short-chain acylcarnitine (ASAC) and AC, both being markers of fatty acid oxidation (Bremer 1997). The serum ASAC and AC are nearly doubled in the supplemented rats (Table 4-2). A decrease in ASAC (29%) and AC (20%) in the exercised rats without supplement (nonsupplemented, exercised vs. nonsupplemented, nonexercised) suggests limited production of these metabolites due to lack of supplement. The lower concentrations of ASAC and AC in the nonsupplemented, exercised rats could not be explained by complete oxidation of acetyl groups because RQ was not decreased in the rats (Table 4-1).

The changes in the markers of fatty acid oxidation in serum were complimentary to the changes in skeletal muscle where supplement increased accretion of triglyceride and carnitine, particularly the ASACs, which are indicators of fatty acid oxidation (Table 4-3). The ASACs were increased about 1.4-fold by the supplement and by exercise in the gastrocnemius muscle, which is consistent with the changes in serum and urine, and supports the argument that the supplement promoted fatty acid oxidation.

So why there was no decrease in the RQ of the supplemented animals if fatty acid oxidation was enhanced? To reconcile the lack of concert between the RQ and biochemical markers of fat oxidation, we postulate the "fatty acid dumping" hypothesis with the following rationale. The activation and subsequent oxidation (via β-oxidation) of fatty acids produces acyl-CoAs of various chain lengths, including acetyl-CoA

(Hiatt et al. 1989). As the ratio of acetyl-CoA/CoA rises in muscle mitochondria after exercise (Friolet et al. 1994, Hülsmann et al. 1964), the acetyl moiety is readily transferred to carnitine (Harris et al. 1987, Sahlin 1990) in the presence of readily available carnitine in the muscle, promoted by choline in the supplement, as reported elsewhere (Daily and Sachan 1995, Daily et al 1998). The AC is not a substrate for condensing enzyme, therefore it can not enter the tricarboxylic acid (TCA) cycle, and must exit mitochondria (Bremer 1997). The cytosolic AC enters blood and is filtered out in urine. This is supported by the rise in AC concentrations by 2-fold in serum (Table 4-2) and 25-fold in urine (Table 4-4) but not in the muscle (Table 4-3) of the supplemented rats. The AC did not accumulate in the muscle, as it was allowed to exit into serum and urine where it makes up 88-98 % of ASAC (Table 4-4). A similar scenario holds true for the fatty acids of various chain lengths, particularly the short-chain fatty acids (ASAC). The rise of ASAC content of skeletal muscle (2-fold), serum (2-fold) and urine (35-45-fold) of the supplemented rats (Tables 4-2-4-4) is consistent with our hypothesis. Thus, a significant amount of fatty acid carbons do not get oxidized to CO_2 and the RQ remains unchanged. Instead, the fatty acid carbons are loaded on to carnitine and dumped in urine as ACs.

Could this be an effect of caffeine or carnitine alone? Caffeine promotes release of fatty acids from adipose tissue, which does not necessarily mean that it enhances fatty acid oxidation. There is paucity of clean biochemical data resulting in a great deal of controversy about the promotion of fat oxidation by caffeine alone (Astrup et al. 1992, Casal and Leon 1985). With regard to carnitine alone, there is reasonable evidence that it

promotes fatty acid oxidation in intact animals; however, the magnitude of the effect is small and controversial. Negrao et al. (1987) found no significant increase in plasma ASAC of rats given i.p. carnitine for 8 wk. In one of our studies (Sachan and Mynatt 1992), we fed 0.5% carnitine-supplemented diet to rats for 5,10, 20, 30 and 40 d and found no significant increase in serum ASAC at any of these time points; however, there was about 2-fold increase in serum ASAC and AIAC after 40 d of treatment. In another study (Berger and Sachan 1992), when rats were fed diets supplemented with various doses of carnitine for 10 d, there was about 2- and 10-fold increase in the ASAC concentrations of blood and urine, respectively. So carnitine alone brings about respectable increases in urinary ASAC (10-fold) but nowhere near the 34–45-fold increase in ASAC and 56–80-fold increase in AIAC seen in the current study (Table 4-4). The magnitude of ASAC and AC loss in urine by the combination of carnitine, caffeine and choline compared to that by carnitine alone is a very important point. A sustained loss of fatty acid carbons, even in smaller amounts, are analogues to a dripping water faucet. Thus, we call this phenomenon "fatty acid dumping". The shift in the normal direction of flow of fatty acid carbons must perturb the metabolic pathways and modulate normal homeostasis and regulation of energy substrate utilization.

In conclusion, the combination of caffeine, carnitine and choline supplement increases aerobic work capacity by enhancing fatty acid oxidation in the skeletal muscle. The simultaneous presence of caffeine, carnitine and choline is the key for promotion of facilitated availability and transport of fat to the site of oxidation as well as disposal of the end products of β-oxidation of fatty acids, i.e., acetyl groups. The loss of fatty acids

as ASAC or AC leaves RQ unchanged, even though fatty acids are oxidized but not to CO_2 as conventionally expected.

CHAPTER V

TISSUE CARNITINE ACCRETION AND FAT METABOLISM IN RATS SUPPLEMENTED WITH CARNITINE, CHOLINE AND CAFFEINE REGARDLESS OF EXERCISE

This chapter is a slightly revised version of a manuscript, whish has been accepted for publication with co-author Dileep S. Sachan in the Journal of Medical Sciences 2: 2002 (in press).

ABSTRACT

The objective of this study was to determine if a combination of carnitine, choline and caffeine supplementation would alter the concentrations of carnitine in various rat tissues (i.e. liver, heart, kidneys, brain, and testes) with or without exercise. Male, 7-wk-old Sprague-Dawley rats were given free access to a nonpurified diet with or without supplementation of carnitine, choline and caffeine at concentrations of 5, 11.5, and 0.1 g/kg diet, respectively for 4 wk. One-half of each dietary group was assigned to exercise on a treadmill for 3 wk. Results showed that the concentration of nonesterified and total carnitine was higher in serum, urine and all tissues of the supplemented rats. Exercise further promoted this effect in the liver and kidneys. There were variable effects of supplement and exercise on the other fractions of carnitine in tissues. However, short-chain acylcarnitines were consistently higher in the skeletal and cardiac muscles and reflected in the serum and urine of the supplemented rats. Serum aspartate amino

transferase (AST) and alanin amino transferase (ALT), liver proteins and DNA were not adversely altered by the supplement. In conclusion, oral feeding of carnitine, choline, and caffeine (CCC) supplement to rats promotes carnitine influx in all tissues and changes in acylcarnitines of skeletal and cardiac muscles, indicating enhanced fatty acid oxidation. This is supported by the changes in serum and urinary acylcarnitine profiles and loss of adipose tissue mass.

INTRODUCTION

Carnitine is an endogenous quaternary amine that is synthesized from the essential amino acids, lysine and methionine (Cox and Hoppel 1973). It is present in all mammalian tissues, with higher concentrations in heart, skeletal muscle, and with lower concentrations in blood plasma, liver and kidney (Bieber 1988). Carnitine is essential in the oxidation of fatty acids, serving as a "carrier" of activated fatty acids, fatty acyl-CoA esters, across the mitochondrial inner membrane to the site of β-oxidation. Carnitine is also necessary for maintaining intramitochondrial acyl-CoA/CoA ratio by accepting acyl moieties from acyl-CoA (Bieber et al. 1982). A positive outcome of this process is the removal of toxic acyl-CoAs generated from the catabolism of branched chain amino acids (Bieber 1988).

Carnitine is present in tissues as free (NEC) and as esterified carnitine (acylcarnitine). Many pathophysiological conditions affect serum and tissue carnitine concentrations and their excretion in urine. Exercise training has been reported to increase NEC and short-chain acylcarnitine or ASAC in skeletal muscle and heart of

swim-trained female rats (Lennon and Mance 1986). There was an increase in NEC in skeletal muscle and long-chain acylcarnitine (AIAC) in plasma and liver of treadmill trained male Sprague-Dawley rats (Negrao et al. 1987). Experiments in humans indicate increased carnitine concentrations in serum and skeletal muscle of trained individuals (Lennon and Mance 1986, Arenas et al. 1991). A number of studies have shown increased carnitine concentrations in different tissues as a result of carnitine supplementation and/or exercise; however, the magnitudes of the changes have been highly variable in some studies (Negrao et al. 1987, Paulson et al. 1984, Simi et al. 1990).

We have reported that choline supplementation promotes tissue carnitine conservation in humans and animals (Daily and Sachan 1995, Daily et al. 1998, Dodson and Sachan 1996). Choline supplementation causes significant accumulation of carnitine in the skeletal muscle of guinea pigs; where it was associated with leaner body composition, improved exercise performance, but showed no change in RQ (Daily et al. 1998). A dosage of choline similar to that given to humans and guinea pigs did not result in carnitine conservation in adult rats (Daily and Sachan 1995, Daily et al. 1998). However, relatively higher doses of choline in combination with caffeine plus carnitine given to rats for 4 wk resulted in significant increase in carnitine status of serum, skeletal muscle and urine (Sachan and Hongu 2000). Functional consequences of the changes due to the supplementation were significant loss of fat pad mass (Hongu and Sachan 2000), increase in VO_2max and enhanced fat oxidation (Sachan and Hongu 2000). The present report addresses distribution of carnitine, acylcarnitine and acetylcarnitine among the tissues of rats fed diets supplemented with CCC under sedentary or exercising regimen.

MATERIALS AND METHODS

Animals and treatment

The study was conducted at the University of Tennessee and all animal procedures were approved by the Institutional Animal Care and Use Committee, and were in accordance with the NIH guidelines (National Research Council, 1985). Twenty, 7-wk-old, male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN), weighing 210-230 g, were individually housed in wire mesh cages in a room with controlled temperature (20-22°C), relative humidity (50%), and light cycle (12-hr light/dark cycle) for the entire 5 wk study period. The rats were randomly divided into two dietary groups to receive either the nonsupplemented or supplemented diet with caffeine, carnitine and choline at concentrations of 0.1, 5 and 11.5 g/kg nonpurified diet, respectively. Food intake and body weight were determined daily. Average food intake (Table 3-1) was calculated by using the daily food intake throughout the 4 wk.

After 1 wk of dietary treatment, one-half of each dietary group (n = 5) were put to exercise on the rodent treadmill (Columbus Instrument Internationals, Inc., Columbus, OH) for 10 min at 15% grade, 6 d/wk. The running speed and duration were increased gradually during the experimental period (3 wk) to maximize at 18 m/min for 25 min/d.

Sample collection and assays

In all animals 24-h urine was collected after 3 wk of the dietary treatment by placing rats in the stainless steel metabolic cages. For the exercised rats urine was collected after completing the last bout of the 2-wk exercise period even though they were kept on the exercise regimen for another week, i.e. 24-h prior to being killed. Urine

samples were quantified, centrifuged, and an aliquot was stored at -80°C for later analysis. After 4 wk of dietary treatment and 3 wk of the exercise regimen, the rats were anesthetized with methoxyflurane (Pitman-Moore, Mundelein, IL) and killed by exsanguination after cardiocentesis. The blood samples were immediately centrifuged and serum was stored at -80°C (Hongu and Sachan 2000). The tissues were removed and immediately frozen in liquid nitrogen and stored at -80°C. Only the heart samples were freeze-dried and stored at -80°C. In the exercise groups, blood and tissue were collected 24-hr after the last bout of exercise (Sachan and Hongu 2000).

Carnitine concentrations in tissues, serum and urine were determined by a radioenzymatic assay originally described by Cederblad and Lindstedt (1972) and modified by Sachan et al. (1984). All samples were fractionated in perchloric acid (PCA) to allow determination of NEC, acid soluble carnitine (ASC) and AIAC.

In brief, the deproteinization of samples with PCA separates the TC content into an acid-soluble fraction and an acid-insoluble fraction, which are removed by centrifugation (1500 X g for 10 min). NEC and ASC remain in the soluble fraction, while AIAC is precipitated with its protein. Following a PCA deproteinization, both the supernatant (acid-soluble fraction) and the pellet (acid-insoluble fraction) are alkalinized using potassium hydroxide (KOH) to release NEC from it's ester linkage. The samples are incubated in a water bath for the hydrolysis at 37°C for 30 min or at 65°C for 60 min. After PCA or KOH treatment, samples are neutralized prior to assay. The neutralized samples are added to the reagent mix, which contains [1-^{14}C] acetyl-CoA, unlabelled acetyl-CoA, and other buffer solutions. The reaction is started by adding carnitine acetyl

transferease (CAT) (EC 2.3.1.7). Carnitine in a sample can incorporate the acetyl moiety to [1-^{14}C] acetyl-CoA to form the [1-^{14}C] acetyl-carnitine. The unreacted, negatively charged [1-^{14}C] acetyl-CoA is then separated by means of anion exchange resin (column). Carnitine esters carrying the positive charge of the quaternary ammonium are not retained by the resin. The radioactivity of the solution due to the eluted [1-^{14}C] acetylcarnitine from the anion exchange column is measured (**Figure 6-2**). This assay gives the fraction values of NEC, ASC and AIAC. The ASAC fraction is calculated by subtracting NEC from the ASC. TC refers to the sum of the NEC, ASAC and AIAC. AC in all samples was determined according to the method of Pande and Caramancion (1981). Detailed procedure of AC assay is described in Appendix A.

Tissue protein was determined using the Bio-Rad Coomassie dye binding assay (Hercules, CA) with bovine serum albumin as the standard (Bradford, 1976). The concentration of DNA in a liver sample was determined using bisbenzimide dye with a calf thymus DNA standard (Sigma, St. Louis, MO). The DNA-dye complex was measured at 425 nm by DyNAQuant 200 fluorometer (Pharmacia Biotech, Piscataway, NJ). The activities of serum AST and ALT were determined spectrophotometrically using Sigma diagnostic kit no. 158 and 159, respectively (Sigma Diagnostics®, St. Louis, MO).

Statistical analysis

All results are presented as group means ± SEM. Data were analyzed using two-way ANOVA to test the effects of exercise, supplementation and their interaction using SAS (SAS Institute, 1998). The main effects of diet and exercise were tested using

specific linear contrasts, as was the interaction. Statistical significance level was set at $P < 0.05$.

RESULTS

The concentration of all carnitine fractions including the AC in serum and urine were higher in the supplemented rats with or without exercise (**Table 5-1**). Significant supplement and exercise interactions were observed in the serum TC, NEC and ASAC. These carnitine fractions were lower in the exercised, nonsupplemented rats but higher in the serum of exercised, supplemented rats; however, the differences were not statistically significant.

Supplement and exercise resulted in lower liver weight (**Table 5-2**). However, no significant difference was found when liver weight was expressed as a percentage of the body weight (data not shown). Therefore, the liver weight may be simply reflecting the body weight, which was significantly affected only by exercise (Table 3-1). Total protein in liver was not affected by either supplement or exercise. DNA concentration in liver was significantly higher in the exercise group, and there was significant interaction between supplement and exercise, but the supplement alone had no significant effect on hepatic DNA concentration. Although no differences were observed in AST activities, supplement and exercise resulted in significantly lower ALT activity (Table 5-2). The concentrations of TC, NEC and AIAC in liver were markedly higher in the supplemented rats (Table 5-2). There was no significant difference in the ASAC of the groups, even though the ASAC was 53% higher in the supplemented, exercised rats

TABLE 5-1

Serum and urinary carnitine profiles of rats with or without supplement and exercise[1-2]

Carnitine	Nonsupplement		Supplement		Statistical significance[3]		
	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[4]
Serum TC (μmol/L)	82.1 ± 1.6	66.5 ± 0.9	106.9 ± 0.9	117.6 ± 4.9	0.0001	NS[5]	0.0003
Serum NEC (μmol/L)	64.8 ± 2.3	53.7 ± 1.4	85.6 ± 2.0	95.0 ± 3.5	0.0001	NS	0.0007
Serum ASAC (μmol/L)	14.4 ± 1.7	10.2 ± 1.7	16.1 ± 1.1	18.3 ± 1.2	0.0035	NS	0.0380
Serum AIAC (μmol/L)	3.0 ± 0.2	2.4 ± 0.3	5.2 ± 0.3	4.3 ± 0.5	0.0001	NS	NS
Serum AC (nmol/L)	42.7 ± 1.1	34.3 ± 0.7	7 1.9 ± 5.7	73.7 ± 4.7	0.0001	NS	NS
Urine TC (μmol/d)	1.0 ± 0.3	0.9 ± 0.1	100.4 ± 7.9	105.6 ± 8.7	0.0001	NS	NS
Urine NEC (μmol/d)	0.6 ± 0.2	0.5 ± 0.0	85.2 ± 7.2	87.5 ± 9.2	0.0001	NS	NS
Urine ASAC (μmol/L)	0.4 ± 0.1	0.4 ± 0.0	13.6 ± 3.2	16.4 ± 2.2	0.0001	NS	NS
Urine AIAC (μmol/L)	0.02 ± 0.00	0.03 ± 0.01	1.6 ± 0.1	1.7 ± 0.1	0.0001	NS	NS
Urine AC (μmol/L)	0.7 ± 0.1	0.6 ± 0.03	13.4 ± 1.1	14.4 ± 1.2	0.0001	NS	NS

[1]Values are means ± SEM, $n = 5$. Rats were fed diet without supplemented or supplemented with caffeine, carnitine and choline at concentrations of 0.1, 5 and 11.5 g/kg diet, respectively.
[2]Abbreviation used: AC, acetylcarnitine; AIAC, acid-insoluble acylcarnitine; ASAC, acid-soluble acylcarnitine; NEC, nonesterified carnitine; TC, total carnitine. [3]*P* values from two-way ANOVA, using the factors, supplementation and exercise. [4]S X E; supplement and exercise interaction. [5]NS: not significant, $P > 0.05$.

TABLE 5-2

Serum enzymes, liver weight, protein, DNA and carnitine profiles of rats with or without supplement and exercise[1-2]

Parameters	Nonsupplement		Supplement		Statistical significance[3]		
	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[4]
Serum AST (U/L)	40.0 ± 4.7	31.9 ± 1.9	42.4 ± 8.6	29.0 ± 1.1	NS[6]	NS	NS
Serum ALT (U/L)	35.7 ± 1.5	26.8 ± 3.4	27.7 ± 2.0	22.0 ± 2.1	0.0158	0.0071	NS
Weight of liver (g[5])	13.6 ± 0.5	12.2 ± 0.1	12.5 ± 0.4	11.2 ± 0.4	0.0123	0.0030	NS
Liver protein (mg/g)	109.7 ± 8.8	145.6 ± 7.9	128.2 ± 8.4	126.9 ± 14.5	NS	NS	NS
Liver DNA (mg/g)	0.66 ± 0.02	0.99 ± 0.04	0.85 ± 0.06	0.84 ± 0.08	NS	0.0085	0.0051
Liver TC (nmol/g)	290.2 ± 16.3	316.7 ± 14.2	356.3 ± 30.4	452.2 ± 30.0	0.0006	0.0205	NS
Liver NEC (nmol/g)	256.0 ± 9.0	265.2 ± 15.4	309.2 ± 26.5	383.9 ± 21.0	0.0004	0.0431	NS
Liver ASAC (nmol/g)	28.0 ± 8.5	46.7 ± 12.5	39.3 ± 14.2	59.3 ± 8.1	NS	NS	NS
Liver AIAC (nmol/g)	6.2 ± 0.9	4.9 ± 0.7	7.9 ± 0.9	9.0 ± 1.1	0.0054	NS	NS
Liver AC (nmol/g)	10.2 ± 2.4	21.4 ± 2.6	11.4 ± 1.6	25.6 ± 6.8	NS	0.0050	NS

[1]Values are means ± SEM, $n = 5$. Rats were fed diet without supplemented or supplemented with caffeine, carnitine and choline at concentrations of 0.1, 5 and 11.5 g/kg diet, respectively.
[2]Abbreviation used: AC, acetylcarnitine; AIAC, acid-insoluble acylcarnitine; ASAC, acid-soluble acylcarnitine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NEC, nonesterified carnitine;TC, total carnitine.
[3]*P* values from two-way ANOVA, using the factors, supplementation and exercise.
[4]S X E; supplement and exercise interaction. [5]g: gram of wet weight of tissue.[6]NS: not significant, $P > 0.05$.

compared to the nonsupplemented, nonexercised rats. AC in liver was higher in the exercised rats with or without supplement. Both exercise groups had twice as much AC concentration compared to their nonexercised counterparts.

Supplementation resulted in significantly higher concentration of TC, NEC and ASAC in the skeletal and cardiac muscles (**Table 5-3**). In the skeletal muscle, supplement and exercise interaction was significant for the ASAC and AIAC. Exercise without supplementation produced slightly lower concentration of AIAC, but exercise with supplementation had the opposite effect. In the cardiac muscle, supplement caused significantly higher concentrations of TC, NEC and ASAC but there was no significant supplement and exercise interaction. The AIAC in cardiac muscle of the exercised rats increased 48-84% compared to their nonexercised counterparts; however, these were statistically not significant. In contrast to skeletal muscle, the AC in cardiac muscle was significantly higher in the exercise groups particularly in the supplemented groups, where it was almost doubled. The decrease in the ASAC in the cardiac muscle of exercised rats without supplement was proportional to the increase in the AC, which amounted to more than 90 % of the entire ASAC.

Supplement resulted in significantly higher concentration of TC and NEC in the testes, brain (**Table 5-4**) and kidney (**Table 5-5**), and AC in the testes (Table 5-4) and kidney (Table 5-5). There were no significant differences in ASAC and AIAC of the testes, brain or kidney (Table 5-4 & Table 5-5).

TABLE 5-3

Skeletal and cardiac muscle carnitine profiles of rats with or without supplement and exercise[1-2]

Carnitine (nmol/g of wet wt. (Skeletal) or nmol/g of freeze-dried wt. (Cardiac)	Nonsupplement		Supplement		Statistical significance[3]		
	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[4]
Skeletal m. TC	1024.8 ± 35.4	965.8 ± 59.6	1269.6 ± 26.2	1282.0 ± 56.5	0.0001	NS[5]	NS
Skeletal m. NEC	576.4 ± 32.2	541.8 ± 33.7	636.8 ± 31.4	727.0 ± 49.8	0.0001	NS	NS
Skeletal m. ASAC	270.0 ± 29.8	295.4 ± 24.5	533.0 ± 30.6	419.2 ± 24.0	0.0001	NS	0.0218
Skeletal m. AIAC	164.4 ± 16.5	138.0 ± 6.3	109.8 ± 8.7	147.8 ± 5.6	NS	NS	0.0165
Skeletal m. AC	244.7 ± 14.9	204.4 ± 19.9	275.6 ± 15.6	254.3 ± 22.1	NS	NS	NS
Cardiac m. TC	1725.6 ± 128	1465.9 ± 100	2101.5 ± 28.9	2573.1 ± 172	0.0001	NS	0.0075
Cardiac m. NEC	1236.2 ± 73.2	1113.8 ± 68.4	1590.4 ± 21.6	1930.0 ± 168	0.0001	NS	0.0318
Cardiac m. ASAC	453.8 ± 70.9	286.6 ± 59.1	475.5 ± 45.3	578.5 ± 48.0	0.0200	NS	0.0350
Cardiac m. AIAC	35.6 ± 8.1	65.5 ± 11.0	43.3 ± 8.2	64.6 ± 12.9	NS	NS	NS
Cardiac m. AC	216.6 ± 50.1	265.3 ± 52.3	207.8 ± 25.5	399.1 ± 63.2	NS	0.0245	NS

[1]Values are means ± SEM, $n = 5$. Rats were fed diet without supplemented or supplemented with caffeine, carnitine and choline at concentrations of 0.1, 5 and 11.5 g/kg diet, respectively.
[2]Abbreviation used: AC, acetylcarnitine; AIAC, acid-insoluble acylcarnitine; ASAC, acid-soluble acylcarnitine; m., muscle; NEC, nonesterified carnitine; TC, total carnitine.
[3]*P* values from two-way ANOVA, using the factors, supplementation and exercise.
[4]S X E; supplement and exercise interaction. [5]NS: not significant, $P > 0.05$.

TABLE 5-4

Testes and brain carnitine profiles of rats with or without supplement and exercise [1-2]

Carnitine (nmol/g wet weight)	Nonsupplement		Supplement		Statistical significance[3]		
	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[4]
Testes TC	185.5 ± 4.2	184.7 ± 6.9	204.9 ± 14.7	235.8 ± 6.6	0.0012	NS[5]	NS
Testes NEC	137.5 ± 4.1	134.9 ± 2.5	158.8 ± 12.3	188.7 ± 7.0	0.0001	NS	0.0442
Testes ASAC	45.4 ± 2.7	47.5 ± 7.7	43.9 ± 4.8	43.7 ± 5.7	NS	NS	NS
Testes AIAC	2.6 ± 0.6	2.2 ± 0.7	2.2 ± 0.4	3.4 ± 0.3	NS	NS	NS
Testes AC	32.7 ± 1.1	30.0 ± 2.0	47.2 ± 3.4	46.3 ± 1.7	0.0001	NS	NS
Brain TC	110.5 ± 6.1	103.4 ± 2.6	160.5 ± 3.5	165.8 ± 9.7	0.0001	NS	NS
Brain NEC	88.0 ± 4.8	79.1 ± 2.2	135.9 ± 1.0	141.8 ± 9.5	0.0001	NS	NS
Brain ASAC	16.7 ± 4.6	17.6 ± 5.2	18.1 ± 2.4	15.9 ± 3.1	NS	NS	NS
Brain AIAC	5.8 ± 1.3	6.7 ± 0.9	6.5 ± 0.6	8.1 ± 0.8	NS	NS	NS
Brain AC	14.9 ± 2.2	17.8 ± 3.0	17.7 ± 2.1	19.8 ± 1.6	NS	NS	NS

[1]Values are means ± SEM, $n = 5$. Rats were fed diet without supplemented or supplemented with caffeine, carnitine and choline at concentrations of 0.1, 5 and 11.5 g/kg diet, respectively.
[2]Abbreviation used: AC, acetylcarnitine; AIAC, acid-insoluble acylcarnitine; ASAC, acid-soluble acylcarnitine; NEC, nonesterified carnitine; TC, total carnitine.
[3]*P* values from two-way ANOVA, using the factors, supplementation and exercise.
[4]S X E; supplement and exercise interaction.
[5]NS: not significant, $P > 0.05$.

TABLE 5-5

Kidney carnitine profiles of rats with or without supplement and exercise[1-2]

Carnitine (nmol/g wet weight)	Nonsupplement		Supplement		Statistical significance[3]		
	Nonexercise	Exercise	Nonexercise	Exercise	Supplement	Exercise	S x E[4]
Kidney TC	874.1 ± 52.1	873.1 ± 36.9	1307.1 ± 62.4	1607.0 ± 19.8	0.0001	0.0036	0.0034
Kidney NEC	743.8 ± 48.2	732.8 ± 32.3	1172.1 ± 47.6	1454.2 ± 26.8	0.0001	0.0071	0.0041
Kidney ASAC	119.1 ± 13.4	130.0 ± 8.5	126.5 ± 22.8	139.8 ± 17.8	NS[5]	NS	NS
Kidney AIAC	11.2 ± 1.4	10.2 ± 1.7	8.5 ± 0.8	13.0 ± 1.7	NS	NS	NS
Kidney AC	90.7 ± 2.3	88.3 ± 6.2	122.2 ± 2.3	126.1 ± 3.9	0.0001	NS	NS

[1]Values are means ± SEM, $n = 5$. Rats were fed diet without supplemented or supplemented with caffeine, carnitine and choline at concentrations of 0.1, 5 and 11.5 g/kg diet, respectively.
[2]Abbreviation used: AC, acetylcarnitine; AIAC, acid-insoluble acylcarnitine; ASAC, acid-soluble acylcarnitine; NEC, nonesterified carnitine; TC, total carnitine.
[3]*P* values from two-way ANOVA, using the factors, supplementation and exercise.
[4]S X E; supplement and exercise interaction.
[5]NS: not significant, $P > 0.05$.

DISCUSSION

In our earlier reports it was shown that a combination of choline, carnitine and caffeine (CCC) supplementation produced carnitine conservation and fat oxidation in intact rats (Hongu and Sachan 2000, Sachan and Hongu 2000). We now report that the CCC supplementation produced positive carnitine balance in all tissues examined (Table 5-3–Table 5-5) and the dosages did not have a toxic effect (Table 5-2). Further, the supplementation increased tissue content of short-chain and/or AC, which are considered to be the biochemical markers of fatty acid mobilization and oxidation (Bremer 1997).

It is important to recognize that unlike any other study reported in the literature outside of our laboratory exogenous CCC were fed in the diet simultaneously. Most of the carnitine was concentrated in skeletal muscle of these rats very much like that reported by other investigators, suggesting normal transport and redistribution of the supplemented carnitine (Rebouche 1977, Paulson et al. 1984). The difference we found was that the concentrations of carnitine were consistently higher in muscles of the supplemented rats (Table 5-3). The muscle carnitine pool exists in free and acylated forms through a rather complex equilibrium with the carnitine pools of serum, liver, kidney and other tissues. It has been suggested that carnitine fractions of the body compartment change in size, because of the increased esterification of the muscle-free carnitine (Lennon et al. 1983). The muscle and other tissue carnitine pools remained at a high state of repletion (Table 5-2–Table 5-5) in the supplemented rats in spite of the significant losses in the urine (Table 5-1) of these rats. The size of acylcarnitine pools within the muscle tissue must depend on its metabolic activity and supply of fatty acids to

this tissue. The supplemented rats put on exercise regimen showed significant interactive effect on size of the acylcarnitine (ASAC, AIAC) pool under the conditions of our study (Table 5-3). The presence of caffeine in the supplement promoted lipolysis and therefore, supply of fatty acids.

The AC pool was increased in serum, urine and kidney of the supplemented animals and in the liver and heart of the exercised animals (Table 5-1 – Table 5-3 & Table 5-5). The sources of acetyl moiety can be pyruvate and/or fatty acids depending on the fed or unfed state (Harris et al. 1987, Bremer 1997, Sachan and Hongu 2000). As the rate of acetyl-CoA production exceeds the rate of its utilization, the carnitine acetyl transferase facilitates transfer of acetyl moiety of acetyl-CoA to carnitine (Bieber et al. 1982, Harris et al. 1987). This process can be augmented by enhanced lipolysis induced by hormones or chemicals, supply of carnitine, and energy demands of animals. These conditions were present in our supplemented, exercised rats. It is apparent that the supplement also promotes formation of ASAC and AIAC in tissue specific manner. These metabolites appear in serum and are excreted in urine, a process termed "fatty acid dumping" (Sachan and Hongu 2000). None of these rats were starved, and only a half of them were exercised and yet there was an increase in the pool size of acylcarnitine in all rats. These observations suggest that there are undefined points of control of fat metabolism that can be manipulated by dietary means.

The supplement enlarged pools of total and acylcarnitine in liver similar to that seen in livers of trained female rats (Lennon and Mance 1986) and Streptozotocin-diabetic rats (Fogle and Bieber 1979). Exercise also enlarged carnitine pools particularly

the AC pools of liver (Table 5-2). An increased cardiac TC pool of exercised rats has been reported (Ciman et al. 1980, Lennon and Mance 1986), however; in the heart of our rats, this was not the case unless they were supplemented with CCC (Table 5-3). This is perhaps because of the differences in the type and intensity of exercise regimen (Lennon and Mance 1986) or the strain of animals (Ciman et al. 1979). The supplement prevented a fall in the size of cardiac NEC pool, in spite of the enlargement of acylcarnitine (ASAC) pool (Table 5-3) which is contrary to the fall in NEC pool observed in starved and diabetic rat hearts (Fogle and Bieber 1979, Bremer 1997). The enlargement of cardiac AC pool by exercise especially in the supplemented rats suggests up-regulation of fatty acid oxidation in this tissue. This and possible modulating of carnitine carrier proteins of cardiac myocytes (Cantrell and Borum 1982) by the supplement remains to be investigated. In any case the supplement may be of benefit to ischemic and aged hearts, where reduced carnitine concentrations have been observed (Shug et al. 1978, Costell and Grisolia 1993).

Carnitine is important for reproductive system in males, where it has been shown that oral carnitine supplementation improved total number of spermatozoa per ejaculate and sperm motility in men (Costa et al. 1994, Vitali et al. 1995). The supplement significantly enlarged the TC, NEC and AC pools in the testes (without epididymus) of the supplemented rats, but the exercise had no effect on testicular carnitine fraction (Table 5-4). The improved reproductive functions in the CCC supplemented males is entirely speculative at this time.

The role of carnitine in brain function has been vigorously investigated because

carnitine is found in the brain (Bresolin et al. 1982, Shug et al. 1982); it crosses blood-brain barrier (Mroczkowska et al. 1996, Mroczkowska et al. 2000); and it has a novel carnitine transport protein in cerebral cortex neurons (Wawrzenczyk et al. 2001). There is some evidence that carnitine may play different roles in the central nervous system, where the fatty acid oxidation is relatively lower than in the peripheral tissues (Warshaw and Terry, 1976). Clinical studies have shown that carnitine and AC are effective in slowing down the progression of mental deterioration in Alzheimer's disease (Forloni et al. 1994). The mechanisms of the therapeutic properties are unknown, except the postulate that it provides activated acetyl moiety for the synthesis of acetylcholine (Rebouche 1992, Nalecz and Nalecz 1996). Addition of carnitine and choline together has been shown to promote acetylcholine synthesis in rat cerebral cortex cells (Wawrzenczyk et al. 1995). The supplement augmented only brain TC and NEC pools in our study, where acylcarnitines were essentially unchanged (Table 5-4). Relative concentrations of ASAC plus AIAC in all groups were about 15-20 % of TC, which is in accord with 10% reported in the supplemented animals (Wawrzenczyk et al. 1995).

Kidney participates in the biosynthesis, acylation and excretion of carnitine (Carter and Frenkel 1979, Guder and Wagner, 1990). The CCC supplement enlarged the pool of NEC and AC (Table 5-5) similar to the streptozotocin-diabetic rats kidney (Fogle and Bieber 1979). Kidney TC and NEC pools were also enlarged not only by the supplement, but also by the exercise regimen. There was also significant supplement and exercise interaction on these carnitine species. Such an effect on kidney is not produced by choline alone in rats (Rein et al. 1997) or guinea pigs (Daily et al. 1998). As a matter

of fact, choline supplementation decreased carnitine concentration in the kidneys and urine of rats and guinea pigs (Daily and Sachan 1995, Daily et al. 1998, Rein et al. 1997), and urine of humans (Daily and Sachan 1995, Dodson and Sachan 1996). It is unlikely that the 5 fold higher dosage of choline (Table 3-1) in the present study compared to the earlier reports (Daily and Sachan, 1995, Daily et al. 1998, Rein et al. 1997) would have caused this shift. Perhaps the caffeine of the supplement mixture may contribute to the change in the carnitine profile of kidney. There is no carnitine data on caffeine supplemented animals, however there are a few reports of a close relative of caffeine, theophylline, being studied (Al-Jafari et al. 1996, Alhomida 1998). In these studies, there was a significant increase in NEC, ASAC, AIAC and TC in the kidney (Alhomida 1998), and in the heart and skeletal muscle (Al-Jafari et al. 1996) of the adult male rats given theophylline. There is little explanation of the data except that it indicates enhanced transport and oxidation of fatty acids, however no clear mechanism has been proposed. Of course, there can be other functions of carnitine in tissues, such as protection against lipid peroxidation (Dayanandan et al. 2001) and the like.

In conclusion, we found that oral supplementation of carnitine, choline and caffeine did not influence dietary intake and produced normal growth of rats, but decreased the fat pad weight. The combination of supplementation increased the total carnitine concentrations in all tissues, serum and urine.

CHAPTER VI

EFFECTS OF CARNITINE-CHOLINE SUPPLEMENTATION ON CARNITINE PROFILES AND INDICES OF FAT METABOLISM IN HEALTHY ADULTS

Some parts of this Chapter has been prepared for manuscripts for publication with co-author Dileep S. Sachan in the American Journal of Clinical Nutrition and Journal of the American College of Nutrition.

ABSTRACT

We have previously shown that dietary choline supplementation promotes carnitine conservation in humans by decreasing daily urinary carnitine excretion. However, there were no consistent changes in the serum carnitine concentration and there was no determination of functional consequence of choline-induced carnitine conservation. Studies in the animal models showed choline-carnitine interactions similar to those seen in humans and allowed the determination of tissue accretion and functional significance such as altered body composition and fatty acid oxidation. The aim of this study was to determine the effects of choline or carnitine pre-loading, followed by a combination of the two and exercise on serum and urinary carnitine fractions, lipid profiles, body weight, percentage body fat (%BF) and indices of fatty acid oxidation in healthy humans. Twenty-three healthy adults, 5 men and 18 women, were given placebo or choline or carnitine for 1 wk; and placebo or choline plus carnitine for an additional wk. Body weight, dietary analysis, serum lipids, carnitines, ketones, leptin, and urinary

carnitine were determined before and after each treatment. While there was no change in the placebo group, serum and urinary carnitine and acylcarnitine were decreased in the choline supplemented group. Introduction of carnitine to the choline group improved the serum carnitine profile, but the daily urinary carnitine excretion continued to fall. On the contrary, serum and urinary carnitine and acylcarnitine increased in the carnitine supplemented group and introduction of choline to the carnitine group decreased serum carnitine, but the urinary carnitine excretion remained high. The concentrations of serum β-hydroxybutyrate and serum and urinary acetylcarnitine were elevated by the supplements and accentuated, particularly by the introduction of carnitine. The supplements reduced the concentrations of serum leptin which was correlated with the body weight, body mass index (BMI; in kg/m^2), percentage body fat (%BF) and serum triglyceride. A mild exercise regimen further increased concentration of serum β-hydroxybutyrate and serum and urinary acetylcarnitines, and decreased leptin levels across the groups. It is concluded that a choline-induced decrease in serum and urinary carnitine is buffered by carnitine pre-loading. The combination of carnitine and choline supplementation promotes fat mobilization, oxidation and disposal of acetylcarnitine in urine and this effect of the supplements is accentuated by a mild exercise regimen as well as sustained until 2 wk following cessation of supplement and exercise interventions.

INTRODUCTION

In our earlier discourse, we have introduced essentiality of carnitine for fatty acid translocation (Bremer 1997), muscle function (Brass and Hiatt 1994) and exercise

performance (Decombaz et al. 1992, Lennon et al. 1983, Neumann 1996). We have talked about choline being a lipotropic agent (Best et al. 1935, Leiber et al. 1994), promoter of synthesis and release of acetylcholine (Zeisel and Blusztajn 1994, Buyukuysal et al. 1995) and need for supplementation in athletes running a marathon (Conlay et al. 1992). We also addressed usage of caffeine as a general stimulant, fatty acids mobilizer from adipose tissues (Bellet et al. 1968, Costill et al. 1978, Denadai 1994) and booster of endurance exercise (Nehlig and Debry 1994).

Recently, we have reported interactive effects of choline and carnitine in normal healthy humans and animals. Choline supplementation resulted in significant conservation of carnitine in humans and guinea pigs (Daily and Sachan 1995, Dodson and Sachan 1996); however, this effect of choline was not seen in the adult rats given choline dosage similar to that of humans and guinea pigs (Daily and Sachan 1995, Rein et al. 1997). When rats were given relatively higher doses of choline in combination with caffeine plus carnitine for 4 wk, there was significant conservation of carnitine (Sachan and Hongu, 2000, Hongu and Sachan 2002). The functional consequences of choline-mediated carnitine accretion in tissues have been studied in animal models. Choline supplementation alone resulted in a significant increase in carnitine concentration in skeletal muscle of guinea pigs (Daily and Sachan 1995) and loss of carcass fat (Daily et al. 1998). In rats, choline plus carnitine along with caffeine supplementation resulted in significant reduction in fat pad mass, serum leptin concentration (Hongu and Sachan 2000), and other biochemical markers indicative of enhanced fatty acid oxidation to acetate (Sachan and Hongu 2000). While exercise endurance was increased in both

guinea pigs and rats by the respective supplements, the RQ was not significantly affected to support fat being energy substrate; for which a novel explanation has been postulated (Sachan and Hongu 2000).

In light of these observations, it was logical to ask if humans respond in a manner akin to animal models when their diets are supplemented with choline and carnitine with or without caffeine. As early as 1988, Sachan and his associates (Mynatt et al. 1988, Dodson and Sachan 1992) reported effects of choline plus pantothenic acid on reduced urinary carnitine excretion in humans which was later documented to be due to choline and not pantothenic acid supplementation. These observations went unappreciated resulting in the delay of the full length publication in 1996, wherein it was clearly demonstrated that choline reduced carnitine excretion in urine without significantly affecting serum carnitine concentrations in all subjects (Dodson and Sachan, 1996). This was especially significant because the subjects were international, multiethnic, free-living who are eating their traditional foods. In one of the studies described in the paper (Dodson and Sachan, 1996), subjects were given a supplement of choline for the first 7 d followed by addition of carnitine for 3 d to see if the urinary carnitine excretions would be restored to normal. The urinary carnitine concentrations were restored, but only to about 40% of the control values. This suggested that there was deficit of carnitine due to choline, and perhaps a longer duration of carnitine supplementation was needed to fully replete the system. This is all the literature on the choline-carnitine interactions in humans and many questions remain to be addressed. Therefore, the main objective of this segment of our studies was to determine effects of choline and carnitine

supplementation with and without exercise on carnitine status, body weight, body fat, lipid profiles and biochemical markers of fat oxidation in free-living men and women.

SUBJECTS AND METHODS

Subjects

The participants were 5 men and 18 women who met the inclusion criteria of the study protocol approved by the Institutional Review Board for the Protection of Human Subjects in Research. Most of the participants were from the faculty and student population of the University of Tennessee, Knoxville, only 4 subjects from the community of Knoxville, TN. They had a mean (± SEM) age of 28.7 ± 2.2 y (range 18 to 54 y), body weight of 72.4 ± 3.6 kg (range 47.5 to 109.3 kg), BMI (kg/m^2) of 25.7 ± 1.0, %BF of 25.7 ± 1.6 %, and waist to hip ratio (WHR) of 0.79 ± 0.02 (**Table 6- 1**). All of the participants were apparently healthy individuals according to the criteria of the American College of Sports Medicine (2000).

Expected participants were verbally screened to ensure that they were willing to maintain their extra-physical activity during the exercise intervention period. They met the following inclusion criteria: 1) no clinical diagnosis of cancer, cardiovascular, gastrointestinal, hepatic, renal disease, hypertension or diabetes; 2) no current use of antibiotic, prescription medication; 3) no use of vitamin or mineral supplements within 2 wk of the start of the experimental periods; 4) no specific dietary practice for weight loss, such as low fat diet or reduced calorie intakes within the past year. Further, women were specifically excluded from the study if they were pregnant, lactating, or using exogenous

TABLE 6-1

Anthropometric measurements of participants in three groups[1-2]

Variables	Day	Placebo (n = 7)	Supplement 1 (n = 8)	Supplement 2 (n = 8)	Statistical Significance[3] Group	Time	G x T[4]
Age (y)		28.1 ± 5.0	29.3 ± 3.5	28.8 ± 3.6	NS[5]	NS	NS
Height (cm)		163.9 ± 3.2	166.0 ± 3.2	170.7 ± 2.9	NS	NS	NS
Weight (kg)	0-d	69.1 ± 6.4	72.3 ± 6.8	76.8 ± 6.6	NS	0.0176	NS
	21-d	69.5 ± 5.7	72.2 ± 6.5	76.3 ± 6.7			
	35-d	68.4 ± 6.0	71.1 ± 6.6	75.8 ± 6.8			
BMI (kg/m^2)	0-d	25.4 ± 1.5	25.5 ± 1.6	26.3 ± 2.1	NS	NS	NS
	21-d	25.4 ± 1.4	25.3 ± 1.8	26.1 ± 2.1			
	35-d	25.3 ± 1.4	25.0 ± 1.8	26.7 ± 2.1			
Sum skinfold (mm)	0-d	100.6 ± 10.9	109.6 ± 10.6	105.2 ± 14.7	NS	NS	NS
	21-d	101.1 ± 12.9	106.5 ± 10.8	100.4 ± 14.9			
	35-d	104.4 ± 14.3	105.4 ± 10.8	101.3 ± 14.8			

[1]Values are expressed as mean ±SEM. [2]Abbreviation used: BIA, bioelectrical impedance analysis; BMI, body mass index; WHR, waist to hip ratio. [3]*P* values from 2-way ANOVA, using the factors group and time. [4]G x T, group and time interaction. [5]NS, no significant differences, $P > 0.05$.

TABLE 6-1

Anthropometric measurements of participants in three groups[1-2] (Continued)

Variables	Day	Placebo (n = 7)	Supplement 1 (n = 8)	Supplement 2 (n = 8)	Statistical Significance[3] Group	Time	G x T[4]
% body fat (Skinfold)	0-d	26.9 ± 2.5	26.0 ± 1.9	23.3 ± 3.8	NS[5]	NS	NS
	21-d	27.0 ± 2.6	25.5 ± 1.8	22.1 ± 3.9			
	35-d	27.2 ± 2.9	25.0 ± 1.8	22.6 ± 3.7			
% body fat (BIA)	21-d	27.8 ± 1.7	25.4 ± 1.7	23.3 ± 3.9	NS	NS	NS
	35-d	27.6 ± 1.6	25.8 ± 1.9	23.5 ± 3.8			
Waist (cm)	0-d	80.4 ± 4.8	83.5 ± 7.7	83.5 ± 5.1	NS	NS	NS
	21-d	79.7 ± 4.9	83.5 ± 7.6	83.4 ± 5.1			
WHR	0-d	0.77 ± 0.03	0.79 ± 0.04	0.80 ± 0.03	NS	NS	NS
	21-d	0.77 ± 0.03	0.79 ± 0.04	0.80 ± 0.03			

[1]Values are expressed as mean ±SEM. [2]Abbreviation used: BIA, bioelectrical impedance analysis; BMI, body mass index; WHR, waist to hip ratio. [3]*P* values from 2-way ANOVA, using the factors group and time. [4]G x T, group and time interaction. [5]NS, no significant differences, $P > 0.05$.

hormones. Subjects who met eligibility requirements participated in an orientation meeting. Each subject was informed about the nature and the purpose of the study and was required to provide written informed consent to participate in the study.

Study design

The randomized placebo-control study with 3 groups lasted a period of 35 d during which interventions were carried out as shown in **Figure 6-1**. At baseline, habitual dietary intake was assessed and anthropometric measures were taken. Fasting venous blood and 24-hr urine samples from 2 consecutive days were collected before the intervention. All participants were specifically instructed to follow their usual dietary and life-style patterns, including exercise, work and recreation; but not to indulge in sexual intercourse a day before or during the 24 h urine collection. They were also asked not to introduce unusual foods to their diets or any nutrient supplements for the duration of the experiment. Following the baseline period, participants were randomly assigned to the placebo (n = 7) or supplement groups. Supplement groups were randomly divided into two subgroups; supplement 1 (S1) and supplement 2 (S2). The S1 group (n = 8) received only choline supplementation for the 1st wk followed by addition of carnitine supplement in the 2^{nd} through 3^{rd} wk. The S2 group (n = 8) received only carnitine supplement for the 1^{st} wk followed by addition of choline in the 2^{nd} through 3^{rd} wk. Neither group received any supplement in 4^{th} and 5^{th} wk (wash out period). Choline was supplied as choline bitartrate (Pharmachem Laboratories, South Hackensack, NJ) and administered at the dosage of 7.8 mmol (0.94 g) choline/d. Carnitine was L-carnitine L-tartrate (Lonza, Switzerland) supplied by Daily Manufacturing, Inc. (Rockwell, NC) and administered at

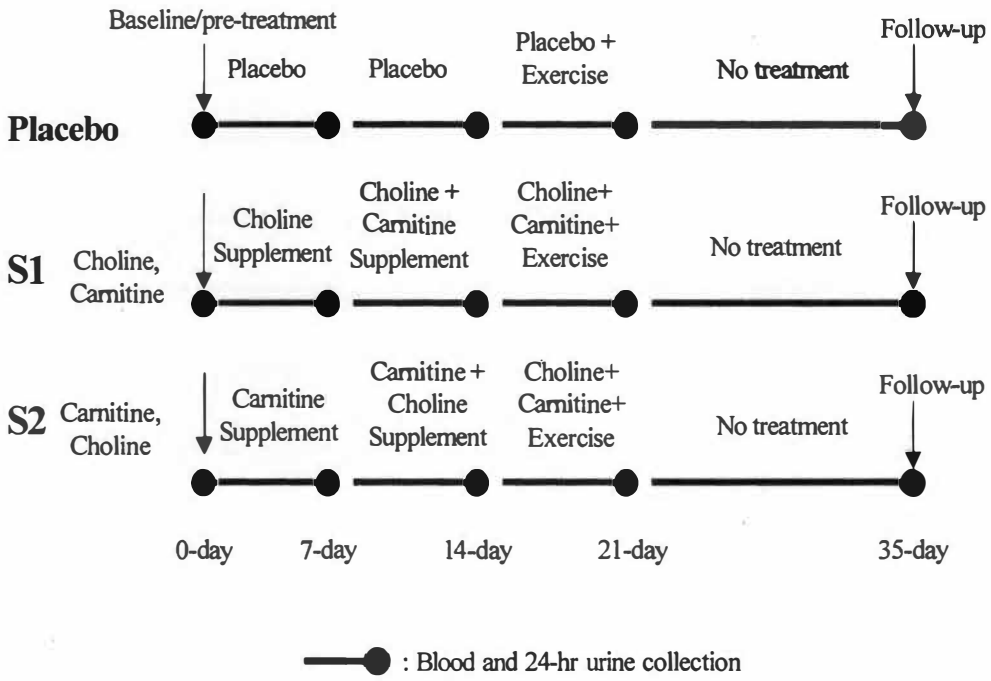


FIGURE 6-1 *Schematic presentation of study design showing dietary and exercise intervention time periods.* S1: supplement group 1, S2: Supplement group 2.

the dosage of 4.2 mmol (0.68 g) carnitine/d (Dodson and Sachan 1996). Both supplements were taken with daily meals. The supplements are US Pharmacopeic (USP)-grade and have purity ≥98.6%. Each supplement was dissolved in distilled water and 1 wk supply dispensed in amber-colored bottles with a coded label and dosing instruction. The placebo bottle contained citric acid (0.2 mmol, 1 g/d) in equivalent dosage. All participants were given daily-supplement sheets and asked to record timing of taking the contents of given bottles and return the sheets and empty bottles on the following visit.

The coordinator of the program reviewed the sheet and bottles at weekly visits to the clinical study facility. From d 7 until d 21, both choline and carnitine were taken by both S1 and S2 groups, so the difference was pre-loading with choline or carnitine in the first wk of the trial.

At the 3rd week of the d 14 to d 21 period, all groups were instructed to perform light to moderate exercise training to increase their daily energy expenditure approximately 100 to 300 kcal/wk above the pre-exercise intervention period regardless of dietary supplementation. All participants were given an exercise log sheet and an electronic pedometer (Yamax Inc., Japan) to record their daily steps, distance and calorie output. The last 2 wk were the follow-up or washout period in which they were asked to go back to their normal activity level and not to take the placebo or supplement.

Physical activity intervention

The physical activity intervention consisted of light to moderate exercise as recommended by American College of Sports Medicine for cardiorespiratory fitness in healthy adults (Pollock et al. 1998). All participants were asked to have ≥ 20 min of aerobic activity such as walking, jogging, stair-climbing, 3-5 d/wk over and above their normal activity, which may equal to an extra 300-500 kcal energy output per wk. In the 2nd wk of this study, before starting exercise intervention, all subjects were instructed how to record their baseline physical activity by using an electronic pedometer, the Yamax Digi-Walker SW-701 (Yamax Inc., Japan) for 5-7 d. They recorded daily estimated calorie output (based on their body weight), walking distance (estimated from their strides) and walking steps, including non-exercise related physical activities, such as

occupational and household activities. All participants were asked to wear a pedometer all the time during the 3rd wk to keep track for their daily physical activity. There was no attempt made to monitor exercise intensity.

Assessment of body composition

The assessments of body composition were made after an overnight fast on d 1, 21 and 35. Body weight was measured at each visit with a Physician Mechanical scale. Barefoot standing height was measured to the nearest 0.1 cm with a vertical height scale. BMI was calculated by dividing weight in kg by height in meters squared.

Skinfold thicknesses were measured 3 times to the nearest 0.1 mm with a Lange skinfold caliper (Cambridge Scientific Industries, Inc., Cambridge Maryland) on the one side of the body at the triceps, subscapular, suprailiac, thigh, abdomen in both men and women, and chest in only male subjects. These sites are described in the Health Fitness: Instructor's Handbook (Bubb 1992). The skinfold thickness values from all these sites were added up and termed the "sum of skinfold thickness".

Percentage BF was estimated using the mean of 3 times reading of triceps, abdomen and suprailiac measurements for women, and triceps, chest and subscapular for men, as described by Jackson and Pollock (1985). Percentage BF was also estimated by bioelectrical impedance analysis (BIA) only on d 21 and 35. Bioelectrical impedance was measured using a BIA-RJL system analyzer (RJL-systems, Detroit) with a 50 kHz, 800 μA device. Two signaling electrodes were placed on the dorsal surface of the right foot at the space between the metatarsal-phalangeal joints of the great and second toes, as well as on the dorsal right hand at the space between the metacarpal-phalangeal joints of the

index and middle fingers. The 2 detecting electrodes were placed between the styloid processes of the right radius and ulna and between the medial and lateral malleoli of the right ankle (Heitmann 1990). During the measurement, subjects remained still in a supine position with their hands and feet slightly abducted from the midline to ensure that no contact was made between the extremities and the torso. Percentage BF was calculated by using an equation of Segal et al. (1988). Equation: BF = a BW-Ht^2/resistance- b Ht + c Age + 14.941, where fractions varies by gender.

Waist circumference was measured, with a measuring tape, midway between the inferior angle of the ribs and the suprailiac crest, whereas hip circumference was measured at the outermost points of the greater trochanters (WHO 1995). The waist-to-hip ratio (WHR) was then calculated. All the anthropometric measurements described above were performed by the same trained investigator throughout the study.

Dietary assessment

All subjects were interviewed and completed a questionnaire about the type of diet consumed, the length of time they had been following the diet, reasons motivating their choice of diet, nutritional supplement use, smoking habits, oral contraceptive use and whether they had received dietary advice. Dietary intake was assessed at baseline and every wk of the intervention by 3-d dietary records. Each of the five 3-d periods consisted of the 1 weekend day and 2 weekdays to control for day-of-the-week effects. At the baseline period each participant was given food record sheets and instructed, by a registered dietitian, how to keep dietary intake. Subjects were asked to record the exact description of all food and drink consumed during each of the 3-d period. Food portion

sizes were estimated by using standard household measures. All completed dietary records were reviewed by the dietitian, and were discussed with the subject, if necessary, to resolve any ambiguities regarding accuracy or completeness. Food records were coded, entered, and analyzed using the Nutritionist IV program (version 4.1, 1997, First Data Bank, San Bruno, Calif).

Blood collection and analysis

Venous blood samples (13 ml) were collected following overnight fasting (8-12 hr) at the beginning of baseline (0 day) and at each visit (Figure 6-1). Whole blood was collected in vacutainer tubes without anticoagulants and kept on ice. After clotting, serum was separated by centrifugation at 1500 X g for 10 min at 4°C and stored at -80°C until used for determination of cholesterol, triglycerides, free fatty acids, β-hydroxybutyrate, leptin and carnitine concentrations.

Total serum cholesterol was determined by a enzymatic procedure using the Sigma kit no.352 (Sigma, St. Louise, MO). Briefly, cholesterol esters are first hydrolyzed by cholesterol esterase (EC 3.1.1.13) to cholesterol, which is then oxidized by cholesterol oxidase (EC 1.1.3.6) and the resulting hydrogen peroxide. The produced hydrogen peroxide is then coupled with the 4-aminoantipyrine and hydroxybenzenesulfonate in the presence of peroxidase (EC 1.11.1.7) to yield a quinoneimine dye which has an absorbance at 500 nm. The intensity of the color produced is proportional to the total cholesterol concentration in the sample. This is a modification of the method of Allain et al. (1974).

Serum high density lipoprotein (HDL) was determined by the same enzymatic

method (Sigma kit no.352, Sigma, St. Louise, MO). First, VLDL and LDL (low density lipoprotein) cholesterol were precipitated, leaving the HDL fraction in solution by using phosphotungstic acid and magnesium chloride (Assmann et al. 1983). The LDL concentration was calculated using the following equation (Friedewald et al. 1972):

LDL (mg/dL) = total cholesterol - HDL - [triglycerides/5]

This equation loses accuracy when the serum triglyceride concentration exceeds 400 mg/dL. However, in this study, there was no individual, whose TG was over 400 mg/dl. Serum triglycerides and nonesterified fatty acids were determined by the methods of Giegel et al. (1975) and Novak (1965) respectively.

Serum leptin concentration was determined using a commercial radioimmunoassay kit (Linco Research, St. Louis, MO). The assay use ^{125}I-labeled human leptin and a human leptin antiserum to determine the level of leptin in serum.

Serum β-hydroxybutyrate was determined spectrophotometrically at 340 nm by NAD linked enzymatic reactions (Sigma kit no.310, Sigma, St. Louise, MO). All serum samples used for β-hydroxybutyrate went through one freeze-thaw cycle during a 1.5 y of storage at - 80 °C before β-hydroxybutyrate was measured.

Serum carnitine was determined by the radioisotopic-enzymatic procedure originally described by Cederblad and Lindstedt (1972) as modified by Sachan et al. (1984). When carnitine is quantified, NEC and acylcarnitine must be distinguished in order to evaluate the metabolic nature of carnitine. First, serum samples were treated with 0.6 M PCA and centrifuged at 1500 X g for 10 min at 4 °C to extract acid soluble forms of carnitine, i.e. free or NEC and short-chain or acid soluble acylcarnitines

(ASAC). The long-chain or acid insoluble acylcarnitines (AIAC) remained with the pellet. An aliquot of the supernatant is assayed for the NEC concentration. Another aliquot of supernatant alkalinized with 0.5 N KOH is assayed for the ASC, which is incubated in a water bath for 30 min at 37°C, thus releasing all carnitine from its ester linkage to the fatty acids. For AIAC concentration, the pellet was treated with 0.5 N KOH to about pH 10 and hydrolysis carried out at 65°C for 60 min for the release of carnitine from the long-chain fatty acid esters. After PCA and KOH treatments, the samples were neutralized with the 3-[N-morpholino] propanesulfonic acid (MOPS) buffer prior to the radioenzymatic assay. Enzymatic assays for carnitine involve the use of the enzyme, CAT, which facilitates transfer of acetyl moiety of [1-^{14}C]acetyl-CoA (Moravek Biochemicals, Brea, CA) to carnitine, resulting in radioactive carnitine species. The unreacted negatively charged [1-^{14}C]acetyl-CoA is then removed from the reaction mixture by running through anion exchange resin (Dowex 1X8, 200-400 mesh, Cl^- form) (Doe & Ingalls, Durham, NC). Carnitine esters carrying the positive charge of the quaternary ammonium are not retained by the resin and are collected into scintillation vials. The radioactivity of samples, the eluted [1-^{14}C]acetyl-CoA from the anion exchange column, was determined using a Beckman LS-3801 liquid scintillation counter (Beckman, Fullerton, CA). Four different carnitine fractions are determined by this procedure: 1) NEC; 2) ASAC, total acid soluble minus the NEC ; 3) AIAC; and 4) Total carnitine, the sum of NEC, ASAC and AIAC (**Figure 6-2**).

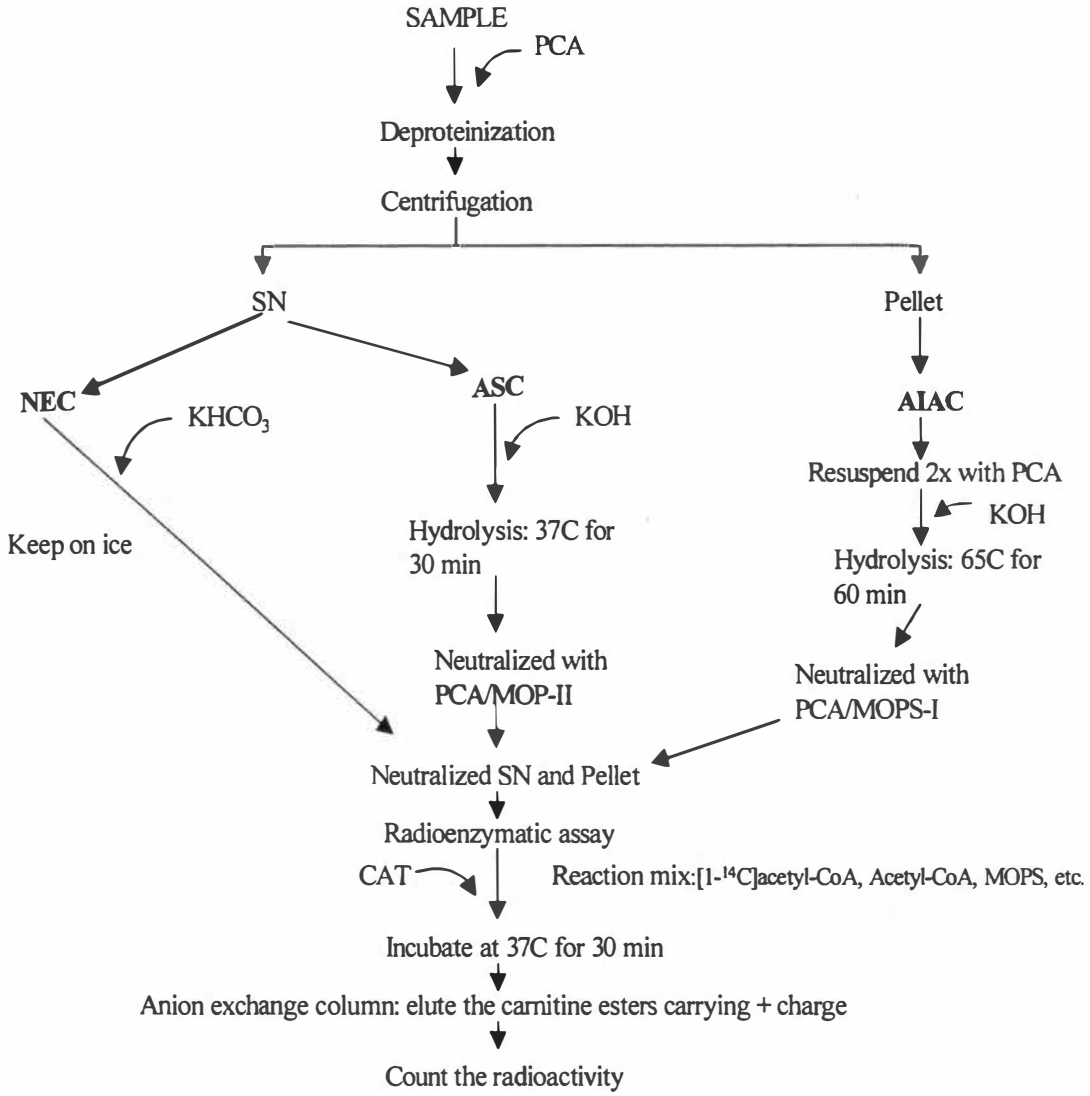


FIGURE 6-2 *Radioenzymatic assay of the various carnitine fractions.*
Sample is treated with PCA to separate acid-soluble and acid-insoluble fraction of carnitine. NEC and ASC remain in supernatant (SN), but AIAC is precipitated with pellet. KOH releases NEC from it ester linkage. After hydrolysis, a neutralized sample is added to reaction mix, which contains [1-^{14}C] acetyl-CoA. The reaction is started by adding carnitine acetyl transferase (CAT). Carnitine in a sample forms [1-^{14}C] acetyl carnitine, which is eluted from the column that is packed with anion exchange resin. Four different values can be obtained: 1) NEC, 2) ASAC = ASC - NEC, 3) AIAC and 4)TC = NEC + (ASC-NEC) + AIAC.

Acetylcarnitine (AC) was determined separately according to the method of Pande and Caramancion (1981) and the modification described below. In the procedure, the acetyl portion of acetylcarnitine is converted to [U-^{14}C]citrate, using an excess of [U-^{14}C]oxaloacetate generated from [U-^{14}C]aspartate and citrate synthase (CS) (EC 4.1.3.7). The radioactivity of the citrate formed is measured after removal of the leftover [U-^{14}C]oxaloacetate which is converted to [U-^{14}C]aspartate by the addition of glutamate oxaloacetate transaminase (GOT) (EC 2.6.1.1) plus glutamate. The [U-^{14}C]aspartate formed is removed from the solution by the adding of a cation exchange resin and leaving the [U-^{14}C]citrate for scintillation counting (**Figure 6-3**).

For estimation of serum acetylcarnitine, samples were first deproteinized by 0.6M PCA and centrifugation as described in the method for carnitine determination. The supernatant of PCA extracts were neutralized with 0.5 M Hepes-KOH and 3M $KHCO_3$ and centrifuged at 2000 X g for 10 min at 4°C. The supernatant was then transferred to the microcentrifuge tube containing 0.5 M Hepes-KOH-6mM EDTA, pH 7.0. This mixture was again transferred to the glass tubes (12 x 75 mm) containing 0.5 M Hepes-KOH, pH 8.5. About 200 mg of anion exchange resin, Dowex 1X8 (200-400 mesh, Cl^- form) (Doe & Ingalls, Durham NC) with methanol and double distilled water (DDW) were added to the glass tubes and vortex 2-3 times at room temperature, and then centrifuged at 2000 X g for 5 min. Endogenous citrate was removed through anion exchange resin and used for acetylcarnitine assay. The reaction mixtures containing freshly prepared CoASH, dithiothreitol, α-ketoglutarate, GOT, and [U-^{14}C]aspartate in Hepes-KOH-EDTA buffer solution was added to the supernatant for starting the reaction

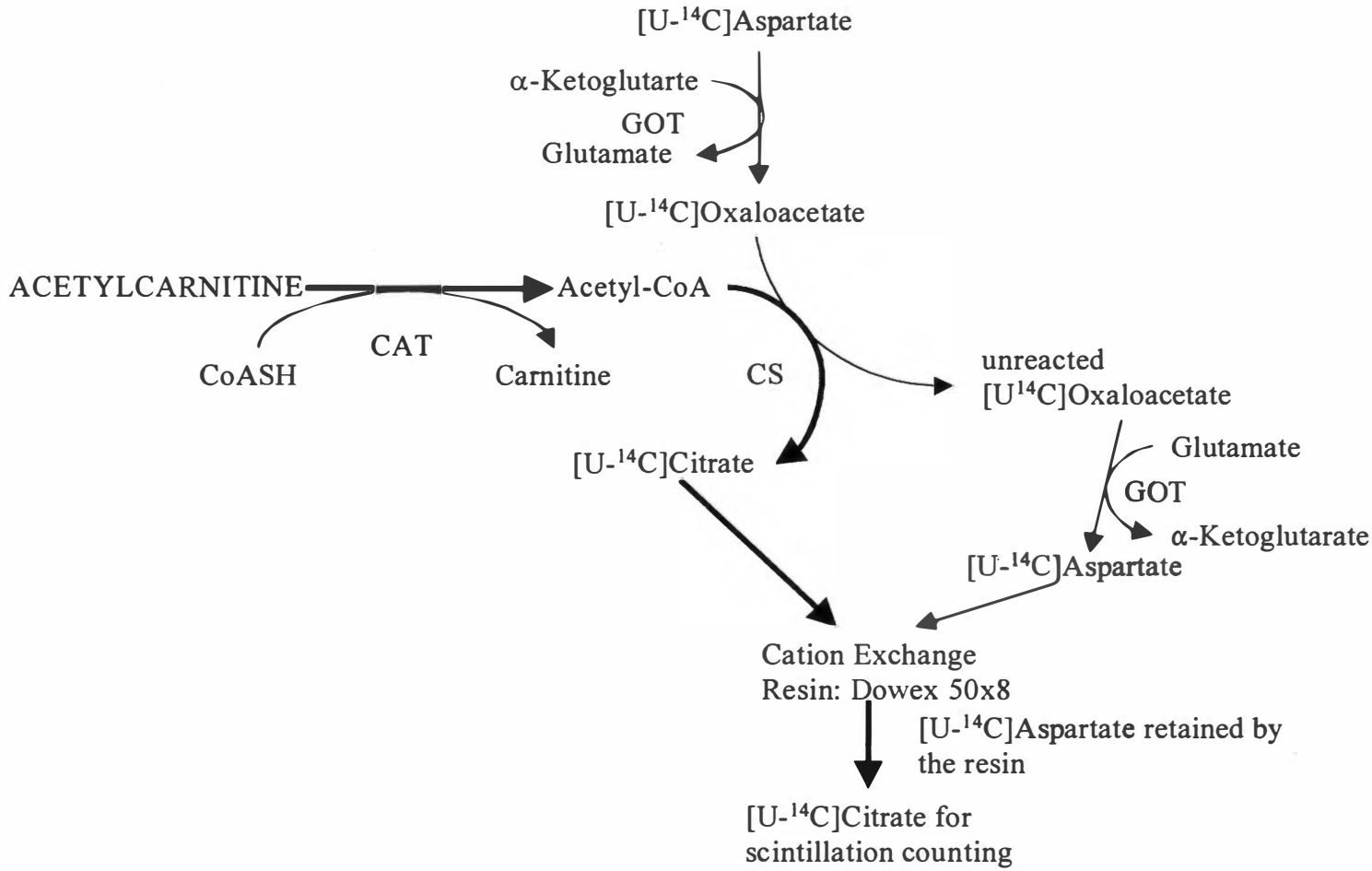


FIGURE 6-3 *Radioenzymatic assay of acetylcarnitine.* Bold arrows indicate the reactions directly involved in the formation of [^{14}C]Citrate from acetylcarnitine.

of [U-^{14}C]aspartate to form [U-^{14}C]oxaloacetate. Subsequently, CAT and CS were added and incubated at room temperature for 20 min. At the end of the incubation, glutamate and GOT mixture was added and incubated for an additional 20 min. Then, a slurry of cation exchange resin, Dowex 50X8 (200-400 mesh, H^+ form, Bio-Rad, Hercules, CA) was added. The tubes were vortexed and placed on ice for 2-3 time over 20 min. After the vortex, the tubes were centrifuged at 2000 X g for 5 min at 4°C. The supernatant was transferred to scintillation vials and count by a Beckman LS counter (Beckman, Fullerton, CA). A detailed procedure is shown in Appendix 1.

Urine collection and analysis

All subjects collected two 24-hr urine samples during the baseline period and one 24-hr urine sample every wk thereafter. Oral and written instructions on the collection of 24-hr urine samples were given to the subjects before and during the study. They were asked to record the date and time of collections. They were instructed to exclude the first morning void of the first day and include the first morning void of the second day. Polyethylene collection bottles containing thymol as a preservative were provided. The volume of urine was measured, then throughly mixed and portions of urine were centrifuged at 1500 X g for 10 min at 4°C and placed in 13 ml of plastic tubes, stored immediately at -80°C until analyzed.

The 24-hr urine was analyzed for creatinine and carnitine. Urinary creatinine was determined by the alkaline-picrate method (Henry 1967, Taussky 1954). Creatinine produces a certain definite color in the presence of an alkaline picric acid solution. This reaction is due to the formation of a red tautomer of creatinine picrate, which has an

absorbance at 520 nm.

Carnitine in urine was determined by the same method as used for the serum (Cederblad and Lindstedt 1972, Sachan et al. 1984). Samples of 20- 30 μl urine were added to the tubes containing 200 μl 0.6 M PCA. In order to form pellets, 100 μl of 8% bovine serum albumin and enough DDW to make the total volume of 400 μl were added. The tubes were centrifuged at 1500 X g for 10 min at 4 °C. An aliquot of supernatant and pellets were used for carnitine assay as described above. Four different carnitine fractions were also obtained in the urine. For the assay of the urinary acetylcarnitine the urine samples of carnitine supplemented subjects were diluted 2 to 10 times, depending on the concentration of the acetylcarnitine in urine.

Statistical analysis

All statistical analyses were done using the Personal Computer Statistical Analysis System for Windows (Version 8e, 2000, SAS Institute, Cary, NC). Two-way ANOVA with repeated measures was performed to determine whether there were any significant differences on variables listed in the Tables. If there was a significant main effect (group, time, interaction of group and time), Duncan's multiple range test was performed to determine how the groups differed. To evaluate treatment effects on each time point (0, 7, 14, 21 and 35 d) separately, two-way ANOVA without repeated measures was also performed to determine whether there were any significant differences (group, gender, interaction of group and gender) on all the variables listed in the Tables, except anthropometric measurement, across three dietary intervention groups. The data in all tables are expressed as means ± standard error of means (SEM). Simple regression

analysis was performed to calculate correlations between the serum leptin concentration and anthropometric parameters. Statistical significance was set at $P < 0.05$.

RESULTS

Initially, 23 biologically unrelated subjects (5 men and 20 women), who met the inclusion criteria, were recruited. Two of these subjects withdrew from the study prior to completion due to the illness in their family. Thus, a total of 23 subjects completed the study as a part of 3 groups: placebo, all women (n=7); supplement-1 (S1), 6 women and 2 men (n=8); and supplement-2 (S2), 6 women and 2 men (n=8). Anthropometric measurements, 24-h urine collection, 3-d diet diary, exercise log, and supplement intake were recorded in all subjects. The anthropometric parameters are shown in Table 6-1. Age and height were recorded only on day 0 and were not significantly different among the groups.

The body weights were not significantly different among the groups; however, there was a significant time effect on body weight between 0 and 35 d when the subjects of the 3 groups were taken together (Table 6-1). The net change in body weights of the groups at 3 different time points is shown in **Table 6-2**. After wk 3 of treatment (at 21-d), the S1 and S2 groups lost on an average 0.14 and 0.45 kg body weight, respectively, and the placebo group lost 0.03 kg body weight. All groups showed loss of body weight between 21 and 35 d (wash out period). Neither group nor time showed significant effects on the remaining parameters listed in Table 6-1, i.e. BMI, sum of skinfold thickness, %BF, waist circumference and WHR.

TABLE 6-2

Net changes in body weight of the groups at 3 time points[1]

	Day	Placebo	Supplement 1	Supplement 2
Weight (kg)	(21-d) - (0-d)	-0.03	-0.14	-0.45
	(35-d) - (21-d)	-0.65	-1.07	-0.49
	(35-d) - (0-d)	-0.68	-1.21	-0.94

[1]Values are mean differences of two time points. No statistical analysis was applied due to the too large individual differences to make any prediction.

Nutrient and caffeine intakes at the pre-treatment (0-d) and on days 7, 14, 21, and 35 are summarized in **Table 6-3 and Table 6-4**, respectively. Two-way ANOVA with repeated measures showed no significant differences in mean nutrient intakes including, energy intakes of the groups throughout the experiment. To determine if there were any differences across the groups due to the total calorie and body size of subjects, intakes of the macro-nutrients (protein, carbohydrate, fat) were calculated on the basis of per 1000 kcal and per kg body weight of each subject. However, there were no significant group differences on any of the macro-nutrients per 1000 kcal and kg of their body weight (Appendix D).

TABLE 6-3

Mean nutrient intakes of participants in three groups[1]

Variables	Placebo	%kcal	Supplement 1	%kcal	Supplement 2	%kcal
Energy (kcal)						
0-d	1748 ± 88		1879 ± 185		2007 ± 278	
7-d	1840 ± 153		1925 ± 264		2223 ± 136	
14-d	1658 ± 164		1826 ± 386		2296 ± 235	
21-d	1758 ± 335		2186 ± 383		2085 ± 262	
35-d	1857 ± 154		1974 ± 199		2122 ± 153	
Protein (g)						
0-d	61 ± 6.0	14 ± 0.9	71 ± 7.8	15 ± 1.5	75 ± 10	15 ± 1.6
7-d	80 ± 13	17 ± 1.7	70 ± 8.0	15 ± 1.9	75 ± 9.3	13 ± 1.5
14-d	59 ± 8.8	14 ± 0.9	72 ± 15	16 ± 1.0	78 ± 9.6	14 ± 1.1
21-d	59 ± 10	14 ± 0.5	80 ± 13	15 ± 1.4	76 ± 13	15 ± 2.0
35-d	70 ± 9.4	15 ± 1.8	69 ± 9.0	15 ± 1.8	78 ± 10	15 ± 1.9
Carbohydrate (g)						
0-d	258 ± 22	59 ± 2.5	260 ± 30	55 ± 2.7	278 ± 38	56 ± 2.2
7-d	239 ± 11	53 ± 3.6	271 ± 39	56 ± 3.2	322 ± 25	58 ± 2.9
14-d	224 ± 14	56 ± 4.1	261 ± 41	61 ± 3.5	307 ± 25	55 ± 4.8
21-d	226 ± 42	52 ± 2.5	307 ± 41	58 ± 3.3	280 ± 43	52 ± 4.2
35-d	253 ± 28	55 ± 5.6	285 ± 34	57 ± 4.3	291 ± 33	54 ± 2.8
Fat (g)						
0-d	50 ± 4.8	25 ± 1.6	64 ± 6.8	31 ± 2.5	67 ± 12	29 ± 1.8
7-d	61 ± 8.7	30 ± 2.2	66 ± 13	30 ± 2.3	76 ± 7.5	31 ± 2.0
14-d	55 ± 11	29 ± 3.7	56 ± 19	24 ± 3.8	87 ± 18	33 ± 3.5
21-d	69 ± 15	34 ± 2.2	69 ± 19	26 ± 2.8	78 ± 10	35 ± 2.7
35-d	59 ± 9.5	28 ± 2.9	64 ± 11	29 ± 3.9	75 ± 6.7	32 ± 2.6
Cholesterol (mg)						
0-d	142 ± 22		196 ± 42		170 ± 42	
7-d	202 ± 36		162 ± 28		212 ± 42	
14-d	138 ± 58		145 ± 48		256 ± 65	
21-d	177 ± 67		241 ± 83		222 ± 57	
35-d	163 ± 37		114 ± 27		199 ± 22	

[1]Values are expressed as mean ± SEM.

TABLE 6-3

Mean nutrient intakes of participants in three groups[1] (continued)

Variables	Placebo	%kcal	Supplement 1	%kcal	Supplement 2	%kcal
Saturated fat (g)						
0-d	15 ± 1.9	7.5 ± 0.8	17 ± 1.1	8.5 ± 0.9	21 ± 4.7	9.0 ± 1.0
7-d	20 ± 3.3	9.4 ± 1.0	22 ± 4.8	10 ± 1.5	23 ± 3.1	9.1 ± 0.8
14-d	16 ± 2.6	8.2 ± 0.9	16 ± 5.5	7.0 ± 1.4	29 ± 7.7	11 ± 1.6
21-d	20 ± 4.9	9.8 ± 1.3	20 ± 5.2	7.7 ± 0.9	26 ± 5.2	11 ± 1.4
35-d	18 ± 2.8	8.6 ± 1.3	20 ± 3.8	9.0 ± 1.5	22 ± 3.2	9.3 ± 1.1
Monounsaturated fat (g)						
0-d	12 ± 1.4	6.3 ± 0.6	17 ± 1.9	8.4 ± 0.7	16 ± 1.5	7.6 ± 0.8
7-d	15 ± 1.8	7.5 ± 0.6	12 ± 1.3	5.8 ± 1.0	18 ± 3.1	7.3 ± 1.2
14-d	17 ± 4.4	9.2 ± 1.8	16 ± 7.4	6.0 ± 1.8	26 ± 2.3	11 ± 0.8
21-d	17 ± 5.6	8.0 ± 1.3	17 ± 8.0	6.1 ± 1.7	23 ± 3.0	10 ± 1.1
35-d	14 ± 2.4	7.0 ± 1.0	19 ± 2.4	8.5 ± 0.7	22 ± 2.9	9.5 ± 1.1
Polyunsaturated fat (g)						
0-d	7.2 ± 1.1	3.7 ± 0.5	18 ± 3.2	8.5 ± 1.5	13 ± 2.9	6.0 ± 0.7
7-d	14 ± 3.8	6.5 ± 1.3	12 ± 4.6	5.1 ± 1.2	19 ± 3.0	7.6 ± 1.1
14-d	10 ± 1.9	5.7 ± 0.9	11 ± 4.3	4.3 ± 1.2	16 ± 3.9	6.1 ± 1.2
21-d	16 ± 4.7	7.4 ± 1.5	10 ± 3.5	3.9 ± 0.6	14 ± 2.0	6.2 ± 1.0
35-d	11 ± 2.4	4.9 ± 0.8	13 ± 5.4	5.8 ± 2.1	13 ± 2.5	5.5 ± 1.0
Dietary fiber (g)						
0-d	10 ± 2.2		11 ± 2.5		13 ± 1.8	
7-d	10 ± 1.5		13 ± 2.5		13 ± 2.0	
14-d	10 ± 1.6		11 ± 1.9		15 ± 2.2	
21-d	10 ± 2.0		12 ± 2.8		12 ± 2.3	
35-d	12 ± 1.7		14 ± 2.7		13 ± 2.3	
Sugar (g)						
0-d	91 ± 15		69 ± 15		89 ± 22	
7-d	85 ± 10		112 ± 22		135 ± 28	
14-d	60 ± 14		77 ± 14		92 ± 29	
21-d	72 ± 18		100 ± 12		97 ± 19	
35-d	74 ± 14		89 ± 17		93 ± 22	

[1]Values are expressed as mean ± SEM.

TABLE 6-3

Mean nutrient intakes of participants in three groups[1] (continued)

Variables	Placebo	Supplement 1	Supplement 2
Vitamin A (μg RE)			
0-d	795 ± 159	744 ± 101	848 ± 132
7-d	537 ± 81	483 ± 118	738 ± 139
14-d	721 ± 157	621 ± 254	597 ± 116
21-d	908 ± 209	888 ± 244	940 ± 346
35-d	847 ± 451	1126 ± 253	1068 ± 570
β-carotene (μg)			
0-d	55 ± 34	164 ± 68	336 ± 107
7-d	92 ± 31	161 ± 92	212 ± 141
14-d	123 ± 50	224 ± 173	65 ± 20
21-d	47 ± 28	132 ± 53	126 ± 48
35-d	221 ± 80	225 ± 102	346 ± 215
Thiamin (mg)			
0-d	1.1 ± 0.2	1.6 ± 0.2	1.7 ± 0.3
7-d	1.3 ± 0.2	1.3 ± 0.3	1.6 ± 0.3
14-d	1.2 ± 0.2	1.2 ± 0.3	2.3 ± 0.8
21-d	1.6 ± 0.4	1.6 ± 0.3	1.2 ± 0.1
35-d	1.2 ± 0.3	1.4 ± 0.3	2.5 ± 0.8
Riboflavin (mg)			
0-d	1.4 ± 0.2	1.6 ± 0.2	1.7 ± 0.2
7-d	1.4 ± 0.2	1.1 ± 0.3	1.8 ± 0.3
14-d	1.3 ± 0.1	1.2 ± 0.4	1.5 ± 0.1
21-d	1.7 ± 0.3	1.6 ± 0.3	1.5 ± 0.2
35-d	1.4 ± 0.2	1.6 ± 0.2	1.7 ± 0.3
Niacin (mg)			
0-d	16 ± 2.0	19 ± 3.0	21 ± 2.8
7-d	27 ± 3.6	20 ± 2.2	20 ± 3.0
14-d	16 ± 1.9	17 ± 4.4	25 ± 4.3
21-d	17 ± 2.2	19 ± 3.8	22 ± 4.1
35-d	19 ± 5.1	19 ± 2.6	22 ± 2.9

[1]Values are expressed as mean ± SEM.

TABLE 6-3

Mean nutrient intakes of participants in three groups[1] (continued)

Variables	Placebo	Supplement 1	Supplement 2
Vitamin B_6 : pyridoxine (mg)			
0-d	1.0 ± 0.2	1.4 ± 0.2	1.3 ± 0.2
7-d	1.4 ± 0.2	1.1 ± 0.2	1.3 ± 0.2
14-d	1.0 ± 0.2	1.0 ± 0.2	1.7 ± 0.3
21-d	1.1 ± 0.1	1.1 ± 0.2	1.3 ± 0.1
35-d	1.2 ± 0.3	1.5 ± 0.3	1.7 ± 0.4
Vitamin B_{12} (μg)			
0-d	2.7 ± 0.7	2.9 ± 0.6	2.7 ± 0.6
7-d	3.4 ± 0.7	2.7 ± 0.8	2.4 ± 0.5
14-d	1.8 ± 0.3	2.5 ± 1.1	3.1 ± 1.0
21-d	3.6 ± 0.9	2.6 ± 0.7	2.6 ± 0.5
35-d	1.8 ± 0.4	2.3 ± 0.6	2.4 ± 0.9
Vitamin C (mg)			
0-d	99 ± 15	79 ± 13	77 ± 16
7-d	83 ± 17	114 ± 18	90 ± 18
14-d	80 ± 31	58 ± 27	103 ± 25
21-d	84 ± 21	94 ± 35	141 ± 56
35-d	77 ± 18	109 ± 28	111 ± 38
Vitamin D (μg)			
0-d	2.2 ± 0.9	3.1 ± 1.3	2.7 ± 1.0
7-d	1.8 ± 0.7	1.1 ± 0.6	1.8 ± 0.9
14-d	1.1 ± 0.4	2.5 ± 1.7	1.6 ± 0.5
21-d	2.9 ± 0.9	3.5 ± 1.4	1.9 ± 0.4
35-d	1.5 ± 0.5	2.4 ± 0.9	2.2 ± 1.3
Vitamin E (mg α-TE)			
0-d	3.7 ± 1.3	13 ± 2.9	5.4 ± 1.9
7-d	12 ± 3.3	6.6 ± 1.5	9.6 ± 3.0
14-d	7.7 ± 3.3	6.1 ± 2.0	11 ± 4.1
21-d	14 ± 4.8	3.4 ± 1.2	8.4 ± 2.2
35-d	5.5 ± 3.3	8.6 ± 2.5	4.3 ± 1.2

[1]Values are expressed as mean ± SEM.

TABLE 6-3

Mean nutrient intakes of participants in three groups[1] (continued)

Variables	Placebo	Supplement 1	Supplement 2
α-Tocopherol (mg)			
0-d	2.4 ± 0.6	11 ± 4.6	3.2 ± 0.8
7-d	4.3 ± 0.6	4.1 ± 0.9	6.1 ± 1.4
14-d	3.9 ± 0.8	3.8 ± 1.2	3.8 ± 0.8
21-d	4.3 ± 1.2	4.4 ± 1.6	4.0 ± 0.8
35-d	3.4 ± 1.2	9.2 ± 5.7	4.0 ± 0.8
Folate (μg)			
0-d	213 ± 45	214 ± 45	186 ± 39
7-d	186 ± 40	204 ± 22	201 ± 28
14-d	214 ± 44	170 ± 44	233 ± 44
21-d	232 ± 37	241 ± 77	184 ± 33
35-d	183 ± 28	242 ± 40	228 ± 57
Pantothenic acid (mg)			
0-d	2.0 ± 0.4	4.1 ± 0.7	2.4 ± 0.5
7-d	2.8 ± 0.2	2.0 ± 0.3	2.5 ± 0.4
14-d	2.0 ± 0.4	2.5 ± 0.7	3.5 ± 0.5
21-d	2.7 ± 0.7	3.2 ± 1.0	2.8 ± 0.6
35-d	1.9 ± 0.4	4.2 ± 0.7	3.3 ± 1.0
Biotin (μg)			
0-d	4.3 ± 1.8	7.6 ± 2.5	7.8 ± 2.7
7-d	7.2 ± 2.2	5.1 ± 1.7	8.9 ± 2.5
14-d	6.5 ± 1.9	4.7 ± 1.8	14 ± 4.4
21-d	12 ± 3.2	12 ± 4.9	9.0 ± 3.1
35-d	4.4 ± 1.5	5.7 ± 1.6	6.5 ± 2.7
Vitamin K (μg)			
0-d	237 ± 104	138 ± 61	137 ± 62
7-d	240 ± 68	122 ± 21	138 ± 64
14-d	178 ± 44	148 ± 42	118 ± 50
21-d	234 ± 48	227 ± 59	143 ± 43
35-d	161 ± 61	171 ± 42	114 ± 54

[1]Values are expressed as mean ± SEM.

TABLE 6-3

Mean nutrient intakes of participants in three groups[1] (continued)

Variables	Placebo	Supplement 1	Supplement 2
Sodium (mg)			
0-d	2909 ± 397	2729 ± 212	3877 ± 253
7-d	2627 ± 269	2873 ± 481	3437 ± 468
14-d	2646 ± 227	2893 ± 563	3075 ± 314
21-d	2579 ± 504	3415 ± 672	3058 ± 410
35-d	2978 ± 238	3690 ± 545	3010 ± 369
Potassium (mg)			
0-d	2044 ± 244	2110 ± 320	2402 ± 318
7-d	2388 ± 273	2158 ± 295	2594 ± 142
14-d	1848 ± 197	2041 ± 466	2465 ± 215
21-d	1989 ± 233	2594 ± 592	2468 ± 257
35-d	2209 ± 138	2578 ± 358	2538 ± 323
Iron (mg)			
0-d	10 ± 1.1	12 ± 2.6	13 ± 1.7
7-d	12 ± 1.5	12 ± 2.1	14 ± 1.3
14-d	11 ± 1.3	11 ± 3.0	15 ± 2.1
21-d	16 ± 2.5	14 ± 3.8	11 ± 1.2
35-d	14 ± 3.4	14 ± 1.9	14 ± 1.1
Calcium (mg)			
0-d	664 ± 68	779 ± 151	888 ± 119
7-d	601 ± 109	656 ± 202	856 ± 103
14-d	545 ± 81	701 ± 214	779 ± 114
21-d	750 ± 152	831 ± 198	788 ± 104
35-d	766 ± 74	743 ± 86	873 ± 191
Magnesium (mg)			
0-d	189 ± 26	254 ± 40	221 ± 28
7-d	225 ± 27	195 ± 23	226 ± 20
14-d	186 ± 21	208 ± 57	273 ± 42
21-d	197 ± 25	251 ± 54	194 ± 28
35-d	180 ± 25	274 ± 33	257 ± 45

[1]Values are expressed as mean ± SEM.

TABLE 6-3

Mean nutrient intakes of participants in three groups[1] (continued)

Variables	Placebo	Supplement 1	Supplement 2
Phosphorus (mg)			
0-d	871 ± 117	1253 ± 142	1316 ± 189
7-d	1106 ± 133	977 ± 182	1140 ± 159
14-d	793 ± 78	932 ± 228	1098 ± 118
21-d	1030 ± 166	1285 ± 288	1186 ± 189
35-d	746 ± 112	1215 ± 161	1192 ± 147
Zinc (mg)			
0-d	6.0 ± 0.8	8.6 ± 1.3	7.7 ± 1.1
7-d	7.9 ± 1.4	6.0 ± 1.2	7.4 ± 1.2
14-d	6.6 ± 0.8	6.2 ± 2.1	8.3 ± 0.9
21-d	9.5 ± 3.9	7.2 ± 1.7	13 ± 5.4
35-d	5.4 ± 1.0	7.9 ± 1.2	8.0 ± 1.9
Copper (mg)			
0-d	0.7 ± 0.1	1.4 ± 0.4	0.9 ± 0.1
7-d	1.3 ± 0.4	0.8 ± 0.1	0.9 ± 0.04
14-d	0.7 ± 0.1	1.0 ± 0.3	1.2 ± 0.2
21-d	1.1 ± 0.3	1.0 ± 0.2	0.9 ± 0.1
35-d	0.8 ± 0.2	1.3 ± 0.1	1.1 ± 0.2
Selenium (μg)			
0-d	30 ± 7.9	92 ± 25	42 ± 14
7-d	51 ± 11	58 ± 15	33 ± 11
14-d*	23 ± 7.3	35 ± 7.4	79 ± 20
21-d	23 ± 6.0	56 ± 17	53 ± 11
35-d	51 ± 23	119 ± 49	80 ± 24
Chromium (μg)			
0-d	42 ± 16	47 ± 9.8	33 ± 13
7-d	54 ± 7.6	70 ± 15	33 ± 11
14-d	33 ± 10	36 ± 14	92 ± 31
21-d	77 ± 19	81 ± 19	50 ± 8.3
35-d	37 ± 16	33 ± 9.5	37 ± 15

[1]Values are expressed as mean ± SEM.

TABLE 6-4

Mean caffeine intakes of participants in three groups[1]

Variables	Placebo	Supplement 1	Supplement 2
Caffeine (mg)			
0-d	234 ± 46	167 ± 55	196 ± 64
7-d	256 ± 38	174 ± 46	262 ± 58
14-d	269 ± 67	226 ± 67	236 ± 66
21-d	309 ± 69	297 ± 93	259 ± 73
35-d	268 ± 97	231 ± 71	223 ± 68

[1]Values are expressed as mean ± SEM.

For all nutrient intakes, factorial analysis was used to evaluate gender differences in the entire population (all 3 groups together). Results of energy and nutrients that are significantly different between male (n = 5) and female (n = 17) subjects are summarized in **Table 6-5.** Total energy, protein and carbohydrate intake were affected by gender at all time periods, except on d 35. More nutrients were affected by the gender on d 14 and 21 than on any other time period. The gender differences were eliminated by d 35, except for the sugar and sodium. On d 7 and 14 there were some nutrients that were significantly different among the groups, when excluding the values of males and only the values of female subjects were compared.

Resting metabolic rate (RMR) of each subject was predicted using the equation formulated by Harris and Benedict (1919). The estimates of total energy expenditure included the additional energy cost associated with activity which was based on

TABLE 6-5

Gender differences on nutrient intakes

0-d	7-d	14-d	21-d	35-d
Energy	Energy	Energy*	Energy	----
Protein	Protein	Protein	Protein	----
Carbohydrate	Carbohydrate*	Carbohydrate*	Carbohydrate	----
Fat	----	Fat*	Fat	----
----	----	----	Cholesterol	----
----	----	Saturated fat*	Saturated fat	----
----	----	Mono- Fat*	Mono-Fat	----
----	----	Poly-Fat*	Poly-Fat	----
Sugar	----	----	Sugar	Sugar
Niacin	Niacin*	Niacin	Niacin	----
----	Sodium	Sodium	Sodium	Sodium
----	----	Potassium	Potassium	----
----	Iron	Iron*	Iron	----
Calcium	Calcium	Calcium	Calcium	----
Magnesium	-----	Magnesium	Magnesium	----
Phosphorus	Phosphorus	Phosphorus	Phosphorus	----
----	----	Zinc*	----	----
----	----	Copper	----	----
----	Chromium	----	----	----
Caffeine	----	----	----	----

* Indicates significant differences ($P < 0.05$) between the groups when compared with only female participants

individual activity level for sedentary to moderately active life style (Whitney et al. 1994). Daily energy balance of each individual for each wk was estimated by subtracting energy intake determined from 3-day food dietary and total energy expenditure. Estimated energy balance of each group for d 0, 7, 14,21 and 35 is summarized in **Table 6-6**. Energy balance was negative in the subjects of the placebo group throughout the study

TABLE 6-6

Energy balance estimated from total energy expenditure and calorie intake in three groups[1]

Variables	Day	Placebo	Supplement 1	Supplement 2
Energy Balance (kcal)	0-d	-142 ± 99	-161 ± 74	12 ± 160
	7-d	-89 ± 154	23 ± 139	205 ± 128
	14-d	-230 ± 161	-357 ± 137	169 ± 166
	21-d	-298 ± 200	-19 ± 128	-14 ± 213
	35-d	-21 ± 111	71 ± 114	96 ± 179

[1]Values are expressed as mean ±SEM. No statistical analysis was applied due to the too large individual differences to make any prediction.

period. There was negative energy balance of ≥ 300 kcal in the S1 group on d 14. The estimates of energy balance were positive in the S2 group during the study period. The difference in energy balance between the S1 and S2 was more than 500 kcal on d 14 but it was 25 kcal on d 35. The placebo group had the largest calorie deficit (~ 300 kcal) following the exercise period (on d 21), but the estimated total energy expenditures and calorie intakes were almost equal in the S1 and S2 during the exercise period.

Serum lipid profiles of the subjects are shown in **Table 6-7**. Significant group effects were observed for HDL on d 14, and for triglycerides on d 21 and 35. The placebo group had significantly higher HDL than the 2 supplement groups on d 14. Serum triglyceride of the S2 group was lower than that of the other two groups during the entire study period, and the differences were statistically significant on d 21 and 35. Men

TABLE 6-7

Serum lipids of participants in three groups[1]

Variables	Day	Placebo (n = 7)	Supplement 1 (n = 8)	Supplement 2 (n = 8)	Statistical Significance[2] Group	Gender	GxG
Total-C (mg/dl)	0-d	197.5 ± 26.2	163.2 ± 8.1	174.2 ± 4.5	NS	NS	NS
	7-d	189.0 ± 9.7	162.1 ± 9.3	162.8 ± 6.0	NS	NS	NS
	14-d	195.7 ± 23.2	178.4 ± 9.7	168.0 ± 5.5	NS	NS	NS
	21-d	203.6 ± 25.3	184.2 ± 9.8	158.7 ± 5.3	NS	NS	NS
	35-d	191.0 ± 24.8	179.7 ± 8.7	169.4 ± 8.5	NS	NS	NS
LDL (mg/dl)	0-d	122.4 ± 22.4	104.4 ± 7.9	114.4 ± 5.4	NS	NS	NS
	7-d	114.9 ± 21.4	105.0 ± 11.7	103.0 ± 3.9	NS	NS	NS
	14-d	124.3 ± 22.0	119.0 ± 10.6	108.2 ± 6.2	NS	NS	NS
	21-d	125.3 ± 21.6	119.6 ± 10.2	99.7 ± 6.3	NS	NS	NS
	35-d	113.9 ± 20.9	112.1 ± 10.2	107.9 ± 9.4	NS	NS	NS
HDL (mg/dl)	0-d	52.6 ± 4.9	39.5 ± 2.7	41.5 ± 4.3	NS	NS	NS
	7-d	49.5 ± 4.3	37.4 ± 1.7	41.3 ± 3.7	NS	NS	NS
	14-d	49.8 ± 3.0	37.2 ± 1.8	40.2 ± 4.0	0.0427	NS	NS
	21-d	54.2 ± 3.8	40.0 ± 2.4	42.7 ± 4.5	NS	NS	NS
	35-d	53.6 ± 4.7	43.1 ± 2.4	44.4 ± 4.8	NS	NS	NS
TG (mg/dl)	0-d	112.7 ± 19.2	96.1 ± 13.5	91.2 ± 8.4	NS	NS	NS
	7-d	122.9 ± 20.1	98.6 ± 18.7	92.6 ± 14.7	NS	NS	NS
	14-d	107.9 ± 13.3	110.8 ± 18.4	99.6 ± 9.7	NS	0.0150	NS
	21-d	120.2 ± 17.1	123.4 ± 13.6	80.9 ± 8.8	0.0371	NS	NS
	35-d	117.3 ± 16.4	116.2 ± 13.1	86.0 ± 8.3	0.0347	NS	NS
FFA (μmol/L)	0-d	479.8 ± 23.0	423.9 ± 35.8	531.4 ± 55.5	NS	NS	NS
	7-d	498.7 ± 56.8	375.3 ± 26.3	584.7 ± 66.9	NS	NS	NS
	14-d	471.5 ± 57.5	396.2 ± 59.0	519.7 ± 49.8	NS	NS	NS
	21-d	453.8 ± 27.6	321.2 ± 48.5	470.5 ± 58.6	NS	NS	NS
	35-d	453.4 ± 33.4	427.3 ± 30.2	494.0 ± 55.0	NS	NS	NS

[1]Values are expressed as mean ± SEM. [2]*P* values from 2-way ANOVA. Abbreviation used: FFA, free fatty acids; NS, no significance differences, *P*> 0.05; TG, triglycerides; Total-C, total cholesterol; GxG, group and gender interaction.

had higher serum triglycerides (134.1 ± 15.0 mg/dl, n = 5) than women (97.5 ± 8.1, n = 17) on d 14. Two-way ANOVA with repeated measures showed significant group differences in HDL ($P = 0.044$) and FFA ($P = 0.033$). The placebo group had higher HDL than the S1 group throughout the study. Duncan's multiple range test revealed that the serum FFA was significantly lower in the S1 than S2 group, although there were no significant group differences from week to week.

Serum concentrations of β-hydroxybutyrate and leptin are shown in **Table 6-8**. The S2 group, after having been on carnitine supplementation for 7 d, showed higher concentration of β-hydroxybutyrate compared to other treatments. Two-way ANOVA with repeated measures also showed group and time effects on the serum concentration of β-hydroxybutyrate. The Duncan Multiple range test revealed that the placebo group was significantly different from the S2 group, and the baseline was significantly different from the d 21. The placebo and S1 group had a peak of β-hydroxybutyrate on d 21, that is after the exercise intervention. The S2 group on the other hand had a peak β-hydroxybutyrate concentration after carnitine supplementation (d 7) and the concentration remained higher than other groups throughout the study period.

No significant group effects were found on serum leptin concentration after any treatment. However, two-way ANOVA with repeated measurements revealed that there was significant time effect in all group ($P = 0.0001$), indicated by reduced serum leptin concentration after 1 wk of exercise intervention. Serum leptin concentration showed significant positive correlations to all the anthropometric measurement, except the WHR, before and after the treatment (**Table 6-9**). Serum leptin concentration was not

TABLE 6-8

Serum β-hydroxybutyrate and leptin concentrations in three groups[1]

Variables	Day	Placebo (n = 7)	Supplement 1 (n = 8)	Supplement 2 (n = 8)	Statistical Significance[2] Group	Gender	GxG[3]
β-Hydroxybutyrate (μmol/L)	0-d	25.2 ± 7.3	27.6 ± 13.1	20.2 ± 4.5	NS	NS	NS[4]
	7-d	26.4 ± 6.6	35.7 ± 10.4	81.1 ± 15.1	0.0169	NS	NS
	14-d	22.3 ± 7.0	41.2 ± 11.5	52.1 ± 14.1	NS	NS	NS
	21-d	42.3 ± 4.8	65.0 ± 17.9	61.8 ± 13.0	NS	NS	NS
	35-d	28.0 ± 6.4	33.4 ± 10.2	38.0 ± 9.5	NS	NS	NS
2-way ANOVA repeated measure:							
Group: 0.0099 ..... P^a, $S1^{a,b}$, $S2^b$							
Week: 0.0198 ..... 0-d^a, 7-d^{bc}, 14-d^{abc}, 21-d^b, 35-d^{ac}							
Group x Week: NS							
Leptin (ng/ml)	0-d	28.8 ± 5.4	25.2 ± 5.6	24.5 ± 9.5	NS	NS	NS
	7-d	28.7 ± 4.3	27.7 ± 5.7	25.4 ± 10.7	NS	NS	NS
	14-d	27.2 ± 4.7	21.7 ± 3.8	21.0 ± 7.6	NS	NS	NS
	21-d	12.8 ± 3.0	11.0 ± 2.7	8.5 ± 3.6	NS	NS	NS
	35-d	—	—	---			
2-way ANOVA repeated measure:							
Group: NS							
Week: 0.0001 .... 0-d^a, 7-d^a, 14-d^a, 21-d^b							
Group x Week: NS							

[1]Values are expressed as mean ± SEM. [2]*P* values from 2-way ANOVA. [3]GxG, group and gender interaction.
[4]NS, no significance differences, $P > 0.05$.

TABLE 6-9

Correlation between serum leptin, anthropometric measurements, serum triglyceride and free fatty acids before and after the exercise intervention

Measurements	All subjects (n = 23)	
	r	*P*-value
Body Weight		
before[1]	0.587	0.0032
after[2]	0.549	0.0066
BMI		
before	0.690	0.0003
after	0.654	0.0007
Waist Circumference		
before	0.493	0.0168
after	0.496	0.0160
Hip Circumference		
before	0.735	0.0001
after	0.730	0.0001
WHR		
before	0.194	0.3755
after	0.236	0.2781
Sum of skinfold thickness		
before	0.713	0.0002
after	0.716	0.0002
% BF		
before	0.723	0.0001
after	0.779	0.0001
Serum triglyceride		
before	0.343	0.1175
after	0.592	0.0036
Serum free fatty acid		
before	0.398	0.0668
after	0.088	0.6979

Pearson correlation coefficients were calculated using data from all 23 subjects.
[1]before: correlation at the beginning of the baseline (0-d),
[2]after: correlation after the exercise intervention week (21-d).

significantly correlated with serum triglyceride before the treatments (0-d), but it became significantly correlated after the treatments (21-d) in all subjects.

Serum carnitine concentrations in the 3 groups after each treatment and the wash-out period are shown in **Table 6 -10**. On d 0 (base line) there were no significant differences among the groups in any of the carnitine fractions. There was a significant gender effect on the ASAC. The mean serum ASAC concentrations in female and male were 10.40 ±1.2 (n = 17) and 5.7 ± 0.5 (n = 3) μmol/L, respectively. Comparisons of the pooled ASAC values of all female showed no significant differences among the groups. After the first treatment period (d 7) all fractions of carnitine, except AIAC, were significantly different among the groups. The S2 group showed significantly higher serum carnitine concentrations after 7 d of carnitine supplementation. After 7 d of the combination of carnitine and choline supplementation (d 14) the group effect disappeared and no significance was found among the groups. After 1 wk of exercise intervention the group effect was observed only in TC. Both supplement groups maintained significantly higher serum carnitine concentration than the placebo group, and the concentrations in the supplemented groups had peaked after the exercise intervention period. The placebo group maintained a very stable serum TC concentration, i.e. within about 2 μmol/L throughout experimental period. After 2 wk of wash-out period (d 35) there was a significant group effect on the AC in that the S2 group had significantly higher serum AC than other 2 groups. Two-way ANOVA with repeated measures showed significant group effect in all the fractions of serum carnitine, except the AIAC.

TABLE 6 -10

Serum carnitine concentrations in three groups[1]

Variables	Day	Placebo	Supplement 1	Supplement 2	Statistical Significance[2] Group	Gender	GxG
Total carnitine (μmol/L)	0-d	37.2 ± 2.8	41.3 ± 3.5	44.6 ± 2.7	NS	NS	NS
	7-d	35.9 ± 2.2	36.1 ± 6.7	55.2 ± 4.1	0.0108	NS	NS
	14-d	39.6 ± 3.3	43.8 ± 2.0	48.1 ± 6.5	NS	NS	NS
	21-d	39.2 ± 3.2	53.5 ± 3.2	55.7 ± 5.3	0.0165	NS	NS
	35-d	37.6 ± 4.1	47.6 ± 3.8	50.8 ± 4.8	NS	NS	NS
NEC (μmol/L)	0-d	25.6 ± 2.1	31.2 ± 2.4	31.2 ± 2.4	NS	NS	NS
	7-d	25.5 ± 1.7	26.0 ± 5.9	39.5 ± 4.1	0.0413	NS	NS
	14-d	30.3 ± 3.0	32.0 ± 1.6	35.9 ± 4.9	NS	NS	NS
	21-d	31.2 ± 2.3	41.0 ± 3.6	43.6 ± 4.7	NS	NS	NS
	35-d	28.1 ± 2.9	36.1 ± 3.5	36.3 ± 2.9	NS	NS	NS
ASAC (μmol/L)	0-d	9.2 ± 1.1	8.9 ± 1.5	10.9 ± 1.2	NS	0.0028	NS
	7-d	8.3 ± 1.0	8.0 ± 1.4	13.3 ± 1.8	0.0413	NS	NS
	14-d	7.2 ± 1.0	10.0 ± 1.6	10.3 ± 2.1	NS	NS	NS
	21-d	6.2 ± 1.2	10.1 ± 2.1	9.3 ± 1.1	NS	NS	NS
	35-d	7.1 ± 1.2	9.3 ± 1.5	11.7 ± 2.5	NS	NS	NS
AIAC (μmol/L)	0-d	2.3 ± 0.30	2.5 ± 0.24	2.5 ± 0.15	NS	NS	NS
	7-d	2.0 ± 0.29	2.0 ± 0.26	2.4 ± 0.14	NS	NS	NS
	14-d	2.1 ± 0.37	1.8 ± 0.14	2.0 ± 0.15	NS	NS	NS
	21-d	1.9 ± 0.33	2.5 ± 0.17	2.8 ± 0.18	NS	NS	NS
	35-d	2.4 ± 0.43	2.2 ± 0.21	2.8 ± 0.31	NS	NS	NS
AC (μmol/L)	0-d	3.9 ± 0.42	4.1 ± 0.38	4.8 ± 0.44	NS	NS	0.0246
	7-d	4.9 ± 0.55	5.0 ± 0.50	7.8 ± 0.91	0.0465	NS	0.0452
	14-d	4.9 ± 0.53	6.3 ± 0.38	6.7 ± 0.60	NS	NS	NS
	21-d	5.2 ± 0.79	6.6 ± 0.63	7.2 ± 0.73	NS	NS	NS
	35-d	4.9 ± 0.47	5.9 ± 0.43	8.2 ± 1.1	0.0346	NS	NS

[1]Values are expressed as mean ± SEM. [2]*P* values from 2-way ANOVA. Abbreviation used: AC, acetylcarnitine; AIAC, acid- insoluble acylcarnitine; ASAC, acid-soluble acylcarnitine, NEC, nonesterified carnitine; NS, no significance differences.

Urinary excretions of carnitine are shown in **Table 6 -11**. There was no significant difference in the fractions of carnitine, except on AIAC, among the groups on d 0. There were significant gender and gender-and-group interactions on AIAC. The mean daily AIAC excretion for females and males were 10.71 ±0.7 (n = 17) and 15.73 ± 1.3 (n = 3) μmol/d, respectively. However, there were no significant differences among the groups if, analyzed without male subjects. As it was observed in the serum, the urinary concentrations of all carnitine fractions were significantly different among the groups after the first treatment. The S2 group showed significantly higher urinary carnitine concentrations after 7 d of carnitine supplementation and the magnitude of changes are much greater (3-4- fold) than those in serum carnitine (1.2-fold). After 7 d of the combination of carnitine and choline supplementation (d 14) the group effect disappeared and no significance was found among the groups. After 1 wk of exercise intervention the group effects and gender effects were observed for all carnitine fractions. The exercise reduced urinary total carnitine excretion to more than half of the previous weeks in the placebo group this effect continued at the wash-out period for fractions of carnitine (d 35). This decline in carnitine excretion has magnified the difference between the placebo and supplemented groups and the differences between the base line and wash-out period. Two-way ANOVA with repeated measures showed significant group effects in the TC, NEC, and AC.

Mean walking steps, distance and estimated caloric output per wk before and during the 1 wk of exercise intervention, including percentage changes of those measurements between the two periods, are summarized in **Table 6-12**. Subjects in the

TABLE 6-11

Daily urinary carnitine excretions in three groups[1]

Variables	Day	Placebo	Supplement 1	Supplement 2	Statistical Significance Group	Gender	GxG
Total carnitine (μmol/d)	0-d	142.1 ± 24.3	113.9 ± 14.6	112.7 ± 24.9	NS	NS	NS
	7-d	132.5 ± 20.7	57.7 ± 15.4	427.9 ± 55.6	0.0001	NS	NS
	14-d	144.3 ± 30.7	234.2 ± 56.8	210.5 ± 53.5	NS	NS	NS
	21-d	66.2 ± 14.8	195.4 ± 41.0	218.7 ± 48.4	0.0098	0.0291	NS
	35-d	55.9 ± 10.2	219.2 ± 43.9	210.4 ± 38.2	0.0161	NS	NS
NEC (μmol/d)	0-d	76.2 ± 18.2	65.5 ± 6.5	63.3 ± 17.1	NS	NS	NS
	7-d	70.0 ± 16.0	18.1 ± 8.3	297.5 ± 48.3	0.0001	NS	NS
	14-d	90.3 ± 23.0	163.6 ± 48.4	149.8 ± 44.9	NS	NS	NS
	21-d	32.5 ± 8.1	144.4 ± 33.3	150.3 ± 42.3	0.0129	0.0412	NS
	35-d	24.8 ± 8.0	129.2 ± 35.9	151.1 ± 25.5	0.0121	0.0111	NS
ASAC (μmol/d)	0-d	54.6 ± 8.9	36.6 ± 7.0	38.7 ± 8.0	NS	NS	NS
	7-d	50.6 ± 8.1	30.5 ± 7.6	111.7 ± 10.7	0.0009	NS	NS
	14-d	42.5 ± 8.2	56.7 ± 7.7	46.8 ± 7.2	NS	NS	NS
	21-d	23.0 ± 5.8	33.4 ± 6.7	50.5 ± 7.4	0.0221	NS	NS
	35-d	22.8 ± 9.6	76.2 ± 20.6	45.4 ± 20.1	NS	NS	NS
AIAC (μmol/d)	0-d	11.3 ± 1.4	11.8 ± 2.4	11.9 ± 0.86	NS	0.0007	0.0076
	7-d	11.8 ± 1.8	9.1 ± 2.1	18.7 ± 1.7	0.0018	0.0381	NS
	14-d	11.6 ± 2.2	13.9 ± 3.1	13.9 ± 2.4	NS	NS	NS
	21-d	10.7 ± 2.5	17.5 ± 3.4	18.0 ± 2.4	NS	0.0221	NS
	35-d	8.3 ± 1.2	13.8 ± 0.9	13.9 ± 1.7	0.0216	NS	NS
AC (μmol/d)	0-d	22.9 ± 5.2	19.5 ± 1.1	19.2 ± 2.3	NS	NS	NS
	7-d	19.4 ± 4.3	5.1 ± 1.8	52.7 ± 5.9	0.0001	NS	NS
	14-d	22.9 ± 4.4	28.0 ± 4.5	30.5 ± 8.1	NS	NS	NS
	21-d	14.2 ± 5.1	35.6 ± 6.2	36.9 ± 8.0	0.0126	NS	0.0147
	35-d	12.5 ± 2.9	27.9 ± 4.9	38.7 ± 3.5	0.0077	NS	NS

[1]Values are expressed as mean ± SEM. [2]*P* values from 2-way ANOVA. Abbreviation used: AC, acetylcarnitine; AIAC, acid-insoluble acylcarnitine; ASAC, acid-soluble acylcarnitine, NEC, nonesterified carnitine; NS, no significance differences.

TABLE 6-12

Average walking steps, distance and estimated calorie outputs per day before and during the exercise intervention in three groups[1]

Variables	Placebo (n = 7)	Supplement 1 (n = 8)	Supplement 2 (n = 8)
Walking steps			
before	9601 ± 2448	7794 ± 2573	4436 ± 2408
during	12829 ± 3845	12375 ± 2987	8676 ± 2885
% change	25.17	37.01	48.86
Walking distance (mile)			
before	2.54 ± 0.4	2.36 ± 0.9	1.32 ± 0.8
during	3.25 ± 0.6	3.85 ± 1.0	2.41 ± 0.8
% change	21.68	38.64	45.23
Calorie output (Kcal)			
before	378.7 ± 92.8	327.4 ± 76.6	181.1 ± 94.2
during	515.5 ± 97.1	494.5 ± 78.9	358.2 ± 99.8
% change	26.52	38.80	49.45

[1]Values are expressed as mean ±SD. Daily walking steps, estimated walking distance and estimated calorie output were determined by the Yamax Digi-Walker SW-701 (Yamax Inc., Japan) for 5-7 d , before (between 7-14 d) and during the exercise intervention week (between 12-21 d).

placebo group recorded more steps both before and during the exercise intervention periods than the other 2 groups. The mean of steps taken by the supplemented groups before exercise intervention were 46-81% of the steps taken by the placebo group. However, after the exercise intervention the mean steps taken by the supplemented groups was 68-96% of the steps taken by the placebo group. The percentages increase in steps taken before and during exercise intervention were 49, 37 and 25 percent in S2, S1

and the placebo group, respectively (Table 6-12).

DISCUSSION

This study was launched to determine if supplementary carnitine, choline and caffeine with or without a mild exercise regimen would enhance fat oxidation and thereby, reduce body fat in humans as seen in the animal model (discussed earlier). Unlike in the rat study, caffeine was not a part of the supplement mixture given to the human participants; however, the subjects in all 3 groups consumed 200-300 mg caffeine daily from a variety of self-selected foods and beverages as indicated by the nutrient intake data (Table 6-4). This amount of caffeine was slightly lower than that given to rats (~ 360 mg/d) in earlier study (Hongu and Sachan 2000). The drawback was that placebo and treatment groups consumed about equal amounts of caffeine. Thus, the independent variables remained carnitine and choline and the combination of these two. The serum (Table 6-10) and urinary (Table 6-11) carnitine concentrations were decreased by the introduction of choline and increased by the introduction of carnitine as has been reported in earlier human (Dodson and Sachan 1996) and animal studies (Hongu & Sachan 2002). So the effects of choline and carnitine independently (d 7), as well as in combination (d 14) are consistently reproducible; i.e. choline promotes carnitine conservation by decreasing losses in urine and thus increasing carnitine concentrations in the tissues (Chapter 5; Hongu & Sachan 2002).

Fatty acid mobilization, oxidation and disposal were augmented by the supplements. For example serum concentrations of β-hydroxybutyrate (Table 6-8) were

elevated and sustained by the supplements particularly by the introduction of carnitine. The concentration of another marker of fatty acid oxidation, acetylcarnitine, was elevated about 2-fold in serum (Table 6-10) and about 3-fold in urine (Table 6-11). Short-chain acylcarnitines (ASAC) were only modestly elevated in serum but greatly increased in the urine by carnitine supplementation. Serum leptin concentrations were reduced about 15% by the combination of supplements (Table 6-8) and leptin was significantly correlated with the BW, BMI, %BF and serum triglycerides. These changes, however modest, support the hypothesis that dietary supplementation with carnitine and choline promotes fat oxidation and disposal in free-living humans who consumed 200-300 mg caffeine daily from their self selected diets .

Exercise intervention further accentuated choline-carnitine effect on fat oxidation and disposal as evidenced by changes in the biochemical markers. The fall in the concentration of serum leptin in all groups following exercise was quite dramatic (> 50%) and this is particularly so in the S2 group (Table 6-8) where there was complimentary decrease in the serum triglycerides and free fatty acids (Table 6-7). The concentrations of serum β-hydroxybutyrate were elevated beyond the concentrations raised by the supplements (d 21 vs d 14) in both supplemented groups, but not in the placebo group (Table 6-8). The leptin concentration was reduced to less than half in the supplemented (d 21) as well as the placebo groups. This indicates a very clear and strong effect of exercise on serum leptin which is further magnified in the carnitine-primed (S2) individuals (Table 6-8). The effect of exercise on AC was very small, but consistent with the idea of enhanced fatty acid oxidation particularly in the carnitine pre-loaded group.

Whereas there were decreases in the urinary acetylcarnitine excretion in the placebo group, there were 21-27% increases in the S1 and S2 groups (d 21 vs d 14 in Table 6-11). The urinary loss of fatty acids in form of ASAC continued in the S2 group. The loss of fatty acid carbons as ASAC and AC was not only enhanced by exercise in the S2 group, but it was sustained for at lease 2 wk beyond (d 35) the cessation of supplementation (Table 6-11). It can be argued that, in spite of increased demand for energy due to exercise, the rate of fatty acid degradation is high enough to sustain the urinary loss of fatty acids. This perturbation of the metabolic processes is of great significance, even though the loss of energy as fatty acid carbons in quantitative terms is rather small. It appears that the sequence of carnitine-choline loading corrupts the metabolic regulators in favor of energy wasting which is accentuated and sustained by mild exercise regimen.

It is interesting to note that the activity level (numbers of walking step) of the S2 group was lowest prior to the exercise intervention and it nearly doubled during exercise intervention wk (Table 6-12). These changes were modest in the S1 group and least in the placebo group. On the contrary, the caloric intake was consistently higher in the S2 group followed by the S1 and placebo group (Table 6-3). The body weights and BMI reflected caloric intake and followed a similar pattern (Table 6-1). On the contrary, the sum of the skinfolds and percent body fat was lowest in the S2 group followed by the S1 and placebo group (Table 6-1). This suggests that the supplement and exercise regimen reduced body fat without changing body weight and this effect was sustained for at least 2 wk following cessation of the treatments. In spite of fat loss the body weight is maintained because carnitine in known to promote nitrogen accretion and protein/muscle

mass (Daily and Sachan 1995, Daily et al. 1998).

The cumulative evidence presented here allows the conclusion that carnitine-choline supplementation promotes fat mobilization, oxidation and disposal in urine without fully capitalizing the energy content of the lost carbons in urine. This effect of the supplements is accentuated by mild exercise regimen and is sustained for at least 2 wk after the cessation of the interventions. The final verdict in support of this conclusion must wait additional studies involving a larger sample size and controlled feeding condition.

CHAPTER VII

SUMMARY AND CONCLUSIONS

The interactive effect of a diet supplemented with carnitine, choline and caffeine at concentrations of 0.1, 5, and 11.5 g/kg diet, respectively was first evaluated in a 2x2 factorial design in rats where one-half of each dietary group was put on exercise regimen and the other half was sedentary. The mean food intake of the groups was not different, however, the body weight was significantly reduced by exercise in both dietary groups.

Fat pad weights and total lipids of epididymal, inguinal and perirenal regions were significantly reduced by the supplements as well as by exercise. Regardless of exercise, supplements significantly lowered triglycerides in serum, but increased triglycerides in the skeletal muscle. Serum leptin concentrations were equally lowered by supplements and exercise. Serum leptin was correlated with body weight ($r = 0.55$, $P < 0.01$), fat pad weight ($r = 0.82$, $P < 0.001$) and glucose ($r = 0.51$, $P < 0.05$).

VO_2max was increased in the supplemented, exercised group; however, RQ was not affected at rest and exhaustion. Post-exercised concentrations of serum triglycerides were decreased, but β-hydroxybutyrate, acylcarnitine and acetylcarnitine were increased in the supplemented animals. The changes in serum metabolites were complemented by the changes in the muscle and urinary metabolites. The magnitude of increase in urinary acylcarnitines (34-45-fold) is an unique effect of this combination of the supplements.

Cumulative evidence indicates enhanced β-oxidation of fatty acids without a change in the RQ because acetyl units were excreted in urine as acylcarnitine and

acetylcarnitine and not oxidized to carbon dioxide. Partially oxidized fatty acid carbons are loaded on to carnitine and dumped in urine as acylcarnitine and acetylcarnitine. For this phenomenon the term "Fatty acid dumping" was introduced.

The concentrations of nonesterified and total carnitine were higher in serum, urine and all tissues of the supplemented rats. Exercise further promoted this effect in the liver and kidney. There was variable effect of the supplement and exercise on the other fractions of carnitine in tissues, except that short-chain acylcarnitines were consistently higher in the in skeletal and cardiac muscles and reflected in the serum and urine of the supplemented animals. Serum AST and ALT as well as liver proteins and DNA were not adversely altered by the supplement.

In humans, serum and urinary carnitine and acylcarnitine were decreased in choline supplemented group; but, this carnitine profile was improved by the introduction of carnitine to the choline group. The concentrations of serum β-hydroxybutyrate and serum and urinary acetylcarnitine were elevated by the supplements and accentuated by the introduction of mild exercise. The supplements reduced the concentrations of serum leptin which was significantly correlated with the body weight, BMI, %BF and serum triglycerides. Mild exercise regimen further increased indices of fatty acid oxidation and reduced leptin concentrations.

From this data it is concluded that the combination of carnitine, choline, and caffeine supplementation promotes carnitine influx in all tissues and changes in acylcarnitines of skeletal and cardiac muscles of rats, indicating enhanced fatty acid oxidation, which is supported by the changes in serum and urinary acylcarnitine profiles

and loss of adipose tissue mass. The indices of body fat loss by dietary supplements were similar to that by mild exercise and the supplements augmented fatty acid oxidation as well as disposal of acylcarnitine in urine of rats and humans.

LITERATURE CITED

Abdelmalek, M.F., Angulo, O., Jorgensen, R.A., Sylvester, P.B. & Lindor K.D. (2001) Betaine, a promising new agent for patients with nonalcoholic steatohepatitis: Results of a pilot study. Am. J. Gastroenterology 96: 2711-2717.

Abdulla, M., Andersson, I., Asp., N-G, Berthelsen, K., Birkhed, D., Dencker, I., Johansson, C-G., Jägerstad, M, Kolar, K., Nair, B.M., Nilsson-Ehle, P., Nordén, Å., Rassner, S., Åkesson, B. & Öckerman, P-A. (1981) Nutrient intake and health status of vegans. Chemical analyses of diets using the duplicate portion sampling technique. Am. J. Clin. Nutr. 34: 2464-2477.

Acheson, K.J., Zahorska-Markiewica, B., Pittet, P., Anantharaman, K. & Jequier, E. (1980) Caffeine and Coffee: Their influence on metabolic rate and substrate utilization in normal weight and obese individuals. Am. J. Clin. Nutr. 33: 989-997.

Ahrén, B., Månsson, S., Gingerich, R.L. & Havel, P.J. (1997) Regulation of plasma leptin in mice: influence of age, high-fat diet, and fasting. Am. J. Physiol. 273: R113-R120.

Aitken, R.J., Best, F., Richardson, D.W., Schats, R. & Simm, G. (1983) Influence of caffeine on movement characteristics, fertilizing-capacity and ability to penetrate cervical-mucus of human-spermatozoa. J. Reprod. Fertil. 67: 19-27.

Albright, C.D., Liu, R., Bethea, T.C., da Costa, K-A., Salganik, R.I. & Zeisel S.H. (1996) Choline deficiency induces apoptosis in SV40-immortalized CWSV-1 rat hepatocytes in culture. FASEB J. 10: 510-516.

Aldridge, A., Bailey, J. & Neims, A.H. (1981) The disposition of caffeine during and after pregnancy. Semin. Perinatol. 5: 310-314.

Alhomida, A.S. (1998) Investigation of the effect of theophylline administration on total, free, short-chain acyl and long-chain acyl carnitine distributions in rat renal tissues. Cell Biochem. Funct. 16: 165-171.

Al-Jafari, A.A., Junaid, M.A. & Alhomida, A.S. (1996) Investigation of the influence of theophylline feeding on total, free, short-chain acyl and long-chain acyl carnitine levels in skeletal muscle and liver of rats. In Vivo 10: 569-574.

Allain, C.A., Poon, L.S., Chan, C.S.G., Richmond, W. & Fu, P.C. (1974) Enzymatic determination of total serum cholesterol. Clin. Chem. 20:470-475.

Allan, F.N., Bowie, D.J., MacLeod, J.J.R. & Robinson W.L. (1924) Behavior of depancreatized dogs kept alive with insulin. Br. J. Exp. Pathol. 5: 75-83.

American Academy of Pediatrics. Committee on Nutrition. (1985) Pediatric Nutrition Handbook. 2nd ed. American Academy of Pediatrics, Elk Grove Village, IL.

American College of Sports Medicine. (2000) ACSM's guidelines for exercise testing and prescription. 6th ed. (Franklin, B.A., Whaley, M.H. & Howley, E.T., eds.) Lippincott Williams & Wilkins, Baltimore, MD.

Anderson, M.E., Bruce, C.R., Fraser, S.F., Stepto, N.K., Klein, R., Hopkins, W.G. & Hawley, J.A. (2000) Improved 2000-meter rowing performance in competitive oarswomen after caffeine ingestion. Int. J. Sport Nutr. Exerc. Metabol. 10: 464-475.

Applegate, E. (1999) Effective nutritional ergogenic aids. Int. J. Sports Nutr. 9: 229-239.

Aranda, J.V., Collinge, J.M., Zinman, R. & Watters, G. (1979) Maturation of caffeine elimination in infancy. Arch. Disease Child. 54: 946-949.

Arenas, J., Ricoy, J.R,.Encinas, A.R., Pola, P., D'iddio, S., Zeviani, M., DiDonato, S. & Corsi, M. (1991) Carnitine in muscle, serum, and urine of nonprofessional athletes: effects of physical exercise, training, and L-carnitine administration. Muscle Nerve 14: 598-604.

Arnaud, M.J. (1987) The pharmacology of caffeine. Prog. Drug. Res. 31: 273-313.

Askew, E.W., Hecker, A.L. & Wise, Jr. W.R. (1977) Dietary carnitine and adipose tissue turnover rate in exercise trained rats. J. Nutr. 107: 132-142.

Assmann, G., Schriewer, H., Schmitz, G. & Hägele, E-O. (1983) Quantification of high-density lipoprotein cholesterol by precipitation with phosphotungstic acid/$MgCl_2$. Clin. Chem. 29:2026-2030.

Astrup, A., Breum, L., Toubro, S., Hein, P. & Quaade, F. (1992) The effect and safety of an ephedrine/caffeine compound compared to ephedrine, caffeine and placebo in obese subjects on an energy restricted diet. A double blind trial. Int. J. Obes. 16: 269-277.

Astrup, A., Toubro, S., Cannon, S., Hein, P., Breum L. & Madsen, J. (1990) Caffeine: A double-blind, placebo-controlled study of its thermogenic, metabolic and cardiovascular effects in healthy volunteers. Am. J. Clin. Nutr. 51: 759-767.

Augenfeld, J. & Fritz, I.B. (1969) Carnitine palmitoyltransferase activity and fatty acid oxidation by livers from fetal and neonatal rats. Can. J. Biochem. 48: 288-294.

Baeyer, A. (1866) Synthese des Neurins I. Ueber Neurin Ann. Chem. Liebigs. 140: 306-313.

Bahl, J.J. & Bressler, R. (1987) The pharmacology of carnitine. Ann. Rev. Pharmacol. Toxicol. 27: 257-277.

Bairam, A., Boutroy, M.J., Badonnel, Y. & Vert, P. (1987) Theophylline versus caffeine: Comparative effects in treatment of idiopathic apnea in the preterm infant. J Pediatr. 110: 636-639.

Barak, A.J., Beckenhauer, H.C. & Tuma, D.J. (1982) Use of S-adenosylmethionine as an index of methionine recycling in rat liver slices. Anal. Biochem. 127: 372-375.

Barak, A.J. & Tuma, D.J. (1983) Betaine, metabolic by-product or vital methylating agent? Life Sci. 32: 771-774.

Barnett, C., Costill, D., Vukovich, M., Cole, K., Goodpaster, B., Trappe, S. Fink, W. (1994) Effect of L-carnitine supplementation on muscle and blood carnitine content and lactate accumulation during high-intensity sprint cycling. Int. J. Sport Nutr. 4: 280-288.

Barone, J.J. & Roberts, H. (1984) Human consumption of caffeine. In: Caffeine; Perspectives from Recent Research (Dews, P.B., ed.), pp.59-73. Springer-Verlag, Berlin, Germany.

Barone, J.J. & Roberts, H. (1996) Caffeine consumption. Food Chem. Toxicol. 34: 119-129.

Bartholmey, S.J. & Sherman, A.R. (1985) Carnitine levels in iron-deficient rat pups. J. Nutr. 138-145.

Bättig, K. (1985) The physiological effects of coffee consumption. In: Coffee: Botany, Biochemistry and Production of Beans and Beverage (Clifford, M.N. & Willson, K.C., eds.), pp.394-439. Croom-Helm, London, England.

Bazzato, G., Mezzina, C., Ciman, M. & Guarnieri, G. (1979) Myasthenia-like syndrome associated with carnitine in patients on long-term hemodialysis. Lancet 1: 1041-1042.

Beach, C.A., Bianchine, J.R. & Gerber, N. (1984) The excretion of caffeine in the semen of man: Pharmacokinetics and comparison of the concentrations in blood and semen. J. Clin. Pharmacol. 24: 120-126.

Beaver, W.T. (1984) Caffeine revisited. JAMA. 251: 1732-1733.

Bedford, T.G., Tipton, C.M., Wilson, N.C., Oppliger, R.A. & Gisolfi, C.V. (1979) Maximum oxygen consumption of rats and its changes with various experimental procedures. J Appl. Physiol 47: 1278-1283.

Behnke, A.R., Feen, B.G. & Welham, W.G. (1942) Specific gravity of healthy man. JAMA. 118: 496-501.

Bell, D.G., Jacobs, I. & Zamecnik, J. (1998) Effects of caffeine, ephedrine and their combination on time to exhaustion during high-intensity exercise. Eur. J. Appl. Physiol. Occup. Physiol. 77: 427-433.

Bell, D.G., Jacobs, I. & Ellerington, K. (2001) Effect of caffeine and ephedrine ingestion on anaerobic exercise performance. 33: 1399-1403.

Bell, D.G., McLellan, T.M. & Sabiston, C.M. (2002) Effects of ingesting caffeine and ephedrine on 10-km run performance. Med. Sci. Sports Exerc. 34: 344-349.

Bellet, S., Kershbaum, A. & Finck, M.E. (1968) Response of free fatty acids to coffee and caffeine. Metabolism 17: 702-707.

Bellet, S., Roman, L., Decastro, O., Evin-Kim, K. & Kershbaum, A. (1969) Effect of coffee ingestion on catecholamine release. Metabolism 18: 288-291.

Bellville, J.W. (1964) Interaction of caffeine and morphine on respiration. J. Pharmacol. Exp. Ther. 143: 165-168.

Berger, R. & Sachan, D.S. (1992) Effects of supplementary levels of L-carnitine on blood and urinary carnitines and on the portal systemic blood ethanol concentrations in the rats. J. Nutr. Biochem. 2: 382-386.

Best, C.H., Hartroft, S.W., Lucas, C.C. & Ridout J.H. (1949) Liver damage produced by feeding alcohol or sugar and its prevention by choline. Br. Med. J. 2: 1001-1006.

Best, C.H. & Hershey, J.M. (1932) Further observations on the effects of some component of crude lecithin in depancreatized animals. J. Physiol. (London) 75: 49-55.

Best, C.H. & Huntsman, M.E. (1932) The effects of the components of lecithin upon the deposition of fat in the liver. J. Physiol. (London) 75:405-412.

Best, C.H. & Huntsman, M.E. (1935) Effect of choline on liver fat of rats in various states of nutrition. J. Physiol (London) 83:255- 274.

Best, C.H., Lucas, C.C., Ridout, J.H. & Patterson, J.M. (1950) Dose-response curves in the estimation of potency of lipotropic agents. J. Biol. Chem. 186: 317-329.

Besterman, J.M., Duronio, V. & Cuatrecasas, P. (1986) Rapid formation of diacylglycerol from phophatidylcholine: a pathway for generation of a second messenger. Proc. Natl. Acad Sci. USA 83:6785-6789.

Bettelheim, F.A. & March, J. (1991) Table 8.5 : pH values for common materials. In: Introduction to General, Organic & Biochemistry, 3rd ed., p.229. Holt, Rinehart and Winston, Orlando, FL

Bieber, L.L. (1988) Carnitine. Ann. Rev. Biochem. 57: 261-283.

Bieber, L.L. & Choi, Y.R. (1977) Isolation and identification of aliphatic short-chain acylcarnitines from beef heart: possible role of carnitine in branched-chain amino acid metabolism. Proc. Natl. Acad. Sci. USA. 74: 2796-2798.

Bieber, L.L., Emaus, R., Valkner, K. & Farrell, S. (1982) Possible functions of short-chain and medium-chain carnitine acyltransferases. Federation Proc. 41: 2858-2862.

Bieber, L.L, Krahling, J.B., Clarke, P.R.H., Valkner, K.J., Tolbert, N.E. (1981) Carnitine acyltransferases in rat liver peroxisomes. Arch. Biochem. Biophys. 211: 599-604.

Bierkamper, G.G. & Goldberg, A.M. (1980) Release of acetylcholine from the vascular perfused rat phrenic nerve-hemidiaphragm. Brain Res. 202:234-237.

Birkett, D.J. & Miners, J.O. (1991) Caffeine renal clearance and urine caffeine concentrations during steady state dosing. Implications for monitoring caffeine intake during sports events. Br. J. Clin. Pharmacol. 31: 405-408.

Björntorp, P. (1985) Regional patterns of fat distribution. Ann. Intern. Med. 103: 994-995.

Blanchard, J. & Sawers, S.J.A. (1983a) Relationship between urine flow-rate and renal clearance of caffeine in man. J. Clin. Pharmacol. 23: 134-138.

Blanchard, J. & Sawers, S.J.A. (1983b) Comparative pharmacokinetics of caffeine in young and elderly men. J. Pharmacokinet. Biopharm. 11: 109-126.

Blinks, J.R., Olson, C.B., Jewell, B.R. & Braveny, P. (1972) Influence of caffeine and other methylxanthines on mechanical properties of isolated mammalian heart muscle. Circ. Res. 30: 367-392.

Blusztajn, J.K., Liscovitch, M. & Richardson, U.I. (1987) Synthesis of acetylcholine from choline derived from phosphatidylcholine in a human neuronal cell line. Proc. Natl. Acad. Sci. U.S.A. 84: 5474-5477.

Blusztajn, J.K. & Wurtman, R.J. (1983) Choline and cholinergic neurons. Science 221: 614-620.

Blusztajn, J.K., Zeisel, S.H. & Wurtman, R.J. (1979) Synthesis of lecithin (phosphatidylcholine) from phosphatidylethanolamine in bovine brain. Brain Res. 179: 319-327.

Boekema, P.J., Samsom, M., van Berge Henegouwen, G.P. & Smout, A.J.P.M. (1999) Coffee and gastrointestinal function: Facts and Fiction. Scand. J. Gastroenterol. 34 (suppl 230): 35-39.

Boden, G., Chen, X., Mozzoli, M. & Ryan, I. (1996) Effect of fasting on serum leptin in normal human subjects. J. Clin. Endocrinol. Metab. 81: 3419-3423.

Bohmer, T. (1967) Tissue levels of activated fatty acids (acylcarnitines) and the regulation of fatty acid metabolism. Biochim. Biophys. Acta. 144: 259-270.

Bonati, M. & Garattini, S. (1984) Intespecies comparison of caffeine disposition. In: Caffeine; Perspectives from Recent Research (Dews, P.B., ed.), pp.48-56. Springer-Verlag, Berlin, Germany.

Bonati, M., Latini, R., Galletti, F., Young, J.F., Tognoni, G. & Garattini, S. (1982) Caffeine disposition after oral doses. Clin. Pharmacol. Ther. 32: 98-106.

Bond, V., Gresham, K., McRae, J. & Tearney, R.J. (1986) Caffeine ingestion and isokinetic strength. Br. J. Sport Med. 20: 135-137.

Bond, V., Adams, R., Balkisson, B., McRae, J., Knight, E, Robbins, S. & Banks, M. (1987) Effects of caffeine on cardiorespiratory function and glucose metabolism during rest and graded exercise. J. Sports Med. 27: 47-52.

Borum, P.R. (1991) Carnitine. In: Handbook of Vitamins, 2rd ed. (Machlin, L.J., ed), pp.557-563. Marcel Dekker, New York, NY.

Borum, P.R. (2000) Supplements: questions to ask to reduce confusion. Am. J. Cli. Nutr. 72 (suppl.): 538S-540S.

Bowyer, B.W., Fleming, C.R., Ilstrup, D., Nelson, J., Reek, S. & Burnes, J. (1986) Plasma carnitine levels in patients receiving home parenteral nutrition. Am. J. Clin. Nutr. 43: 85-91.

Bracco, D., Ferrarra, J.M., Arnaud, M.J., Jequier, E., Schutz, Y. (1995) Effects of caffeine on energy-metabolism, heart-rate, and methylxanthine metabolism in lean and obese women. Am. J. Physiol. 32: E671-E678.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254.

Brass, E.P. (1992) Carnitine transport. In: L-Carnitine and its Role in Medicine: From Function to Therapy (Ferrari, R. Di Mauro S. & Sherwood, G., eds.), pp.21-36. Academic Press, San Diego, CA.

Brass, E.P. (1994) Overview of coenzyme A metabolism and its role in cellular toxicity. Chemico-Biolog. Interact 90: 203-214.

Brass, E.P. (2000) Supplemental carnitine and exercise. Am. J. Clin. Nutr. 72 (suppl.): 618S-623S.

Brass, E.P., Adler, S., Sietsema, K.E., Hiatt, W.R., Orlando, A.M. & Amato, A. (2001) Intravenous L-carnitine increases plasma carnitine, reduces fatigue, and may preserve exercise capacity in hemodialysis patients. Am. J. Kidney Dis. 37: 1018-1028.

Brass, E.P. & Hiatt, W.R. (1994) Minireview: carnitine metabolism during exercise. Life Sci. 54: 1383-1393.

Brass, E.P. & Hiatt, W.R. (1998) The role of carnitine and carnitine supplementation during exercise in man and in individuals with special needs. J. Am. Coll. Nutr. 17: 207-215.

Brass, E.P. & Hoppel, C.L. (1978) Carnitine metabolism in the fasting rat. J. Biol. Chem. 253: 2688-2693.

Brass, E.P. & Hoppel, C.L. (1980) Relationship between acid-soluble carnitine and coenzyme-A pools in vivo. Biochem. J. 190: 495-504.

Brass, E.P., Hoppel, C.L. & Hiatt, W.R. (1980) Effect of intravenous L-carnitine on carnitine homeostasis and fuel metabolism during exercise in humans. Clin. Pharmacol. Ther. 55: 681-692.

Brass, E.P., Scarrow, A.M., Ruff, L.J., Masterson, K.A. & Van Lunteren, E. (1993) Carnitine delays rat skeletal muscle fatigue in vitro. J. Appl. physiol. 75: 1595-1600.

Bray, G.A. (1994) Commentary on classics in obesity. I. Quetelet: Quantitative medicine. Obes. Res. 2: 68-71.

Bray, G.A. (1998) What is the ideal body weight? J. Nutr. Biochem. 9: 489-492.

Bremer, J. (1961) Biosynthesis of carnitine in vivo. Biochim. Biophys. Acta. 48: 622-624.

Bremer, J. (1962) Carnitine in intermediary metabolism: The metabolism of fatty acid esters of carnitine by mitochondria. J. Biol. Chem. 237: 3628-3632.

Bremer, J. (1983) Carnitine: metabolism and functions. Physiol. Rev. 63: 1420-1480.

Bremer, J. (1997) The role of carnitine in cell metabolism. In: Carnitine Today. (De Simone, C. & Famularo, G., eds.), pp. 1-37. Landes Bioscience, Austin, TX.

Bremer, J. & Greenberg, D.M. (1961) Methyl transfering enzyme system of microsomes in the biosynthesis of lecithin (Phosphatidylcholine). Biochim. Biophys. Acta. 46: 205-216.

Bresolin, N., Freddo, L., Vergani L. & Angelini, C. (1982) Carnitine, carnitine acyltransferases, and rat brain function. Exp. Neurol. 78: 285-292.

Broderick, T.L., Quinney, H.A. & Lopaschuk, G.D. (1992) Carnitine stimulation of glucose oxidation in the fatty acid perfused isolated working rat heart. J. Biol. Chem. 267: 3758-3763.

Brooks, D.E. & McIntosh, J.E.A. (1975) Turnover of carnitine by rat tissues. Biochem. J. 148:439-445.

Brophy, P., Choy, P., Toone, J. & Vance, D. (1977) Choline kinase and ethanolamine kinase are separate soluble enzymes in rat liver. Eur. J. Biochem. 78: 491-496.

Broquist, H.P. (1994) Carnitine. In: Modern Nutrition in Health and Disease, 8th ed. (Shils, M.E., Olson, J.A. & Shike, M., eds.), pp.459-465. Lea & Febiger, Philadelphia, PA.

Brown, S.R., Cann, P.A. & Read, N.W. (1990) Effect of coffee on distal colon function. Gut 31:450-453.

Bubb, W.J. (1992) Relative leanness. In: Health Fitness. Instructor's Handbook, 2nd ed. (Howley, E.T. & Franks, B.D., eds.), pp.115-130. Human Kinetics Books, Champaign, IL.

Buchman, A.L., Dubin, M., Jenden, D., Moukarzel, A., Roch, M.H., Rice, K., Gornbein, J., Ament, M.E. & Eckhert C.D. (1992) Lecithin increases plasma free choline and decreases hepatic steatosis in long-term total parenteral nutrition patients. Gastroenterology 102: 1363-1370.

Buchman, A.L., Jenden, D. & Roch, M. (1999) Plasma free, phospholipid-bound and urinary free choline all decrease during a marathon run and may be associated with impaired performance. J. Am. Coll. Nutr. 18: 598-601.

Bukowiecki, L.J., Lupien, J., Follea, N. & Jahjah, L. (1983) Effects of sucrose, caffeine and cola beverages on obesity, cold resistance, and adipose tissue cellularity. Am. J. Physiol. 244: R500-R507.

Butcher, R.W. & Sutherland, E.W. (1962) Adenosine 3'5'-phosphate in biological materials. J. Biol. Chem. 237: 1244-1250.

Butts, N.K. & Crowell, D. (1985) Effect of caffeine ingestion on cardiorespiratory endurance in men and women. Res. Quarterly 56: 301-305.

Buyukuysal, R.L., Ulus, I.H., Aydin, S. & Kiran, B.K. (1995) 3,4-diaminepyridine and choline increase in vivo acetylcholine release in rat striatum. Eur. J. Pharmacol. 281: 179-185.

Cabot, M.C. & Jaken, S. (1984) Structural and chemical specificity of diradylglycerols for protein kinase C activation. Biochem. Biophys. Res. Commun. 125: 163-169.

Callahan, M.M., Robertson, R.S., Branfman, A.R., McComish, M.F. & Yesair, D.W. (1983) Comparison of caffeine metabolism in three nonsmoking populations after oral administration. Drug. Metab. Dispos. 10: 417-423.

Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R. & Burn, P. (1995) Recombinant mouse OB protein: Evidence for a peripheral signal linking adiposity and central neural networks. Science 269:546-549.

Cantrell, C.R. & Borum, P.R. (1982) Identification of a cardiac carnitine binding protein. J. Biol. Chem. 257: 10599-10604.

Canty, D.J. & Zeisel, S.H. (1994) Lecithin and choline in human health and disease. Nutr. Rev. 52: 327-339.

Carter, H.E., Bhattacharya, P.K., Weidman, K.R. & Fraenkel, G. (1952) Chemical studies on vitamin B_T. Isolation and characterization as carnitine. Arch. Biochem. Biophys. 38: 405-416.

Carter, A.L. & Frenkel, R. (1978) The relationship of choline and carnitine in the choline deficient rat. J. Nutr. 108: 1748-1754.

Carter, A.L. & Frenkel, R. (1979) The role of the kidney in the biosynthesis of carnitine in the rat. J. Biol. Chem. 254: 10670-10674.

Carter, A.L., Lennon, D.L.F. & Stratman, F.W. (1981) Increased acetylcarnitine in rat skeletal muscle as a result of high-intensity short-duration exercise. FEBS. Lett. 126: 21-24.

Carter, A.L. & Stratman, F.W. (1982) Sex steroid regulation of urinary excretion of carnitine in rats. J. Steroid Biochem. 17: 211-215.

Casal, D.C. & Leon, A.S. (1985) Failure of caffeine to affect substrate utilization during prolonged running. Med. Sci. Sports Exerc.17: 174-179.

Cederblad, G. (1987) Effect of diet on plasma carnitine levels and urinary carnitine excretion in humans. Am. J. Clin. Nutr. 45: 725-729.

Cederblad, G., Carlin, J.I., Constantin-Teodosiu, D., Harper, P. & Hultman, E. (1990) Radioisotopic assays of CoASH and carnitine and their acetylated forms in human skeletal muscle. Anal. Biochem. 185: 274-278.

Cederblad, G. & Lindstedt, S. (1971) Excretion of L-carnitine in man. Clin. Chim. Acta. 33: 117-123.

Cederblad, G. & Lindstedt, S. (1972) A method for the determination of carnitine in the picomole range. Clin. Chim. Acta. 37: 253-243.

Cedarblad, G. & Lindstedt, S. (1976) Metabolism of labeled carnitine in the rat. Arch. Biochem. Biophys. 175: 173-180.

Cermak, J.M., Holler, T., Jackson, D.A. & Blusztajn, J.K. (1998) Prenatal availability of choline modifies development of the hippocampal cholinergic system. FASEB J. 12: 349-357.

Chan, M.M. (1991) Choline. In: Handbook of Vitamins, 2rd ed. (Machlin, L.J., ed.), pp.537-556. Marcel Dekker, Inc., New York, NY.

Chapoy, P.R., Angelini, C., Brown, W.J., Stiff, J.E., Shug, A.L. & Cederbaum, S.D. (1980) Systemic carnitine deficiency - a treatable inherited lipid-storage disease presenting as Reye's syndrome. N. Eng. J. Med. 303: 1389-1394.

Chou, C-H. & Waller, G.R.(1980) Possible allelopathic constituents of coffea arabica. J. Chem. Ecol. 6: 643-654.

Christianen, R.Z. & Bremer, J. (1976) Active transport of butyrobetaine and carnitine into isolated liver cells. Biochim. Biophs. Acta. 448: 562-577.

Ciman, M., Rizzoli,V. & Siliprandi, N. (1980) Effect of physical training on carnitine concentration in liver, heart and gastrocnemius muscle of rat. Internat. J. Vit. Nutr. Res. 50: 40-43.

Clarkson, P.M. (1996) Nutrition for improved sports performance: current issues on ergogenic aids. Sports Med. 21: 393-401.

Colombani, P., Wenk, C., Kunz, I., Krahenbuhl, S., Kuhnt, M., Arnold, M., Frey-Rindova, P., Frey, W., Langhans, W. (1996) Effects of L-carnitine supplementaion on physical performance and energy metabolism of endurance-trained athletes: a double-blind crossover field study. Eur. J. Appl. Physiol. Occup. Physiol. 73: 434-439.

Coffey, M.T., Shireman, R.B., Herman, D.L. & Jones, E.E. (1991) Carnitine status and lipid utilization in neonatal piglets fed diets low in carnitine. J. Nutr.121: 1047-1053.

Cohen, S. (1980) Pathogenesis of coffee-induced gastrointestinal symptoms. N. Eng. J. Med. 303: 122-124.

Cohen, E.L. & Wurtman, R.J. (1975) Brain acetylcholine increase after systemic choline administration. Life Sci. 17: 1095-1102.

Collomp, K., Aneslme, F., Audran, M., Gay, J.P., Chanal, J.L. & Prefaut, C. (1996) Effects of moderate exercise on the pharmacokinetics of caffeine. Eur. J. Clin. Pharmacol. 40: 279-282.

Colombani, P., Went, C., Kunz, I., Krahenbuhl, S., Kuhnt, M. Arnold, M., Frey-Rindova, P., Frey, W. & Langhans, W. (1996) Effects of L-carnitine supplementation physical performance and energy metabolism of endurance-trained athletes: a double-blind crossover field study. Eur. J. Appl. Physiol. 73: 434-439.

Conlay, L.A., Sabounjian, L.A. & Wurtman, R.J. (1992) Exercise and neuromodulators: choline and acetylcholine in marathon runners. Int. J. Sports Med. 13: S141-S142.

Considine, R.V., Sinha, M.A., Heiman, M.L., Kriauciunas, A., Stephens, T.W., Nyce, M.R., Ohannesian, J.P., Marco, C.C., McKee, L.J., Bauer, T.L. & Caro, J.F. (1996) Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N. Engl. J. Med. 334: 292-295.

Cook, C.E., Tallent, C.R., Amerson, E.W., Myers, M.W., Kepler, J.A., Taylor, G.F. & Christensen, H.D. (1976) Caffeine in plasma and saliva by a radioimmunoassay procedure. J. Pharmacol. Exp. Ther. 199:679-686.

Copeland, D.H. & Salmon, W.D. (1946) The occurrence of neoplasms in the liver, lungs, and other tissues of rats as a result of prolonged choline deficiency. Am. J. Pathol. 22: 1059-1081.

Cornford, E.M., Braun, L.D. & Oldendorf, W.H. (1978) Carrier mediated blood-brain barrier transport of choline and certain choline analogs. J. Neurochem. 30: 299-308.

Corredor, C., Mansbach, C. & Bressler, R. (1967) Carnitine depletion in the choline-deficient state. Biochim. Biophys. Acta. 144: 366-374.

Costa, M., Canale, D., Filicori, M., D'lddio, S. & Lenzi, A. (1994) L-carnitine in idiopathic asthenozoospermia: a multicenter study. Andrologia, 26: 155-159.

Costell. M. & Grisolia, S. (1993) Effect of carnitine feeding on the levels of heart and skeletal muscle carnitine of elderly mice. FEBS., 315: 43-46.

Costill, D.L., Dalsky, G.P. & Fink, W.J. (1978) Effects of caffeine ingestion on metabolism and exercise performance. Med. Sci. Sports 10: 155-158.

Cox, R.A. & Hoppel, C.L. (1973) Biosynthesis of carnitine and 4-N-trimethylaminobutyrate from lysine. Biochem. J. 136: 1075-1082.

da Costa, K-A., Cochary, E.F., Blusztajn, J.K., Garner, S.C. & Zeisel, S.H. (1993) Accumulation of 1,2-*sn*-diacylglycerol with increased membrane-associated protein kinase C may be the mechanism for spontaneous hepatocarcinogenesis in choline deficient rats. J. Biol. Chem. 268: 2100-2105.

da Costa, K-A., Garner, S.C., Chang, J. & Zeisel, S.H. (1995) Effect of prolonged (1 year) choline deficiency and subsequent refeeding of choline on 1,2-*sn*-diacylglycerol, fatty acids and protein kinase C in rat liver. Carcinogenesis 16: 327-334.

Daily, J.W.III, Hongu, N., Mynatt, R.L. & Sachan, D.S. (1998) Choline supplementation increases tissue concentrations of carnitine and lowers body fat in guinea pigs. J. Nutr. Biochem. 9: 464-470.

Daily, J.W.III & Sachan, D.S. (1995) Choline supplementation alters carnitine homeostasis in humans and guinea pigs. J. Nutr. 125: 1938-1944.

Daniel, L.W., Small, G.W., Schmitt, J.D., Marasco, C.J., Ishaq, K. & Piantidosi, C. (1988) Alkyl-linked diglycerides inhibit protein kinase C activation of diacylglycerols. Biochem. Biophys. Res. Commun. 151: 291-297.

Davis, A.T. (1989) Fractional contributions to total carnitine in the neonatal rat. J. Nutr. 119: 262-267.

Davis, K.L., Berger, P.A. & Hollister, L.E. (1975) Choline for tardive dyskinesia. N. Eng. J. Med. 293:152-153.

Davis, K.L. & Berger, P.A. (1978) Pharmacological investigations of the cholinergic imbalance hypothesis of movement disorders and psychosis. Biol. Psychol. 13:23-49.

Dayanandan, A., Kumar, P. & Panneerselvam, C. (2001) Protective role of L-carnitine on liver and heart lipid peroxidation in atherosclerotic rats. J. Nutr. Biochem., 12: 254-257.

Decombaz, J., Gmuender, B., Sierro, G. & Cerretelli, P. (1992) Muscle carnitine after strenuous endurance exercise. J. Appl. Physiol. 72: 423-427.

Décombaz, J.E., Reffet, B. & Bloemhard, Y. (1987) L-carnitine supplementation, caffeine and fuel oxidation in the exercising rat. Nutr. Res. 7: 923-933.

De La Huerga, J. & Popper, H. (1951) Urinary excretion of choline metabolites following choline administration in normals and patients with hepatobiliary diseases. J. Clin. Invest. 30: 463-470.

Dempster, P. & Aitkens, S. (1995) A new air displacement method for the determination of human body composition. Med. Sci. Sports Exerc. 27: 1692-1697.

Denadai, B.S. (1994) Effect of caffeine on the metabolism of rats exercising by swimming. Braz. J. Med. Biol. Res. 27: 2481-2485.

Desmond, P.V., Patwardhan, R.V., Johnson, R.F. & Schenker, S. (1980) Impaired elimination of caffeine in cirrhosis. Digestive Dis. Sci. 25. 193-197.

Dews, P.B. (1984) Foreword. In: Caffeine: Perspectives from Recent Research (Dews, P.B., ed.), pp. v-vi., Springer-Verlag, Berlin, Germany.

Di Donato, S., Garavaglia, B., Rimoldi, M. & Carrara, F. (1992) Clinical and biomedical phenotypes of carnitine deficiencies. In: L-Carnitine and its Role in Medicine: From Function to Therapy (Ferrari, R. Di Mauro S. & Sherwood, G., eds), pp. 81-98. Academic Press, San Diego, CA.

Di Lisa, F., Bieber, L.L., Kerner, J., Menabò, R., Barbato, R. & Siliprandi, N. (1992) Methods for carnitine assay. In: L-Carnitine and its Role in Medicine: From Function to Therapy (Ferrari, R. Di Mauro S. & Sherwood, G., eds), pp. 37-62. Academic Press, San Diego, CA.

Dodd, S.L., Brooks, E., Powers, S.K. & Tulley, R. (1991) The effects of caffeine on graded-exercise performance in caffeine naive versus habituated subjects. Eur. J. Appl. Physiol. Occup. Physiol. 424-429.

Dodson, W.L. & Sachan, D.S. (1992) Supplementary choline and pantothenate reduce serum and urinary carnitine concentration in humans. In: Current Concepts in Carnitine Research (Carter, L.A., ed), pp.123. CRC Press, Boca Raton, FL.

Dodson, W.L. & Sachan, D.S. (1996) Choline supplementation reduces urinary carnitine excretion in humans. Am. J. Clin. Nutr. 63: 904-910.

Donelly, K. & McNaughton, L. (1992) The effects of two levels of caffeine ingestion on excess postexercise oxygen consumption in untrained women. Eur. J. Appl. Physiol. 65: 459-463.

Dorfman, L.J. & Jarvik, M.E. (1970) Comparative stimulant and diuretic actions of caffeine and theobromine in man. Clin. Pharmacol. Ther. 11: 869-872.

Douglas, B.R., Jansen, J.B., Tham, R.T. & Lamers C.B. (1990) Coffee stimulation of cholecystokinin release and gallbladder contraction in humans. Am. J. Clin. Nutr. 52:553-556.

Drågan, I. G., Vasiliu, A., Georgescu, E. & Eremia, N. (1989) Studies concerning chronic and acute effects of L-Carnitina in elite athletes. Physiol. 26:111-129.

Dubelaar M-L., Lucas, C.M.H.B. & Hulsmann, W.C. (1991) Acute effect of L-carnitine on skeletal muscle force tests in dogs. Am. J. Physiol. 260: E189-E193.

Dubuc, G.R., Phinney, S.D., Stern, J.S., & Havel, P.J. (1998) Changes of serum leptin and endocrine and metabolic parameters after 7 days of energy restriction in men and women. Metabolism. 47: 429-434.

Dudeja, V., Misra, A., Pandey, R.M., Devina, G., Kumar, G. & Vikram, N.K. (2001) BMI does not accurately predict overweight in Asian Indians in northern India. Brit. J. Nutr. 86: 105-112.

Dulloo, A.G., Geissler, C.A., Horton, T., Collins, A. & Miller, D.S. (1989) Normal caffeine consumption: Influence on thermogenesis and daily energy expenditure in lean and postobese human volunteers. Am. J. Clin. Nutr. 49: 44-50.

Dulloo, A.G. & Miller, D.S. (1986) The thermogenic properties of ephedrine/methylxanthine mixtures: human studies. Int. J. Obesity 10: 467-481.

Dunn, W.A. & Englard, S. (1981) Carnitine biosynthesis by the perfused rat liver from exogenous protein-bound trimethyllysine. Metabolism of methylated lysine derivatives arising from the degradation of 6-N-[methyl-^{3}H]lysine-labeled glycoprotein. J. Biol. Chem. 256: 12437-12444.

Egger, G., Cameron-Smith, D. & Stanton, R. (1999) The effectiveness of popular, non-prescription weight loss supplements. Med. J. Aust. 171: 604-608.

Ellefson, R.D. & Caraway, W.T. (1976) Lipids and lipoproteins. In: Fundamentals of Clinical Chemistry. (Tietz, N.W., ed.), pp. 474-541. W.B. Saunder Co., Philadelphia, PA.

Endo, M. (1977) Calcium release from sarcoplasmic-reticulum. Physiol. Rev. 57: 71-108.

Engel, R.W. (1943) The choline content of animal and plant products. J.Nutr. 25:441-446.

Engel, A.G., Rebouche, C.J., Wilson, D.M., Glasgow, A.M., Romshe, C.A. & Cruse, R.P. (1981) Primary systemic carnitine deficiency. II. Renal handling of carnitine. Neurology 31: 819-825.

Essig, D., Costill, D.L. & van Handel, P.J. (1980) Effects of caffeine ingestion on utilization of muscle glycogen and lipid during leg ergometer cycling. Int. J. Sports Med. 1: 86-90.

Exton, J.H. (1990) Signaling through phosphatidylcholine breakdown. J. Biol. Chem. 265:1-4.

Fabiato, A. & Fabiato, F. (1977) Calcium release from sarcoplasmic reticulum. Circ. Res. 40: 119-123.

Fallon, H.J., Gertman, P.M. & Kemp, E.L. (1969) The effects of ethanol ingestion and choline deficiency on hepatic lecithin biosynthesis in the trat. Biochim. Biophys. Acta. 187: 94-104.

Fernstrom, J.D. & Fernstrom M.H. (1984) Effects of caffeine on monoamine neurotransmitters in the central and peripheral nervous system. In: Caffeine: Perspectives from Recent Research (Dews, P.B., ed.), pp.107-118, Springer-Verlag, Berlin, Germany.

Fix, J.A., Engle, K., Porter, P.A. & Alexander, J. (1986) Acylcarnitine: drug absorption-enhancing agents in the gastrointestinal tract. Am. J. Physiol. 251: G332-G340.

Flinn, S., Gregory, J, McNaughton, L.R., Tristram, S. Davies, P. (1990) Caffeine ingestion prior to incremental cycling to exhaustion in recreational cyclists. Int. J. Sports. Med. 11: 188-193.

Flower, R.J. Pollitt, R.J., Sanford, P.A. & Smyth, D.H. (1972) Metabolism and transfer of choline in hamster small-intestine. J. Physiol. (London) 226: 473-89.

Fogle, P.J. & Bieber, L.L. (1979) Effect of streptozotocin on carnitine and carnitine acyltransferases in rat heart, liver, and kidney. Biochem. Med. 22: 119-126.

Food and Drug Administration (1980) Caffeine content of various products. FDA, Washington (FDA Talk paper, T80-45).

Food and Nutrition Board. Institute of Medicine. National Academy of Sciences, Subcommittee on Folate, Other B Vitamins, and Choline. (1998) Dietary Reference Intakes: Thiamin, Riboflavin, Niacin, Vitamin B-6, Folate, Vitamin B-12, Pantothenic Acid, Biotin and Choline. National Academy Press, Washington, DC.

Forloni, G., Angeretti, N. & Smiroldo, S. (1994) Neuroprotective activity of acetyl-L-carnitine: studies in vitro. J. Neurosci. Res. 37: 92-96.

Frederich, R. C., Hamann, A., Anderson, S., Löllmann, B., Lowell, B.B. & Flier, J.S. (1995) Leptin levels reflect body lipid content in mice: Evidence for diet-induced resistance to leptin action. Nature Med. 1: 1311-1314.

Fredholm, B.B. (1984) Cardiovascular and renal actions of methylxanthines. In: The Methylxanthine Beverages and Foods: Chemistry, Consumption, and Health Effects (Spiller, G.A., ed.), pp. 185-214, Alan R. Liss, New York, NY.

Fredholm, B.B. (1985) On the mechanism of action of theophylline and caffeine. Acta. Medica. Scandinavica. 217: 149-153.

Freeman, J.J. & Jenden, D.J. (1976) Minireview: The source of choline for acetylcholine synthesis in brain. Life Sci. 19:949-961.

Friedewald, W.T., Levy, R.I. & Fredrickson, D.S. (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin. Chem. 18: 499-502.

Friedman, S. & Fraenkel, G. (1955) Reversible enzymatic acetylation of carnitine. Arch. Biochem. Biophys. 59: 491-501.

Friolet, R., Hoppeler, H. & Krähenbühl, S. (1994) Relationship between Coenzyme A and the carnitine pools in human skeletal muscle at rest and after exhaustive exercise under normoxic and acutely hypoxic conditions. J. Clin. Invest. 94: 1490-1495.

Fritz, I.B. (1955) The effects of muscle extracts on the oxidation on palmitic acid by liver slices and homogenates. Acta. Physiol. Scand. 34: 367-385.

Fritz, I.B. & Yue, K.T.N. (1963) Long-chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine. J. Lipid Res. 4: 279-288.

Frolich, J.D., Seccombe, D.W., Hahn, P., Dodek, P. & Hynie. (1978) Effect of fasting on free and esterified carnitine levels in human serum and urine: correlation with serum levels of free fatty acids and β-hydroxybutyrate. Metabolism 27: 555-561.

Flores, C.A. & Hu, C., Edmond, J. & Koldovsky, O. (1996) Milk carnitine affects organ carnitine concentration in newborn rats. J. Nutr. 126: 1673-1682.

Fujino, Y. (1952) Studies on the conjugated lipids IV: On the enzymatic hydrolysis of sphingomyelin. J. Biochem. Tokyo 39:55-62.

Ghoshal, A.K. & Farber, E. (1984) The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. Carcinogenesis 5: 1367-1370.

Ghoshal, A.K. & Farber, E. (1993) Choline deficiency, lipotrope deficiency and the development of liver disease including liver cancer: a new perspective. Lab. Invest. 68: 255-260.

Giegel, J.L., Ham A.B. & Clema, W. (1975) Manual and semiautomatic procedure for measurement of triglycerides in serum. Clin. Chim. Acta 21: 1575-1581.

Gilbert, R.M. (1976) Caffeine as a drug of abuse. In: Research Advances in Alcohol and Drug Problems (Gibbins, R.J., Israel, Y., Kalant, H., Pophan, R.E., Schmidt, W. & Smart, R.G., eds.), vol.3, pp. 49-176, Wiley & Sons, New York, NY.

Gilbert, R.M. (1984) Caffeine consumption. In: The Methylxanthine Beverages and Foods: Chemistry, Consumption, and Health Effects (Spiller, G.A., ed.), pp. 185-214, Alan R. Liss, New York, NY.

Gillies, M.E. & Birkbeck, J.A. (1983) Tea and coffee as sources of some minerals in the New-Zealand diet. Am. J. Clin. Nutr. 38: 936-942.

Goasduff, T., Dreano, Y., Guillois, B., Ménez, J-F. & Berthou, F. (1996) Induction of liver and kidney CYP1A1/1A2 by caffeine in rat. Biochem Pharmacol. 52: 1915-1919.

Gorostiaga, E.M., Maurer, C.A. & Eclache, J.P. (1989) Decrease in respiratory quotient during exercise following L-carnitine supplementation. Int. J. Sports Med. 10: 169-174.

Graham, T.E. (2001) Caffeine and exercise: Metabolism, endurance and performance. Sports Med. 31: 785-807.

Graham, T.E., Rush, J.W. & van Soeren, M.H. (1994) Caffeine and exercise: metabolism and performance. Can. J. Appl. Phys. 19:111-138.

Graham, T.E. & Spriet, L.L. (1991) Performance and metabolic responses to a high caffeine dose during prolonged exercise. J. Appl. Physiol. 71:2292-2298.

Graham, T.E. & Spriet, L.L. (1995) Metabolic, catecholamine, and exercise performance responses to various doses of caffeine. J. Appl. Physiol. 78:867-874.

Green, H.J. (1991) How important is endogenous muscle glycogen to fatigue in prolonged exercise? Can. J. Physiol. Pharmacol. 69: 290-297.

Greig, G., Finch, K.M., Jones, D.A., Cooper, M., Sargeant, A.J. & Forte, C.A. (1987) The effects of oral supplementation with L-carnitine on maximum and submaximum exercise capacity. Eur. J. Appl. Physiol. 56: 457-460.

Greten, H., Ractzer, H. Stiehl, A. & Schettler, G. (1980) The effect of polyunsaturated phosphatidylcholine on plasma lipids and fecal sterol excretion. Atherosclerosis 36:81-88.

Gross, C.J. & Henderson, L.M.(1984) Absorption of D- and L-carnitine by the intestine and kidney tubule in the rat. Biochim. Biophys. Acta. 772: 209-219.

Grossman, E.B. & Hebert, S.C. (1989) Renal inner medullary choline dehydrogenase activity: characterization and modulation. Am. J. Physiol. 256: F107-F112.

Growdon, J.H., Hirsch, M.J., Wurtman, R.J. & Wiener, W. (1977) Oral choline administration to patients with tardive dyskinesia. N. Eng. J. Med. 197:524-527.

Grundy, S.M. (1980) Studies on plasma lipoproteins during absorption of exogenous lecithin in man. J. Lipid Res. 21: 525-536.

Guder, W.G. & Wagner, S. (1990) The role of the kidney in carnitine metabolism. J. Clin. Chem. Clin. Biochem. 28: 347-350.

Gudjonsson, H., Li, B.U.K., Shug, A.L. & Olsen, W.A. (1985) Studies of carnitine metabolism in relation to intestinal absorption. Am. J. Physiol. 248: G313-G319.

Gulewitsch, W. & Krimberg, R. (1905) Zur Kenntnis der Extraktivstoffe der Muskeln. 2. Mitteilung über das Carnitine. Hoppe-Seyler's Z Physiol Chem. 45: 326-330.

Gutmann, I. & Wahlefeld, A.W. (1974) L-(+)-lactate determination with lactate dehydrogenase and NAD. In: Methods of Enzymatic Analysis, 2nd ed. (Bergmeyer, H.U.,ed.), pp. 1464-1468. Academic Press, New York, NY.

Hahn, P., Allardyce, D.B. & Frolich, J. (1982) Plasma carnitine levels during total parenteral nutrition of adult surgical patients. Am. J. Clin. Nutr. 36: 569-572.

Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L, Burley, S.K. & Friedman, J.M. (1995) Weight-reducing effects of the plasma protein encoded by the *obese* gene. Science 269: 543-546.

Haldi, J., Bachman, G., Ensor, C. & Wynn, W. (1941) The effect of various amounts of caffeine on the gaseous exchange and the respiratory quotient in man. J. Nutr. 21: 307-320.

Hamilton, J.W., Li, B.U.K., Shug, A.L. & Olsen, W.A. (1986) Carnitine transport in human intestinal biopsy specimens. Demonstration of an active transport system. Gastroenterology 91: 10-16.

Hanada, K., Nishijima, M., Kiso, M., Hasegawa, A., Fujita, S., Ogawa, T. & Akamatsu, Y. (1992) Sphingolipids are essential for the growth of Chinese hamster ovary cells. Restoration of the growth of a mutant defective in sphingoid base biosynthesis by exogenous sphingolipids. J. Biol. Chem. 267:23527-23533.

Hannun, Y.A & Bell, R.M. (1989) Regulation of protein kinase C by sphingosine and lysosphingolipids. Clin. Chim. Acta. 185: 333-345.

Hannun, Y.A., Merrill, A.H. & Bell, R.M. (1991) Use of sphingomyelin as an inhibitor of protein kinase C. Methods Enzymol. 201:316-328.

Harland, B.F. (2000) Caffeine and nutrition. Nutr. 16: 522-526.

Harper, P., Elwin, C-E., & Cederblad, G. (1988) Pharmacokinetics of intravenous and oral bolus doses of L-carnitine in healthy subjects. Eur. J. Clin. Pharmacol. 35: 555-562.

Harris, J.A. & Benedict, F.G. (1919) A biometric study of basal metabolism in man. Publication No. 279. Carnegie Institution of Washington. Washington, D.C.

Harris, R.C., Foster, C.V.L .& Hultman, E. (1987) Acetylcarnitine formation during intense muscular contraction in humans. J. Appl. Physiol. 63: 440-442.

Haubrich, D.R., Wang, P.F.L. & Wedeking, P.W. (1975) Distribution and metabolism of intravenouly administered choline[methyl]-3-H] and synthesis in vivo of acetylcholine in various tissues of guinea pigs. J. Pharmacol. Exper. Ther. 193: 246-255.

Haubrich, D.R. & Pflueger, A.B. (1979) Choline administration: Central effect mediated by stimulation of acetylcholine synthesis. Life Sci. 24: 1083-1090.

Haubrich, D.R., Wedeking, P.W. & Wang, P.F.L. (1974) Increase in tissue concentration of acetylcholine in guinea pigs in vivo induced by administration of choline. Life Sci. 14: 921-929.

Hawley, J.A., Brouns, F. & Jeukendrup, A. (1998) Strategies to enhance fat utilisation during exercise. Sports Med. 25: 241-257.

Havel, P.J. (1998) Leptin production and action: relevance to energy balance in humans. Am. J. Clin. Nutr. 67: 355-356.

Health Communications Australia Pty Limited (2000) The Asia-Pacific perspective: redefining obesity and its treatment. http: www.idi.org.au/obesity_report.htm.

Heinonen, O.J. & Takala, J. (1994) Moderate carnitine depletion and long-chain fatty acid oxidation, exercise capacity, and nitrogen balance in the rat. Pediatr. Res. 36: 288-292.

Heitmann, B.L. (1990a) Evaluation of body fat estimated from body mass index, skinfolds and impedance. A comparative study. Eur. J. Clin. Nutr. 44: 831-837.

Heitmann, B.L. (1990b) Prediction of body water and fat in adult Danes from measurement of electrical impedance. A validation study. Int. J. Obes. 14: 789-802.

Heller, M. & Shapiro, B. (1966) Enzymatic hydrolysis of spingomyelin by rat liver. Biochem. J. 98: 763-769.

Henry, R.J. (1967) Determination of creatine and creatinine. In: Clinical Chemistry: Principles and Techniques. (Henry, R.J. ed.) pp. 292-296. Harper and Row, New York, NY.

Herrmann, G.R. (1947) Some experimental studies in hypercholesterolemic states. Exp. Med. Surg. 5:149-159.

Hershey, J.M. (1930) Substitution of "lecithin" for raw pancreas in the diet of the depancreatized dog. Am. J. Physiol. 93: 657-658.

Heymsfield, S.B., Tighe, A. & Wang, Z-M (1999) Nutritional assessment by anthropometric and biochemical methods. In: Modern Nutrition in Health and Disease, 8th ed. (Shils, M.E., Olson, J.A. & Shike, M., eds.), vol. 1, pp. 812-841. Lea & Febiger, Philadelphia, PA.

Hiatt, W.R., Regensteiner, J.G., Wolfel, E.E., Ruff, L. & Brass, E.P. (1989) Carnitine and acylcarnitine metabolism during exercise in humans; dependence on skeletal muscle metabolic state. J. Clin. Invest. 84: 1167-1173.

Hickey, M.S., Houmard, J.A., Considine, R.V., Tyndall, G.L., Midgette, J.B., Gavigan, K.E., Weidner, M.L., McCammon, M.R., Israel, R.G. & Caro, J.F. (1997) Gender-dependent effects of exercise training on serum leptin levels in humans. Am. J. Physiol. 272: E562-E566.

Hirsche, M.J., Growdon, J.H. & Wurtman, R.J. (1978) Relations between dietary choline or lecithin intake, serum choline levels and various metabolic indices. Metabolism 27: 953-959.

Hoeger, W.W.K., Harris, C., Long, E.M. & Hopkins, D.R. (1998) Four-week supplementation with a natural dietary compound produced favorable changes in body composition. Adv. Therapy 15: 305-314.

Hokland, B.M. & Bremer, J. (1986) Metabolism and excretion of carnitine and acylcarnitines in the perfused rat kidney. Biochim. Biophys. Acta. 886: 223-230.

Hollands, M.A., Arch, J.R.S., & Cawthorne, M.A. (1981) A simple apparatus for comparative measurements of energy expenditure in human subjects: The thermic effect of caffeine. Am. J. Clin. Nutr. 34: 2291-2294.

Hollister, L.E., Jenden, D.J. Amaral, J.R.D., Barchas, J.D., Davis, K. L. & Berger, P.A. (1978) Plasma concentrations of choline in man following choline chloride. Life Sci. 23:17-22.

Holmes-McNary, M.Q., Cheng, W-L., Mar, M-H, Fussell, S. & Zeisel, S.H. (1996) Choline and choline esters in human and rat milk and in infant formulas. Am. J. Clin. Nutr. 64: 572-576.

Hongu, N. & Sachan, D.S. (2000) Caffeine, carnitine and choline supplementation of rats decreases body fat and serum leptin concentration as does exercise. J. Nutr. 130: 152-157.

Hongu, N. & Sachan, D.S. (2002) Tissue carnitine accretion and fat metabolism in rats supplemented with carnitine, choline and caffeine regardless of exercise. J. Med. Sci. 2 (in press).

Hoppel, C. (1992) The physiological role of carnitine. In: L-Carnitine and its Role in Medicine: From Function to Therapy (Ferrari, R. Di Mauro S. & Sherwood, G., eds), pp. 5-19. Academic Press, San Diego, CA.

Horne, D.W., Cook, R.J. & Wagner, C. (1989) Effect of dietary methyl group deficiency on folate metabolism in rats. J. Nutr. 119: 618-621.

Horne, D.W., Tanphaichitr, V. & Broquist, H.P. (1971) Role of lysine in carnitine biosynthesis in *Neurospora Crassa.* J. Biol. Chem. 246: 4373-4375.

Hortobagyi, T., Israel, R.G. & O'Brien, K.F. (1994) Sensitivity and specificity of the Quetelet index to assess obesity in men and women. Eur. J. Clin. Nutr. 48: 769-775.

Horton, T.J. & Geissler, C. (1996) Post-prandial thermogenesis with ephedrine, caffeine and aspirin in lean, pre-disposed obese and obese women. Int. J. Obesity. 20: 91-97.

Houtsmuller, U.M.T. (1979) Metabolic fate of dietary lecithin. In: Nutrition and the Brain, Vol. 5 (Barbeau, A., Growdon, J.H. & Wurtman, R.J., eds), pp. 83-94. Raven Press, New York, NY.

Hulse, J.D., Ellis, S.R. & Henderson, L.M. (1978) Carnitine biosynthesis . β-hydroxylation of trimethyllysine by an α-ketoglutarate-dependent mitochondrial dioxygenase. J. Biol. Che. 253: 1654-1659.

Hülsmann, W.C., Siliprandi, D., Ciman, M. & Siliprandi, N. (1964) Effect of carnitine on the oxidation of α-oxoglutarate to succinate in the presence of acetoacetate or pyruvate. Biochim. Biophys. Acta. 93: 166-168.

Huth, P.J. & Shug, A.L. (1980) Properties of carnitine transport in rat kidney cortex. Biochim Biophys. Acta. 602: 621-634.

Illingsworth, D.R. & Portman, O.W. (1973) Formation of choline from phospholipid precursors: A comparison of the enzymes involved in phospholipid catabolism in the brain of the rhesus monkey. Physiol. Chem. Phys. 5:365-373.

Itoh, T., Ito, T., Ohba, S., Sugiyama, N., Mizuguchi, K., Yamaguchi, S. & Kidouchi, K. (1996) Effect of carnitine administration on glycine metabolism in patients with isovaleric acidemia: significance of acetylcarnitine determination to estimate the proper carnitine dose. Tohoku J. Exp. Med. 179: 101-109.

Ivy, J.L., Costill, D.L., Fink, W.J. & Lower, R.W. (1979) Influence of caffeine and carbohydrate feedings on endurance performance. Med. Sci. Sports 11: 6-11.

Jackson, A.S. & Pollock, M.L. (1985) Practical assessment of body composition. Physician Sportsmed. 13: 76-90.

Jackson, J.M. & Lee, H.A. (1996) L-carnitine and acetyl-L-carnitine status during hemodialysis with acetate in humans: a kinetic analysis. Am. J. Clin. Nutr. 64: 922-927.

Jacobson, B.H. & Edwards, S.W. (1991) Influence of 2 levels of caffeine on maximal torque at selected angular velocities. J. Sports. Med. Phys. Fitness 31: 147-153.

Jacobson, B.H. & Kulling, F.A. (1989) Health and ergogenic effects of caffeine. Br. J. Sports Med. 23: 34-40.

James, J.E. (1991) Historical overview and current use. In: Caffeine and Health, pp. 3-18. Academic Press, San Diego, CA.

Jamil, H., Hatch, G.M. & Vance D.E. (1993) Evidence that binding of CTP-Phosphocholine cytidylyltransferease to membranes in rat hepatocytes is modulated by the ratio of bilayer-forming to non-bilayer-forming lipids. Biochem. J. 291: 419-427.

Jansson, E. (1982) On the significance of the respiratory exchange ratio after different diets during exercise in man. Acta Physiol. Scand. 114: 103-110.

Jones, J.P. III, Meck, W.H., Williams, C.L., Wilson, W.A. & Swartzwelder, H.S. (1999) Choline availability to the developing rat fetus alters adult hippocampal long-term potentiation. Dev. Brain Res. 118: 159-167.

Jung, R.T., Shetty, P.S., James, W.P.T., Barrand, M.A. & Callingham, B.A. (1981) Caffeine: Its effect on catecholamines and metabolism in lean and obese humans. Clin. Sci. 60: 527-535.

Jus, A, Pineau, R., Lachance, R., Pelchat, G., Jus, K., Pires, P., Villeneuve, R. (1976) Epidemiology of tardive dyskinesia. Part 2. Dis. Nerv. Syst. 37:257-261.

Kalow, W. & Tang, B. (1993) The use of caffeine for enzymatic assays: A critical appraisal. Clin. Pharmacol. Ther. 53: 503-514.

Kanter, M.M. & Williams, M.H. (1995) Antioxidants, carnitine, and choline as putative exgogenic aids. Int. J. Sport Nutr. 5: S120-S131.

Kaplan, E., Holmes, J.H. & Sapeika, N. (1974) Caffeine content of tea and coffee. S. Afr. J. Nutr. 10: 32-33.

Kaplan, R.J., Daman, L., Rosenberg, E.W. & Feigenbaum, S. (1978) Topical use of caffeine with hydrocortisone in the treatment of atopic dermatitis. Arch. Dermatol. 114: 60-62.

Kavaler, F., Anderson, T.W. & Fisher, V.J. (1978) Sarcolemmal site of caffeine's inotropic action on ventricular muscle of the fog. Circ. Res. 42: 285-290.

Kennedy, E.P. & Weiss, S.B. (1956) The function of cytidine coenzymes in the biosynthesis of phospholipids. J.Biol.Chem. 222: 193-214.

Kesaniemi, Y.A. & Grundy, S.M. (1986) Effects of dietary polyenylphosphatidylcholine on metabolism of cholesterol and triglycerides in hypertriglyceridemic patients. Am. J. Clin. Nutr. 43: 98-107.

Kesten, H.D. & Silbowitz, R. (1942) Experimental atherosclerosis and soya lecithin. Pro. Soc. Exp. Biol. Med. 49: 71-73.

Kispal, G., Melegh, B., Alkonyi, I. & Sandor, A. (1987) Enhanced uptake of carnitine by perfused liver following starvation. Biochim. Biophys. Acta. 896: 96-102.

Kissebah, A.H. & Krakower, G.W. (1994) Regional adiposity and morbidity. Physiol. Rev. 74: 761-811.

Kissebah, A.H., Vydelingum, N., Murray, R., Evans, D.J., Hartz, A.J., Kalkhoff, R.K. & Adams, P.W. (1982) Relation of body fat distribution to metabolic complications of obesity. J. Clin. Endocrinol. Metab. 54: 254-260.

Kohrt, W.M. (1998) Preliminary evidence that DEXA provides an accurate assessment of body composition. J. Appl. Physiol. 84: 372-377.

Koizumi, T., Nikaido, H., Hayakawa, J., Nonomura, A. & Yoneda, T. (1988) Infantile disease with microvesicular fatty infiltration of viscera spontaneously occurring in the C3H-H-2° strain of mouse with similarities to Reye's syndrome. Lab Anim. 22: 83-87.

Kossel, A. (1888) Über eine neue Base aus dem Pflanzenreich. Ber. Dtsch. Che. Ges. 21: 2164-2167.

Kovacs, E.M.S., Stegen, J.H.C.H. & Brouns, F. (1998) Effects of caffeinated drinks on substrate metabolism, caffeine excretion, and performance. J. Appl. Physiol. 85: 709-715.

Kruk, B. Pekkarinen, H. & Hanninen, O. (1999) Effect of caffeine during repeated explosive vertical jumping. Biol. Sports 16: 217-224.

Kuczmarski, R.J. (1996) Bioelectric impedance analysis measurements as part of a national nutrition survey. Am. J. Clin. Nutr. 64 (suppl.): 453S-458S.

Kuwajima, M., Kono, N., Horiuchi, M., Imamura, Y., Ono, A., Inui, Y., Kawata, S., Koizumi, T., Hayakawa, J., Saheki, T. & Tarui, S. (1991) Animal model of systemic carnitine deficiency: analysis in C3H-H-2° strain of mouse associated with juvenile visceral steatosis. Biochem. Biophys. Res. Commun. 174: 1090-1094.

Lamarine, R.J. (1998) Caffeine as an ergogenic aid. In: Caffeine (Spiller, G.A., ed.), pp. 233-250. CRC press, Boca Raton, Florida.

Landt, M., Lawson, G.M., Helgeson, J.M., Davila-Roman, V.G., Ladenson, J.H., Jaffe, A.S. & Hickner, R.C. (1997) Prolonged exercise decreases serum leptin concentrations. Metabolism 46: 1109-1112.

Lean, M.E., Han, T.S. & Morrison, C.E. (1995) Waist circumference as a measure for indicating need for weight management. Brit. Med. J. 311: 158-161.

Lehniger, A.L., Nelson, D.L. & Cox M.M. (1993) Oxidation of fatty acids. In: Principles of Biochemistry, 2nd ed. pp. 479-505. Worth Publishers, New York, NY.

Leiber, C.S., Robins, S.J., Li, J.J, De Carli, L. M., Mak, K.M., Fasulo, J.M. & Leo, M.A. (1994) Phosphatidylcholine protects against fibrosis and cirrhosis in the baboon. Gastroenterology 106: 152-159.

Lemieux, S., Prud'homme, D., Bouchard, C., Tremblay, A. & Després, J.P. (1996) A single threshold value of waist girth identifies normal-weight and overweight subjects with excess visceral adipose tissue. Am. J. Clin. Nutr. 64: 685-693.

Lennon, D.L.F. & Mance, M.J. (1986) Interorgan cooperativity in carnitine metabolism in the trained state. J. Appl. Physiol. 60: 1659-1664.

Lennon, D.L.F., Stratman, F.W., Shrago, E., Nagle, F.J., Madden, M., Hanson, P. & Carter, A.L. (1983) Effects of acute moderate-intensity exercise on carnitine metabolism in men and women. J. Appl. Physiol. 55:489-495.

Lima, D.R., Andrade, G.D.N., Santos, R.M. & David, C.N. (1989) Cigarettes and caffeine. Chest 95: 255-256.

Lindstedt, G. & Lindstedt, S. (1961) On the biosynthesis and degradation of carnitine. Biochem. Biophs. Res. Commun. 6: 319-323.

Locker, J., Reddy, T.V. & Lombardi, B. (1986) DNA methylation and hepatocarcinogenesis in rats fed a choline-devoid diet. Carcinogenesis 7: 1309-1312.

Lombard, K.A., Olson, A.L., Nelson, S.E. & Rebouche, C.J. (1989) Carnitine status of lactoovovegetarians and strict vegetarian adults and children. Am. J. Clin. Nutr. 50: 301-306.

Lopes, J.M., Aubier, M., Jardim, J., Aranda, J.V. & Macklem, P.T. (1983) Effect of caffeine on skeletal muscle function before and after fatigue. J. Appl. Physiol. 54: 1303-1305.

Lovallo, W.R., Al'Absi, M., Blick, K., Whitsett, T.L. & Wilson, M.F. (1996) Stress-like adrenocorticotropin responses to caffeine in young healthy men. Pharmacol. Biochem Behav. 55: 363-369.

Lundsberg, L.S. (1998) Caffeine consumption. In: Caffeine (Spiller, G.A., ed.), pp.199-224. CRC press, Boca Raton, Florida.

Maebashi, M., Kawamura, N., Sato, M., Yoshinaga, K. & Suzuki, M. (1976) Urinary excretion of carnitine in man. J. Lab. Clin. Med. 87: 760-766.

Marconi, C., Sassi, G., Carpinelli, A. & Ceretelli, P. (1985) Effects of L-carnitine loading on the aerobic and anaerobic performance of endurance athletes. Eur. J. Appl. Physiol. 54:131-135.

Marks, V. & Kelly, J.F. (1973) Absorption of caffeine from tea, coffee, and coca cola. Lancet 1: 827.

Martin, R.J., Miller, M.J. & Carlo, W.A. (1986) Pathogenesis of apnea in preterm infants. J. Pediatr. 109: 733-741.

Mastellone, I., Polichette, E., Gres, S., de la Maisonneuve, C., Domingo, N., Marin, V., Loree, A-M., Famarier, C., Portugal, H., Kaplanski, G. & Chanussot, F. (2000) Dietary soybean phosphatidylcholines lower lipidemia: Mechanisms at the levels of intestine, endothelial cell, and hepato-biliary axis. J. Nutr. Biochem. 11:461-466.

Mathew, R.J., Barr, D.L. & Weinman, M.L. (1983) Caffeine and cerebral blood flow. Br. J. Psychi. 143: 604-608.

Mathew, R.J. & Wilson, W.H. (1985) Caffeine consumption, withdrawal and cerebral blood flow. Headache 25: 305-309.

Mazess, R.B., Barden, H.S., Bisek, J.P. & Hanson, J. (1990) Dual-energy x-ray absorptiometry for total-body and regional bone- mineral and soft-tissue composition. Am. J. Clin. Nutr. 51: 1106-1112.

McArdle, W., Katch, F.I. & Katch, V.I. (1986) Energy transfer in exercise. In: Exercise Physiology: Energy, Nutrition, and Human Performance, 2nd ed. pp 127-144. Lea & Febiger, Philadelphia, PA

McCall, A.L., Millington, W.R. & Wurtman, R.J. (1982) Blood-brain barrier transport of caffeine: Dose-related restriction of adeninetransport. Life Sci. 31: 2709-2715.

McCrory, M.A., Gomez, T.D., Bernauer, E.M. & Molé, P.A. (1995) Evaluation of a new air displacement plethysmograph for measuring human body composition. Med. Sci. Sports Exerc. 27: 1686-1691.

McGarry, J.D. & Foster, D.W. (1979) In support of the roles of malonyl-CoA and carnitine acyltransferase I in the regulation of hepatic fatty acid oxidation and ketogenesis. J. Biol. Chem. 254: 8163-8168.

McGarry, J.D., Robles-Valdes, C. & Foster, D.W. (1975) The role of carnitine in hepatic ketogenesis. Proc. Natl. Acad. Sci. USA 72: 4385-4388.

McNaughton, L. (1987) Two levels of caffeine ingestion on blood lactate and free fatty acid response during incremental exercise. Res. Quarterly Exerc. Sport 58: 255-259.

Meck, W.H., Smith, R.A. & Williams, C.L. (1989) Organizational changes in cholinergic activity and enhanced visuospatial memory as a function of choline administered prenatally or postnatally or both. Behav. Neurosci. 103: 1234-1241.

Meck, W.H. & Williams, C.L. (1999) Choline supplementation during prenatal development reduces proactive interference in spatial memory. Dev. Brain Res. 118: 51-59.

Mean, J.H., Aub, J. C. & Du Bois, E. F. (1917) The effect of caffeine on heat production. Arc. Int. Med. 19: 832-839.

Meeusen, R. & De Meirleir, K. (1995) Exercise and brain neurotransmission. Sports Med. 20: 160-188.

Merrill, A.H. & Jones, D.D. (1990) An update of the enzymology and regulation of sphingomyelin metabolism. Biochim. Biophys. Acta. 1044: 1-12.

Millington, D.S., Bohan, T.P., Roe, C.R. Yergey, A.L. & Liberato, D.J. (1985) Valprolylcarnitine: a novel drug metabolite identified by fast atom bombardment and thermospray liquid chromatography-mass spectrometry. Clin. Chim. Acta. 145: 69-76.

Millington, D.S. & Dubay, G. (1993) Dietary supplement L-carnitine: analysis of different brands to determine bioavailability and content. Clin. Res. Regul. Affairs 10: 71-80.

Montoya, D. & Swartzwelder, H.S. (2000) Prenatal choline supplementation alters hippocampal N-methyl-D-aspartate receptor-mediated neurotransmission in adult rats. Neurosci. Lett. 296:85-88.

Mroczkowska, J.E., Roux, F.S., Galla, H-J., Nałęcz, M.J. & Nałęcz, K.A. (1996) Transport of carnitine in RBE4 cells- an in vitro model of blood-brain barrier. Neurosci. Res. Commun. 19: 153-160.

Mroczkowska, J.E., Roux, F.S., Nałęcz, M.J. & Nałęcz, K.A. (2000) Blood-brain barrier controls carnitine levels in the brain: a study on a model system with RBE4 cells. Biochem. Biophys. Res. Commun. 241: 127-131.

Must, A., Spadano, J., Coakley, E.H., Field, A.E., Colditz, G. & Dietz, W.H. (1999) The disease burden associated with overweight and obesity. JAMA. 282: 1523-1529.

Mynatt, R.L., Dodson, W.L. & Sachan, D.S. (1988) Effects of supplementary choline and pantothenate on carnitine in humans. Fed. Proc. 2: A1421 (Abst.)

Nahata, M.C., Zingarelli, J. & Durrell, D.E. (1989) Stability of caffeine citrate injection in intravenous admixtures and parenteral nutrition solutions. J. Clin. Pharm. Therap: 14: 53-55.

Nakajima, T., Horiuchi, M., Yamanaka, H., Kizaki, Z., Inoue, F., Kodo, N., Kinugasa, A., Saheki, T. & Sawada, T. (1997) The effect of carnitine on ketogenesis in perfused livers from Juvenile Visceral Steatosis mice with systemic carnitine deficiency. Pediatr. Res. 42: 108-113.

Nałęcz, K.A. & Nałęcz, M.J. (1996) Carnitine - a known compound, a novel function in neural cells. Acta. Neurobiol. Exp. 56: 597-609.

Natali, A., Santoro, D., Brandi, L., Faraggiana, D., Ciociaro, D. & Pecori, N. (1993) Effects of acute hypercarnitinemia during increased fatty substrate oxidation in man. Metabolism 42: 594-600.

National Coffee Association of U.S.A. (1991) Coffee drinking study 1991. NCA of U.S.A. New York, NY.

National Institutes of Health (1998) Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults-the evidence report. Obes. Res. 6:51S-209S.

National Research Council (1985) Guide for the care and use of laboratory animals. Publication no. 85-23 (rev.). National Institutes of Health, Bethesda, MD.

Navder, K.P., Baraona, E. & Lieber, C.S. (1997) Polyenylphosphatidylcholine attenuates alcohol-induced fatty liver and hyperlipemia in rats. J. Nutr. 127: 1800-1806.

Negrao, C.E., Ji, L.L., Schauer, J.E., Nagle, F.J. & Lardy, H.A. (1987) Carnitine supplementation and depletion: tissue carnitine and enzymes in fatty acid oxidation. J. Appl. Physiol. 63: 315-321.

Nehling, A., Daval, J.L. & Debry, G. (1992) Caffeine and the central nervous system: Mechanisms of action, biochemical, metabolic and psychostimulant effects. Brain Res. Rev. 17: 139-170.

Nehlig, A. & Debry, G. (1994) Caffeine and sports activity: A review. Int. J. Sports Med. 15:215-223.

Newberne, P.M. & Rogers, A.E. (1986) Labile methyl groups and the promotion of cancer. Ann. Rev. Nutr. 6: 407-432.

Neumann, G. (1996) Effects of L-carnitine on athletic performance. In Carnitine: patho-physiological basics and clinical applications. (Hermann Seim & Heinz Loster., eds.), pp 61-71. Ponte Press, Bochum, Germany.

Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. Science 233: 305-312.

Norgan, N.G. (1994) Population differences in body composition in relation to the body mass index. Eur. J. Clin. Nutr. 48: S10-S25.

Novak, M. (1965) Calorimetric ultramicromethod for the determination of free fatty acids. J. Lipid Res. 6: 431-433.

Novak, M., Monkus, E. F., Buch, M., Lesmes, H. & Silverio, J. (1983) The effect of a L-carnitine supplemented soy formula on the plasma lipids of infants. Acta. Chir. Scand. 517 (Suppl): 149-155.

Obeid, L.M., Linardic, C.M., Karolak, L.A. & Hannun, Y.A. (1993) Programmed cell death induced by ceramide. Science 259: 1769-1771.

Omullane, J.E. & Hawthorne, J.N. (1982) A comparison of the effects of feeding linoleic acid-rich lecithin or corn-oil on cholesterol absorption and metabolism in the rat. Atherosclerosis 45:81-90.

Olah, M.E. & Stiles, G.L. (1995) Adenosine receptor subtypes: Characterization and therapeutic regulation. Ann. Rev. Pharmacol. Toxicol. 35: 581-606.

Olson, R.E., Jablonski, J.R. & Edmund, T. (1958) The effect of dietary protein, fat, and choline upon the serum lipids and lipoproteins of the rats. Am. J. Clin. Nutr. 6: 111-118.

Onrot, J., Goldberg, M.R., Biaggioni, I., Hollister, A.S., Kincaid, D. & Robertson, D. (1985) Hemodynamic and humoral effects of caffeine in autonomic failure: Therapeutic implications for postprandial hypotension. New Engl. J. Med. 313: 549-554.

Oyono-Enguelle, S., Freund, H., Ott, C., Gartner, M., Heitz, A., Marbach, J., Maccari, F., Frey, A., Bigot, H. & Bach, A.C. (1988) Prolonged submaximal exercise and L-carnitine in humans. Eur. J. Appl. Physiol. 58: 53-61.

Pande, S.V. & Caramancion, M.N. (1981) A simple radioisotopic assay of acetylcarnitine and acetyl-CoA at picomolar levels. Anal. Biochem. 112: 30-38.

Parsons, W.D., Aranda, J.V. & Neims, A.H. (1976) Elimination of transplacentally acquired caffeine in full term neonates. Pediatr. Res. 10: 333.

Parsons, W.D. & Neims, A.H. (1978) Effect of smoking on caffeine clearance. Clin. Pharmacol. Ther. 24: 40-45.

Parthasarathy, S., Subbaiah, P.V. & Ganguly, J. (1974) Mechanism of intestinal absorption of phosphatidylcholine in rats. Biochem. J. 140:503-508.

Pasman, W.J., van Baak, M.A., Jeukendrup, A.E., de Haan, A. (1995) The effect of different dosages of caffeine on endurance performance time. Int. J. Sports Med. 16: 225-230.

Pasman, W.J., Westerterp-Plantenga, M.S. & Saris, W.H. (1997) The effectiveness of long-term supplementation of carbohydrate, chromium, fiber and caffeine on weight maintenance. Int. J. Obes. 21: 1143-1151.

Passmore, A.P., Kondowe, G.B., Johnson, G.D. (1987) Renal and cardiovascular effects of caffeine: A dose-response study. Clin. Sci. 72: 749-756.

Paulson, D.J., Schmidt, M.J., Traxler, J.S., Ramacci, M.T. & Shug, A.L. (1984) Improvement of myocardial function in diabetic rats after treatment with L-carnitine. Metab. Clin. Exp. 33: 358-363.

Paulson, D.J. & Shug, A.L. (1981) Tissue specific depletion of L-carnitine in rat heart and skeletal muscle by D-carnitine. Life Sci. 28: 2931-2938.

Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T. & Collins, F. (1995) Effects of the *obese* gene product on body weight regulation in *ob/ob* mice. Science 269: 540-543.

Peluso, G., Nicolai, R., Reda, Benatti, P., Barbarisi, A. & Calvani, M. (2000) Cancer and anticancer therapy-induced modifications on metabolism mediated by carnitine system. J. Cell. Physiol. 182: 339-350.

Penn, D. & Schmidt-Sommerfeld, E. (1988) The carnitine deprived newborn rabbit: potential model to study carnitine deficiency. J. Nutr. 118: 1535-1539.

Penn, D., Schmidt-Sommerfeld, E. & Wolf, H. (1980) Carnitine deficiency in premature infants receiving total parenteral nutrition. Early Hum. Dev. 4: 23-34.

Pearson, D.J. & Tubbs, P.K. (1967) Carnitine and derivatives in rat tissues. Biochem. J. 105: 953-963.

Physicians' Desk Reference, 21 ed. (2000) Medical Economics Company, Montvale, NJ.

Polichetti, E., Diaconescu, N., de la Porte, P.L., Malli, L., Portugal, H., Pauli, A.M., Lafont, H., Tuchweber, B., Yousef, I. & Chanussot, F. (1996) Cholesterol-lowering effect of soyabean lecithin in normolipidemic rats by stimulation of biliary lipid secretion. Brit. J. Nutr. 75: 471-481.

Polichetti, E., Janisson, A., de la Porte, P.L., Portugal, H., Leonardi, J., Luna, A., La Droitte, P. & Chanussot, F. (2000) Dietary polyenylphosphatidylcholine decreases cholesterolemia in hypercholesterolemic rabbits: role of the hepato-biliary axis. Life Sci. 67: 2563-2576.

Polichetti, E., Janisson, A., Iovanna, C., Portugal, H., Mekki, N., Lorec, A-M., Pauli, A-M., Luna, A., Lairon, D., La Droitte, P. Lafont, H. & Chanussot, F. (1998) Stimulation of the apo AI-high density lipoprotein system by dietary soyabean lecithin in humans. J. Nutr. Biochem. 9: 659-664.

Pollock, M.L., Gaesser, G.A., Butcher, J.D., Després, J-P., Dishman, R.K., Franklin, B.A. & Garber, C.E. (1998) The recommended quantity and quality of exercise for developing and maintaining cardiorespiratory and muscular fitness, and flexibility in healthy adults. Med. Sci. Sports. Exerc. 30: 975-991.

Pollock, M.L. & Jackson, A.S. (1984) Research progress in validation of clinical methods of assessing body composition. Med. Sci. Sports Exerc. 16: 606-613.

Powers, S.K., Byrd, R., Tulley, R. & Callendar, T. (1983) Effects of caffeine ingestion on metabolism and performance during graded exercise. Eur. J. Appl. Physiol. 50: 301-305.

Powers, S.K. & Dodd, S.L. (1985) Caffeine and endurance performance. Sports Med. 2: 165-174.

Pyapali, G.K., Turner, D.A., Williams, C.L., Meck, W.H. & Swartzwelder, H.S. (1998) Prenatal dietary choline supplementaiton decreases the threshold for induction of long-term potentiation in young adult rats. J. Neurophysiol. 79: 1790-1796.

Rada, P.V., Mark, G.P. & Hoebel, B.G. (1994) Effects of supplemental choline on extracellular acetylcholine in the nucleus-accumbens during normal behavior and pharmacological acetylcholine depletion. Synapse 16: 211-218.

Rall, T.W. (1985) Central nervous system stimulants. In: Pharmacological Basis of Therapeutics, 7th ed. (Goodman, L.S. & Gilman, A., eds.), pp. 589-603. Macmillan, New York, NY.

Rao, S.S.C., Welcher, K., Zimmerman, B. & Stumbo, P. (1998). Is coffee a colonic stimulant? Eur. J. Gastroenterol Hepatol. 10:113-118.

Roberts, H. & Barone, J.J. (1983) Biological effects of caffeine: History and use. Food Technol. 37: 32-39.

Rebouche, C.J. (1977) Carnitine movement across muscle cell membranes: studies in isolated rat muscle. Biochim. Biophys. Acta. 471: 145-155.

Rebouche, C.J. (1989) Carnitine transport and tissue carnitine accretion in rats. Biochim. Biophys. Acta. 1033:111-113.

Rebouche, C.J. (1991) Ascorbic acid and carnitine biosynthesis. Am. J. Clin. Nutr. 54: 1147S-1152S.

Rebouche, C.J. (1992) Carnitine function and requirements during the life cycle. FASEB J. 6: 3379-3386.

Rebouche, C.J. (1995) Renal handling of carnitine in experimental vitamin C deficiency. Metabolism 44: 1639-1643.

Rebouche, C.J. (1999) Carnitine. In: Modern Nutrition in Health and Disease, 9th ed. (Shils, M.E., Olson, J.A., Shike, M. & Ross, A.C., eds), pp. 505-512. Lippincott Williams & Wilkins, Baltimore, MD.

Rebouche, C.J. & Chenard, C.A. (1991) Metabolic fate of dietary carnitine in human adults: identification and quantification of urinary and fecal metabolites. J. Nutr. 121: 539-546.

Rebouche, C.J. & Engel, A.G. (1984) Kinetic compartmental analysis of carnitine metabolism in the human carnitine deficiency syndromes. Evidence for alterations in tissue carnitine transport. J. Clin. Invest. 73: 857-867.

Rebouche, C.J., Lehman, L.J. & Olson, A.L. (1986) ϵ-N-trimethyllysine availability regulates the rate of carnitine biosynthesis in the growing rats. J. Nutr. 116: 751-759.

Rebouche, C.J. & Mack, D.L. (1984) Sodium gradient-stimulated transport of L-carnitine into renal brush border membrane vesicles: kinetics, specificity, and regulation by dietary carnitine. Arch. Biochem. Biophys. 235: 393-402.

Rebouche, C.J., Mack, D.L. & Edmonson, P.F. (1984) L-carnitine dissimilation in the gastrointestinal tract of the rat. Biochemistry 23: 6422-6426.

Rein, D., Krasin, B., & Sheard, N.F. (1997) Dietary choline supplementation in rats increases carnitine concentration in liver, but decreases plasma and kidney carnitine concentrations. J. Nutr. Biochem. 8: 68-73.

Richter, V., Purschwitz, K., Bohusch, A., Seim, H., Weisbrich, C., Reuter, W., Sorger, D. & Rassoul, F. (1999) Lipoproteins and other clinical-chemistry parameters under the conditions of lacto-ovo-vegetarian nutrition. Nutr. Res. 19: 545-554.

Robins, S.J. & Armstrong, M.J. (1976) Biliary lecithin secretion. II. Effects of dietary choline and biliary lecithin synthesis. Gastroenterology 70:397-402.

Roe, C.R. & Bohan, T.P. (1982) L-carnitine therapy in propionic acidemia. Lancet 1: 1411-1412.

Roe, C.R., Millington, D.S., Maltby, D.A. & Bohan, T.P. (1984a) L-carnitine enhances excretion of propionyl coenzyme A as propionylcarnitine in propionic acidemia. J. Clin. Invest. 73: 1785-1788.

Roe, C.R., Millington, D.S., Maltby, D.A., Kahler, S.G. & Bohan, T.P. (1984b) L-carnitine therapy in isovaleric acidemia. J. Clin. Invest. 74: 2290-2295.

Roller, L. (1981) Caffeinism: Subjective quantitative aspect of withdrawal syndrome. Med. J. Aust. 140: 146.

Romijn, J.A., Coyle, E.F., Sidossis, L.S., Gastaldelli, A., Horowitz, J.F., Endert, E. & Wolfe, R.R. (1993) Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. Am. J. Physiol. 265: E380-E391.

Rowell, L.B. (1974) Human cardiovascular adjustments to exercise and thermal stress. Physiol. Rev. 54: 75-103.

Ruff, L.J., Miller, L.G. & Brass, E.P. (1991) Effect of exogenous carnitine on carnitine homeostasis in the rat. Biochim. Biophys. Acta. 1073: 543-549.

Runge, F. (1820) Phytochemische Entdeckungen, Berlin, p.204.

Rushmore, T.H., Farber, E., Ghoshal, A.K., Parodi S., Pala, K. & Taningher, M. (1986) A choline-devoid diet, carcinogenic in the rat, induces DNA damage and repair. Carcinogenesis 7: 1677-1680.

Rushmore, T.H., Lim, Y.P., Farber, E. & Ghoshal, A.K. (1984) Rapid lipid peroxidaiton in the nuclear fraction of rat liver induced by a diet deficient in choline and methionine. Cancer Lett. 24: 251-255.

Sachan, D.S., Daily, J.W., III, Munroe, S.G. & Beauchene, R.E. (1997) Vegetarian elderly women may risk compromised carnitine status. Int. J. Veg. Nutr. 1:64-69.

Sachan, D.S. & Hongu, N. (2000) Increases in VO_2max and metabolic markers of fat oxidation by caffeine, carnitine, and choline supplementation in rats. J. Nutr. Biochem. 11: 521-526.

Sachan, D.S. & Hoppel, C.L. (1980) Carnitine biosynthesis. Hydroxylation of N^6-trimethyl-lysine to 3-hydroxy-N^6-trimethyl-lysine. Biochem. J. 188: 529-534.

Sachan, D.S. & Mynatt, R.M. (1992) Effects of carnitine and ethanol on the disposal of fatty acids in urine. FASEB J. 6: A1088.(Abst.)

Sachan, D.S., Rhew, T.H. & Ruark, R.A. (1984) Ameliorating effects of carnitine and its precursors on alcohol-induced fatty liver. Am. J. Clin. Nutr. 39: 738-744.

Sachan, D.S. & Ruark, R.A. (1985) Distribution of carnitine and acylcarnitine in small intestine of carnitine-supplemented and fasted rats. J. Nutr. 115: 865-871.

Sahlin K. (1990) Muscle carnitine metabolism during incremental dynamic exercise in humans. Acta. Physiol. Scand. 138: 259-262.

Sandage, B.W., Sabounjian, L., White, R. & Wurtman, R.J. (1992) Choline citrate may enhance athletic performance. Physiologist 35: 236. (Abst.)

Sant'Ambrogio, G., Mognoni, P., Ventrella, L. (1964) Plasma levels of caffeine after oral, intramuscular and intravenous administration. Arch. Int. Pharmacodyn. Ther. 150: 259-263.

Salomon, G. (1883) Über das Paraxanthin, einen neuen Bestandtheil des normalen menschlichen Harns. Ber. Dtsch. Chem. Ges. 16: 195-200.

Sanford, P.A. & Smyth, D.H. (1971) Intestinal transport of choline in the rat and hamster. J. Physiol. 215:769-788.

SAS Institute Inc. (1997) SAS User's Guide: statistics. SAS Institute, Cary, NC.

SAS Institute Inc. (1998) SAS User's Guide: statistics, version 6.12. SAS Institute, Cary, NC.

Schlosberg, A.J., Fernstrom, J.B., Kopczynski, M.C., Cusack, B.M. & Gillis, M.A. (1981) Acute effects of caffeine injection on neural amino acids and brain monoamine levels in rats. Life Sci. 29: 173-183.

Schneider, W.J. & Vance, D.E. (1979) Conversion of phosphatidylethanolamine to phosphatidylcholine in rat-liver-partial-purification and characterization of the enzymatic activities. J. Biol. Chem. 254:3386-3891.

Segal, K.R., Van Loan, M., Fitzgerald, P.I., Hodgdon, J.A. & Van Itallie, T.B. (1988) Lean body mass estimation by bioelectrical impedance analysis: a four-site cross-validation study. Am J. Clin. Nutr. 47: 7-14.

Selhub, J., Seyoum, E., Pomfret, E.A. & Zeisel, S.H. (1991) Effects of choline deficiency and methotrexate treatment upon liver folate content and distribution. Cancer Res. 51: 16-21.

Shapiro, P. (1982) Caffeine for allergic rhinitis. Lancet 144: 793.

Sharp, M.A., Patton, J.F., Knapik, J.J. Hauret, K. Mello, R.P, Ito, M. & Frykman, P.N. (2002) Comparison of the physical fitness of men and women entering the U.S. Army: 1978-1998. Med. Sci. Sports Exerc. 34: 356-363.

Shaw, R.D., Li, BUK., Hamilton, J.W., Shug, A.L. & Olsen, W.A. (1983) Carnitine transport in rat small intestine. Am. J. Physiol. 245: G376-G381.

Sheard, N.F. & Krasin, B. (1994) Restricting food intake does not exacerbate the effects of a choline-deficient diet on tissue carnitine concentrations in rats. J. Nutr. 124: 738-743.

Sheard, N.F., Rein, D., & Krasin, B. (1992) Lack of effect of chronic choline supplementation on carnitine levels in the rat. FASEB J. 6: S1381. (Abst.)

Sheard, N.F., Tayek, J.A., Bistrian, B.R., Blackburn, G.L. & Zeisel, S.H. (1986) Plasma choline concentration in humans fed parenterally. Am. J. Clin. Nutr. 43: 219-224.

Short, S.H. & Marquart, L.F. (1993) Sports nutrition fraud. New York State J. 93: 112-116.

Shronts, E.P. (1997) Essential nature of choline with implications for total parenteral nutrition. J. Am. Diet. Assoc. 97: 639-649.

Shug, A.L., Schmidt, M.J., Golden, G.T. & Fariello, R.G. (1982) The distribution and role of carnitine in the mammalian brain. Life Sci. 31: 2869-2874.

Shug, A.L., Thomsen, J.H., Folts, J.D., Bittar, N., Klein, M.I., Koke, J.R. & Huth, P.J. (1978) Changes in tissue levels of carnitine and other metabolites during myocardial ischemia and anoxia. Arch. Biochem. Biophys.187: 25-33.

Siddiqui, L.K. & Bamji, M.S. (1980) Plasma carnitine levels in adult males in India: effects of high cereal, low fat diet, fat supplementation and nutritional status. Am. J. Clin. Nutr. 33: 1259-12363.

Sidransky, H. & Farber, E. (1960) Liver choline oxidase activity in man and in several species of animals. Arch. Biochem. Biophys. 87: 129-133.

Siliprandi, N., DiLisa, F., Pieralisi, G., Ripari, P., Maccari, F., Menabo, R., Giamberardino, M.A. & Vecchiet, L. (1990) Metabolic changes induced by maximal exercise in human subjects following L-carnitine administration. Biochim Biophys. Acta 1034: 17-21.

Simi, B., Mayet, M.H., Sempore, B. & Favier, R.J. (1990) Large variations in skeletal muscle carnitine level fail to modify energy metabolism in exercising rats. Comp. Biochem. Physiol. 97A: 543-549.

Sinclair, C.J.D. & Geiger, J.D. (2000) Caffeine use in sports: A pharmacological review. J. Sports Med. Phys. Fitness. 40: 71-79.

Skinner, J.D., Carruth, B.R., Houck, K.S., Morris, M., Moran, J. III & Coletta, F. (2000) Caffeine intake in young children differs by family socioeconomic status. J. Am. Diet. Assoc. 100:229-231.

Solberg, H.E. & Bremer, J. (1970) Formation of branched chain acylcarnitines in mitochondria. Biochim. Biophys. Acta. 222: 372-380.

Soop, M., Bjorkman, O., Cederblad, G., Hagenfeldt, L. & Wahren, J. (1988) Influence of carnitine supplementation on muscle substrate and carnitine metabolism during exercise. J. Appl. Physiol. 64: 2394-2399.

Spector, S.A., Jackman, M.R, Sabounjian, L.A., Sakkas, C., Landers, D.M. & Willis, W.T. (1995) Med. Sci. Sports Exerc. 27: 668-673.

Spielman, W.S. & Thompson, C.I. (1982) A proposed role for adenosine in the regulation of renal hemodynamics and renin release. Am. J. Physiol. 242: F423. (Abst.)

Spindel, E. (1984) Action of the methylxanthines on the pituitary and pituitary-dependent hormones. In: The Methylxanthine Beverages and Foods: Chemistry, Consumption, and Health Effects (Spiller, G.A., ed.), pp. 185-214, Alan R. Liss, New York, NY.

Spindel, E.R. & Wurtman, R.J. (1984) Neuroendorcine effects of caffeine in rat and men. In: Caffeine: Perspectives from Recent Research (Dews, P.B., ed.), pp. 119-127, Springer-Verlag, Berlin, Germany.

Spriet, L.L., MacLean, D.A., Dyck, D.J., Hultman, E., Cederblad, G. & Graham T.E. (1992) Caffeine ingestion and muscle metabolism during prolonged exercise in humans. Am. J. Physiol. 262: E891-E898.

Stadler, D.D., Chenard, C.A. & Rebouche, C.J. (1993) Effect of dietary macronutrient content on carnitine excretion and efficiency of carnitine reabsorption. Am. J. Clin. Nutr. 58: 868-872.

Stein, R. & Englard, S. (1982) Properties of rat 6-N-trimethyl-L-lysine hydroxylases: similarities among the kidney, liver, heart, and skeletal muscle activities. Arch. Biochem. Biophys. 217: 324-331.

Steiner, A. & Domanski, B. (1944) Effect of feeding of "soya lecithin" on serum cholesterol level of man. Proc. Soc. Exp. Biol. Med. 55:236-238.

Strecker, A. (1861) Untersuchungen über die chemischen Beziehungen zwischen Guanin, Xanthin, Theobromine, Caffeïn und Kreatinin. Liebigs Ann. Chem. 118: 151-177.

Strecker, A. (1862) Uber einige neue Bestandtheile der Schweingalle. Ann. Chem. Pharmacie. 123: 353-360.

Stumpf, D.A., Parker, W.D. & Angelini, C. (1985) Carnitine deficiency, organic acidemias, and Reye's syndrome. Neurology 35: 1041-1045.

Sugden, M.C. & Holness, M.J. (1994) Interactive regulation of the pyruvate dehydrogenase complex and the carnitine palmitoyltrasnferase system. FASEB J. 8: 54-61.

Sumida, S., Tanaka, K., Kitao, H. & Nakadomo, F. (1989) Exercise-induced lipid peroxidation and and leakage of enzymes before and after vitamin E supplementation. Int. J. Biochem. 21: 835-838.

Sundler, R. & Akesson, B. (1975) Biosynthesis of phosphatidylethanolamines and phosphatidylcholines from ethanolamine and choline in rat-liver. Biochem. J. 146: 309-315.

Sutherland, E.W., Robison, A. & Butcher, R.W. (1968) Some aspects of the biological role of adenosine 3', 5'-monophosphate (cyclic AMP). Circulation 37: 279-306.

Svendsen, O.L., Hassager, C., Bergmann, I. & Christiansen, C. (1993) Measurement of abdominal and intra-abdominal fat in postmenopausal women by dual energy x-ray absorptiometry and anthropometry - comparison with computerized-tomography. Int. J. Obes. 17: 45-51.

Tanphaichitr, V. & Broquist, H.P. (1973) Role of lysine and ϵ-N-trimethyllysine in carnitine biosynthesis. II. Studies in the rat. J. Biol. Chem. 248:2176-2181.

Tanzer, F., Hizel, S., Çetinkaya, Ö., Sekreter, E. (2001) Serum free carnitine and total triglyceride levels in children with iron deficiency anemia. Int. J. Vitam. Nutr. Res. 71: 66-69.

Taussky, H.H. (1954) A microcolorimetric determination of creatinine in urine by the Jeff reaction. J. Biol. Chem. 208: 853-861.

Tarka, S.M. (1982) The toxicology of cocoa and methylxanthines: A review of the literature. CRC. Critical Rev. Toxicol. 9: 275-311.

Tarnopolsky, M.A., Atkinson, S.A., MacDougall, J.D., Sale, D.G. & Sutton, J.R. (1989) Physiological responses to caffeine during endurance running in habitual caffeine users. Med. Sci. Sports. Exerc. 21: 418-424.

Tein, I., De Vivo, D.C., Ranucci, D. & DiMauro, S. (1993) Skin fibroblast carnitine uptake in secondary carnitine deficiency disorders. J. Inherit. Metab. Dis. 16: 135-146.

Tomita, K. & Tsuchiya, H. (1989) Caffeine enhancement of the effect of anticancer agents on human sarcoma cells. Jap. J. Cancer Res. 80: 83-88.

Trang, J.M., Blanchard, J. Conrad, K.A. & Harrison, G.G. (1982) The effect of vitamin C on the pharmacokinetics of caffeine in elderly men. Am. J. Clin. Nutr. 35: 487-494.

Trappe, S.W., Costill, D.L., Goodpaster, B., Vukovich, M.D. & Fink, W.J. (1994) The effects of L-carnitine supplementation on performance during interval swimming. Int. J. Sports Med. 15: 181-185.

Tsai, A.C., Romsos, D.R. & Leveille, G.A.(1975) Significance of dietary carnitine for growth and carnitine turnover in rats. J. Nutr. 104:782-792.

Tsai, A.C., Romsos, D.R. & Leveille, G.A. (1974) Determination of carnitine turnover in choline-deficient and cold-exposed rats. J. Nutr. 105: 301-307.

United States Department of Agriculture. (1995) World coffee situation. Foreign Agriculture Service. Circular Series, FCOF 1-93.

United States Department of Agriculture & United States Department of Health and Human Services. (1990) Nutrition and your health: Dietary guidelines for Americans. 3rd ed. Washington, D.C. US Government Printing Office. Publication 261-495/20124.

United States Department of Agriculture & United States Department of Health and Human Services. (2000) Nutrition and your health: Dietary guidelines for Americans. 5th ed. Washington, D.C. US Government Printing Office. (Home and Garden Bulletin no.232)

United States Department of Health and Human Services. (2000) Healthy people 2010: Understanding and improving health. Washington, D.C. US Government Printing Office. http://www.health.gov/healthypeople/.

Uthus, E.O., Skurdal, D.N. & Cornatzer W.E. (1976) Effect of ethanol ingestion on choline phosphotransferase and phosphatidyl ethanolamine methyltransferase activities in liver-microsomes. Lipids 11: 641-644.

Uziel, G., Garavaglia, B. & Di Donato, S. (1988) Carnitine stimulation of pyruvate dehydrogenase complex (PDHC) in isolated human skeletal muscle mitochondria. Muscle Nerve 11:720-724.

Vague, J. (1956) The degree of masculine differentiation of obesity - a factor determining predisposition to diabetes, atherosclerosis, gout and uric calculus. Am. J. Clin. Nutr. 4: 20-34.

Vanakoski, J., Kosunen, V., Meririnne, E. & Seppala, T. (1998) Creatine and caffeine in anaerobic and aerobic exercise: effects on physical performance and pharmacokinetic considerations. Int. J. Clin. Pharmacol. Ther. 36: 258-262.

Vance, D.E. & Ridgway, N.D. (1988) The methylation of phosphatidylethanolamine. Prog. Lipid Res. 27: 61-79.

van Soeren, M.H., Sathasivam, P., Spriet, L.L. & Graham, T.E. (1993) Caffeine metabolism and epinephrine response during exercise in users and nonusers. J. Appl. Phys. 75: 805-812.

Vecchiet, L., Di Lisa, F., Pieralisi, P., Ripari, R., Menabo, M., Giamberardino, M. & Siliparandi, N. (1990) Influence of L-carnitine administraiton on maximal physical exercise. Eur. J. Appl. Physiol. 61: 486-490.

Vickers, S., Duncan, C.A., White, S.D. Ramjit, H.G., Smith, J.L., Walker, R.W., Flynn, H. & Arison, B.H. (1985) Carnitine and glucuronic acid conjugates of pivalic acid. Xenobiotica. 15: 453-458.

Vitali, G., Parente, R. & Melotti, C. (1995) Carnitine supplementation in human idiopathic asthenospermia: clinical results. Drugs Exp. Clin. Res. 21: 157-159.

Vogel, J.A., Patton, J.F., Mello, R.P. & Daniels, W.L. (1986) An analysis of aerobic capacity in a large United States population. J. Appl. Physiol. 60: 494-500.

Volek, J.S., Kraemer, W.J., Rubin, M.R., Gómez, A.L., Ratamess, N.A. & Gaynor, P. (2002) L-carnitine L-tartrate supplementation favorably affects markers of recovery from exercise stress. Am. J. Physiol. Endocrinol. Metab. 282: E474-E482.

von Allwörden, H.N., Horn, S., Kahl, J. & Feldheim, W. (1993) The influence of lecithin on plasma choline concentrations in triathletes and adolescent runners during exercise. Eur. J. Appl. Physiol. 67: 87-91.

von Borstel, W.R. & Wurtman, R.J. (1984) Caffeine and the cardiovascular effects of physiological levels of adenosine. In: Caffeine: Perspectives from Recent Research (Dews, P.B., ed.), pp. 142-150, Springer-Verlag, Berlin, Germany.

von Giese, F. (1820) Vermischte Notizen. 1. Kaffeestoff und salzgehalt des quassia extrakts. Allg. Nord. Ann. Chem. Freunde. Naturkd. Arzneiwiss 4: 240-241.

Vukovich, M.D., Costill, D.L. & Fink, W.J. (1994) Carnitine supplementation: effect on muscle carnitine and glycogen content during exercise.

Wadden, T.A., Considine, R.V., Foster, G.D., Anderson, D.A., Sarwer, D.B. & Caro, J.S. (1998) Short- and long-term changes in serum leptin in dieting obese women: effects of caloric restriction and weight loss. J. Clin. Endocrinol. Metab. 83: 214-218.

Wang, Y. & Lau, C.E. (1998) Caffeine has similar pharmacokinetics and behavioral effects via the i.p. and p.o. routes of administration. Pharmacol. Biochem. Behav. 60: 271-278.

Warber, J.P., Patton, J.F., Tharion, W.J., Zeisel, S.H., Mello, R.P., Kemnitz, C.P. & Lieberman, H.R. (2000) The effects of choline supplementation on physical performance. Int. J. Sport Nutr. Exerc. Metabolism 10:170-181.

Warshaw, J.B. (1972) Cellular energy metabolism during fetal development. IV. Fatty acid activation, acetyltransfer and fatty acid oxidation during development of the chick and rat. Dev. Biol. 28: 537-544.

Warshaw, J.B. & Terry, M.L. (1976) Cellular energy metabolism during fetal development. VI. Fatty acid oxidation by developing brain. Dev. Biol. 52: 161-166.

Wasserman, K. & Whipp, B.J. (1975) Exercise physiology in health and disease. Am. Rev. Resp. Dis. 112: 219-249.

Watkins, J.D. & Kent, C. (1992) Immunolocalization of membrane-associated CTP: phosphocholine cytidyltransferase in phosphatidylcholine-deficient Chinese Hamster ovary cells. J. Biol. Chem. 267: 5686-5692.

Wawrzeńczyk, A., Nałęcz, K.A. & Nałęcz, M.J. (1995) Effect of externally added carnitine on the synthesis of acetylcholine in rat cerebral cortex cells. Neurochem. Int. 26: 635-641.

Wawrzeńczyk, A., Sacher, A., Mac, M., Nałęcz, M.J. & Nałęcz, K.A. (2001) Transport of L-carnitine in isolated cerebral cortex neurons. Eur. J. Biochem. 268: 2091-2098.

Wecker, L. (1986) Neurochemical effects of choline supplementation. Can. J. Physiol. Pharmacol. 64: 329-333.

Wecker, L. (1990a) Choline utilization by central cholinergic neurons. In: Nutrition and the Brain, Vol. 8 (Wurtman, R.J. & Wurtman, J.J., eds.), pp.147-162. Raven Press, New York, NY.

Wecker, L. (1990b) Dietary choline: a limiting factor for the synthesis of acetylcholine by the brain. Adv. Neurol. 51: 139-145.

Weinhold, P.A. & Sanders, R. (1973) The oxidation of choline by liver slices and mitochondria during liver development in the rat. Life Sci. 13:621-29.

Welsch, F. (1976) Studies on accumulation and metabolic fate of (N-Me3h)choline in human term placenta fragments. Biochem. Pharmacol. 25: 1021-1030.

Whitney, E.N., Cataldo, C.B. & Rolfes, S.R. (1994) Nutrition assessment. In: Understanding Normal & Clinical Nutrition. 4th ed., pp.521-566. West Publishing Company, St. Paul, MN.

World Health Organisation (WHO) Expert Committee. (1995) Physical status: the use and interpretation of anthropometry. Teaching Report Series no. 854: Geneva.

World Health Organisation. Report of a WHO consultation on obesity. (1998) Obesity: preventing and managing the global epidemic. WHO, Geneva.

Wolf, G. & Berger, C.R.A. (1961) Studies on the biosynthesis and turnover of carnitine. Arch. Biochem. Biophys. 92: 360-365.

Wolf, H., Stave, U., Novak, M. & Monkus, E.F. (1974) Recent investigations on neonatal fat metabolism. J. Perinatal Med. 2:75-87.

Woskresensky, A. (1984) Über das Theobromin. Liebigs Ann. Chem 41: 125-127.

Wurtman, J.J. (1979) Sources of choline and lecithin in diet. In: Nutrition and the Brain, Vol. 5 (Barbeau, A., Growdon, J.H. & Wurtman, R.J., eds), pp. 73-81. Raven Press, New York, NY.

Wyss, V., Ganzit, G.P. & Rienzi, A. (1990) Effects of L-carnitine administration on VO_2max and aerobic-anaerobic threshold in normoxia and acute hypoxia. Eur. J. Appl. Physiol. 60: 1-6.

Yamamura, H.I. & Snyder, S.H. (1973) High affinity transport of choline into synaptosomes of rat brain. J. Neurochem. 21: 1355-1374.

Yang, E.K., Blusztajn, J.K., Pomfret, E.A. & Zeisel, S.H. (1988) Rat and human mammary tissue can synthesize choline moiety via the methylation of phosphatidylethanolamine. Biochem. J. 256: 821-828.

Yao, Z.M., Jamil, H. & Vance D.E. (1990) Choline deficiency causes translocation of CTP: phosphocholine cytidylyltransferase from cytosol to endoplasmic reticulum in rat liver. J. Biol. Chem. 265: 4326-4331.

Yao, Z.M. & Vance D.E. (1988) The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. J. Biol. Chem. 263:2998-3004.

Yao, Z.M. & Vance D.E. (1989) Head group specificity in the requirement of phosphatidylcholine biosynthesis for very low density lipoprotein secretion from cultured hepatocytes. J. Biol. Chem. 264:11373-11380.

Yesair, D.W., Branfman, A.R. & Callahan, M.M. (1984) Human disposition and some biochemical aspects of methylxanthines. In: The Methylxanthine Beverages and Foods: Chemistry, Consumption, and Health (Spiller, G.A. ed.), pp. 215-234. Alan R. Liss, New York, NY.

Yonug, V.R. & Pellet, P.L. (1994) Plant proteins in relation to human protein and amino acid nutrition. Am. J. Clin. Nutr. 59 (suppl.): 1203S-1212S.

Yunis, J.J., Soreng, A.L. & Bowe, A.E. (1987) Fragile sites are targets of diverse mutagens and carcinogens. Oncogene 1:59-69.

Zając, A., Waśkiewicz, Z., & Nowak, K. (2001) The influence of L-carnitine supplementation on body fat content, speed, explosive strength and VO_2max in elite athletes. Biol. Sport 18:127-135.

Zeisel, S.H. (1981) Dietary choline: Biochemistry, physiology, and pharmacology. Ann. Rev. Nutr. 1: 95-121.

Zeisel, S.H. (1990) Choline deficiency. J. Nutr. Biochem. 1: 332-349.

Zeisel, S.H. (1992) Choline -An important nutrient in brain-development, liver-function and carcinogenesis. J. Am. Coll. Nutr. 11: 473-481.

Zeisel, S.H. (1993) Choline phospholipids: signal transduction and carcinogenesis. FASEB J. 7: 551-557.

Zeisel, S.H. (1999) Choline and phosphatidylcholine. In: Modern Nutrition in Health and Disease, 9th ed. (Shils, M.E., Olson, J.A., Shike, M. & Ross, A.C., eds.), pp. 513-523. Lippincott Williams & Wilkins, Baltimore, MD.

Zeisel, S.H. (2001) Choline. In: Handbook of Vitamins, 3rd ed. (Rucker, R.B., Suttie, J.W., McCormick, D.B., & Machlin, L.J., eds.), pp.513-528. Marcel Dekker, New York, NY.

Zeisel, S.H. & Blusztajn, J.K. (1994) Choline and human nutrition. Ann. Rev. Nutr. 14: 269-296.

Zeisel, S.H. Char, D. & Sheard, N.F. (1986) Choline, phosphatidylcholine and sphingomyelin in human and bovine milk and infant formulas. J. Nutr. 116: 50-58.

Zeisel, S.H., da Costa, K-A, Franklin, P.D., Alexander, E.A., Lamont, J.T., Sheard, N.F.& Beiser, A. (1991) Choline, an essential nutrient for humans. FASEB J. 5: 2093-2098.

Zeisel, S.H., Epstein, M.F. & Wurtman, R.J. (1980a) Elevated choline concentration in neonatal plasma. Life Sci. 26: 1827-1831.

Zeisel, S.H., Growdon, J.H., Wurtman, R.J., Magil, S.G. & Logue, M. (1980b) Normal plasma choline responses to ingested lecithin. Neurology 30: 1226-1229.

Zeisel, S.H., Stanbury, J.B., Wurtman, R.J., Brigida, M. & Fierro-Benitez R. (1982) Choline content of mothers' milk in Ecuador and Boston. N. Engl. J. Med. 306: 175-176.

Zeisel, S.H., Zola, T., da Costa K-A & Pomfret, E.A. (1989) Effect of choline deficiency on S-adenosylmethionine and methionine concentrations in rat liver. Biochem. J. 259:725-729.

Zhang, A.Q., Mitchell, S.C. & Smith, R.L. (1999) Dietary precursors of trimethylamine in man: A pilot study. Food Chem. Toxicol. 37: 515-520.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. & Friedman, J.M. (1994) Positional cloning of the mouse *obese* gene and its human homologue. Nature 372: 425-432.

Zhang, Y. & Wells, J.N. (1990) The effect of chronic caffeine administration on peripheral adenosine receptors. J. Pharmacol. Exp. Therap. 254: 257-263.

Zurbriggen, E. (2000) L-carnitine: historical review. Ann. Nutr. Metab. 44:78-79.

Zylber-Katz, E., Granit, L. & Levy, M. (1984) Relationship between caffeine concentrations in plasma and saliva. Clin. Pharmacol. Ther. 36: 133-137.

APPENDIXES

APPENDIX A

ACETYLCARNITINE ASSAY

Principle

The acetyl portion of acetylcarnitine is converted to [^{14}C]Citrate, with the aid of carnitine acetyltransferease (CAT) (EC 3.2.1.7), citrate synthase (CS) (EC 4.1.3.7), and [^{14}C]Oxaloacetate. Then, the unreacted [^{14}C]Oxaloacetate is converted to [^{14}C]Aspartate by the addition of glutamate oxaloacetate transaminase (GOT) (EC 2.6.1.1) and glutamate. A cation exchange resin is added to absorb the [^{14}C]Aspartate, and the radioactivity of the solution due to [^{14}C]Citrate is measured (Pande and Caramancion 1981).

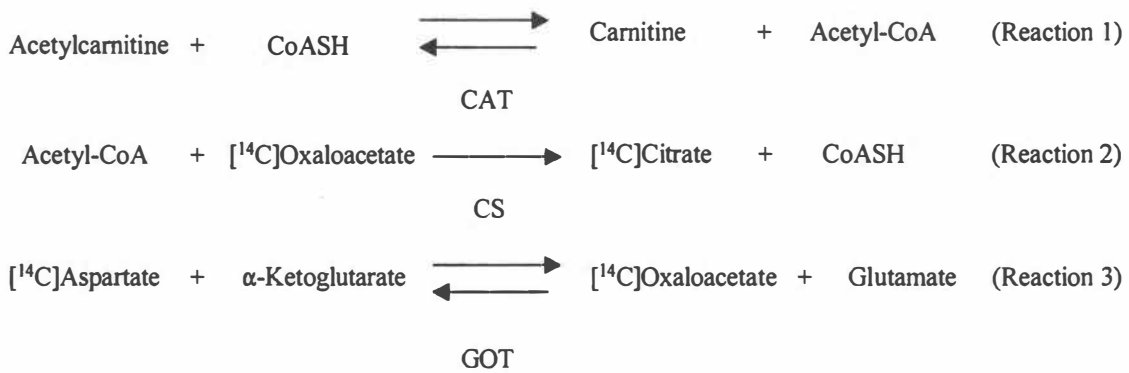


FIGURE A-1. *Principal reactions in acetylcarnitine assay*

In Reaction 1, CAT catalyses the transfer of the acetyl moiety from acetylcarnitne to CoA, forming carnitine and acetyl-CoA. In Reaction 2, acetyl-CoA is then reacted with labeled oxaloacetate to form [^{14}C]Citrate and CoASH, which is catalysed by CS.

[^{14}C]Oxaloacetate is produced from the reaction of [^{14}C]Aspartate with α-ketoglurarate in the presence of GOT. Unreacted [^{14}C]Oxaloacetate is then trasaminated back to the [^{14}C]Aspartate with glutamate with the presence of GOT (Reaction 3). This provides a means for separation of unreacted [^{14}C]Oxaloacetate from [^{14}C]Citrate. Then, a cation exchange resin (i.e. Dowex 50X8, 200-400 mesh, H^+ form) at neutral pH allows the separation of [^{14}C]Citrate from [^{14}C]Aspartate. [^{14}C]Citrate passes through the column (or in supernatant of the reaction mixture) and positively charged [^{14}C]Aspartate is retained in the resin. The [^{14}C]Citrate is counted by a Beckman LS counter (Beckman, Fullerton, CA).

High specific activity of [U-^{14}C]Aspartate (> 200 mci/mmol) allows the estimation of > 10 pmol acetylcarnitine. The assay can determine acetylcarnitine ranging from 20-80 pmol. Thea assay is not affected by carnitine even when the ratio of carnitine:acetylcarnitine is > 500 (Di Lisa et al. 1992).

Reagents

Chemicals involved in the assay are as follows:

1. 0.6 M and 1M PCA
2. 0.5 M N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid](HEPES)-KOH, pH 8.5. (0.5 M HEPES neutralized with KOH to pH 8.5)
3. 0.5M HEPES-KOH-6mM EDTA, pH 7.0
4. 66.7 mM HEPES-KOH-1.96 mM EDTA, pH 7.4
5. 61.6 mM HEPES-KOH-9.87 EDTA, pH 7.4
6. 37.5 mM HEPES-KOH, pH 7.4
7. 3 M $KHCO_3$
8. CoASH (20 μg/μl = 5 mg CoA in 250 μL of DDW)
   Use a 10 mg or 5 mg of vile and add DDW directly to the vile.
9. Dithiothreitol (DTT)
10. α-ketoglutarate
11. GOT

12. CS
13. CAT
14. U-^{14}C-Aspartate (> 200 mci/mmol)
15. Sodium glutamate
16. Dowex 1X8, 200-400 mesh, Cl^- form
17. Dowex 50x8, 200 -400 mesh, H^+ form
18. Methanol
19. Scintillation cocktail

Preparation of samples: PCA extraction

Plasma

1. 0.2 mL in 0.8 mL of 0.6 M $HClO_4$, vortex, place on ice for 10 min.
2. Centrifuge at 2000 X g for 10 min at 4°C. Use supernatant (SN) for acetylcarnitine assay.

Note; Dilute samples, if supplemented animals or humans

Urine

1. Thaw samples, vortex thoroughly.
2. Take 0.1 mL urine, 0.1 mL DDW, and 0.8 mL of 0.6M PCA, vortex, place on ice for 10 min.
3. Centrifuge at 2000 X g for 10 min at 4°C. Use SN for acetylcarnitine assay.

Liver

1. 100 mg wet liver added to 3 mL of 0.6 M PCA, polytron (place in ice water while using polytron).
2. Centrifuge at 10,000 X g for 5 min at 4°C. Use SN for acetylcarnitine assay.

Muscle

1. 50 mg wet (or 10 mg freeze dried) skeletal muscle added to 3 mL of 0.6 M PCA in 1 mM Na-EDTA (i.e. 0.17 mL of 70% PCA in 3mL of 1mM Na-EDTA), polytron (place in ice water while using polytron)
2. Centrifuge at 10,000 X g for 5 min at 4°C. Use SN for acetylcarnitine assay.

Neutralizaiton

1. All PCA extracts (tissues, plasma, or urine) must be neutralized. Transfer 0.8 mL of SN in glass test tubes (12 x 75 mm) containing 75 µL of 0.5 M Hepes-KOH, pH 8.5.
2. Vortex, and neutralize with 125 µL 3 M $KHCO_3$. Need to be freshly prepared each time use.

3. Keep on ice for 10 min.
4. Centrifuge at 2000 X g for 10 min at 4°C.
5. Transfer 0.45 mL of the SN to micro-centrifuge tube containing 20 µL of 0.5 M Hepes-KOH-6mM EDTA, pH 7.0.
6. Mix well, capped and can be stored frozen until assayed for acetylcarnitine.

Anion exchange (remove anion, citrate, by using anion Dowex resin)

1. Make anion exchange resin: 200 mg/1.5 ml (0.5 ml methanol + 1.0 mL DDW/tube)
   Example: 2 gm/5 ml methanol + 10 ml methanol
2. Take a 400 µL of sample into a glass tube and add 1.5 mL of the mix resin, and add 400 µL of DDW
3. Vortex and let stand for 2 min.
4. Centrifuge at 2000 x g for 5 min at 4°C.
5. Take 198 µL of SN to a microcentrifuge tube containing 2 µL of 0.5M HEPES-KOH-6mM EDTA, pH 7.0.

Making Stock Standard:

Make 37.5 µM acetylcarnitine. First, make 375 µM acetylcarnitine. Dissolve 0.0898 gm acetylcarnitine-HCL (239.7g/mol) in 1000 mL DDW, then take 5 ml of this solution, and diluted with DDW to make the volume to 50 ml.

Dilution factor for a sample:

Because 100 µL of samples are diluted to 2.66 µL/Assay, dilution factor is 375 (1000 µL÷2.66 µL = 375)

Example:

a. 100 ul urine sample + 100 DDW + 800 µL PCA = Total volume: 1000 µL (100 µL of urine/1000 µL)
b. Take 800 µL of SN (80 µL of urine) + 75 µL Buffer + 125 µL $KHCO_3$ = Total volume: 1000 µL (80 µL of urine/1000 ul)
c. Take 450 µL of SN (36 µL of urine) + 20 µL buffer = Total voule: 470 µL (36 µL of urine/470 µL)
d. Take 400 µL of SN (30.63 µL of urine) for anion exchange +1.5 mL resin mix + 400 µL DDW = Tv; 2300 µL (30.63 µL of urine/2300 µL)
e. Take 200 µL of SN (2.66 µL of urine) for acetylcarnitine assay
f. 1000 µL ÷ 2.66 µL =375

Preparation for reaction mixtures

a. **Reaction mixture A** (Per 50 assay tubes)
   1. 1.0 ml of 37.5 mM HEPES-KOH, pH 7.4
   2. 10 µl of CoASH (20 µg/µl; 5 mg CoA in 250 µl of DDW)
   3. 15 µl of DTT (20 µg/µl; 5 mg DTT in 250 µl of water; 10 mg DTT in 500 µl of water)
   4. Add 475 µl of 37.5 mM HEPES-KOH, pH 7.4 (Total volume is 1.5 mL.)
   5. Vortex, let stand at room temperature for 20 min and place in ice bath until ready to use

b. **Reaction mixture B** (Per 50 assay tubes)
   1. 277 µl of 66.7 mM HEPES-KOH-1.96 mM EDTA, pH 7.4
   2. 8 µl of α-ketoglutarate (21.2 µg/µl: 5 mg in 234 µl of DDW)
   3. 1 µl of GOT
   4. 40 µl of [U-^{14}C]Aspartate (50 µCi/ 1 ml of DDW)
   5. Vortex and let stand at room temperature for 10 min.
   6. Add, while vortexing, 37 µl of 1 M PCA.
   7. Let stand on ice for 15 min, then add 668 µl of 61.6 mM KOH-9.87 EDTA.
   8. Vortex and use immediately.

c. **Reaction mixture C** (Per 50 assay tubes)
   1. Pipette 878 µl of DDW into a test tube.
   2. Add 22.7 µl of citrate synthetase (CS) to equal 0.5 units/assay tube.
   3. Add 110 µl of carnitine acyl transferase (CAT) to equal 0.5 units/assay tube.
   4. Mix and keep on ice until use.

d. **Reaction mixture D** (Per 50 assay tubes)
   1. Pipette 1.351 ml of water in a test tube.
   2. Add 37.5 mg of sodium glutamate.
   3. Vortex until glutamate is completely dissolved.
   4. Add 125 µl of 1:22 diluted GOT (6 µl in 127 µl of water)
   5. Mix and keep in ice until use.

Reactions

1. Add reaction mixture A to reaction mixture B and mix. Immediately pipette 50 µl of this mixture (A+B) into each assay tube.

2. Add 20 µl of the reaction mixture C and mix. Incubate at room temperature for 20 minutes.

3. Add 30 µl of the reaction mixture D and mix. Incubate at room temperature for an additional 20 min.

Cation Exchange Resin: Use Dowex 50x8, 200-400 mesh, H^+ form.

1. Make Dowex slurry (4 gm resin: 6 ml DDW).
2. Add 0.75 ml of Dowex 50x8 slurry and cap.
3. Vortex and place on ice.
4. Vortex an additional 2 times over 20 min and a maximum time period of 90 min.
5. After the last vortex, centrifuge for 1 min in a micro-centrifuge tube.

Count

1. Transfer 0.2 mL of the SN to scintillation vials. Add 4.5 ml of Fisher BD Scintillation cocktail.

2. Count for 10 min or to 1.0 % error in a Beckman LS counter.

References

1. Cederblad et al. (1990)
2. Di Lisa et al. (1992)
3. Jackson and Lee (1996)
4. Pande and Caramancion (1981)

APPENDIX B

FREE FATTY ACIDS ASSAY

Serum and tissue nonesterified fatty acids are determined by the method of Novak (1965). The free fatty acids are extracted in Dole's Extraction Mixture (DEM) and partitioned into heptane where they formed soaps with cobalt. A color complex was formed with alpha nitroso-beta-naphthol.

Reagents

1. Solution A:
   a. Prepare a saturated potassium sulfate solution by adding several grams of the solute to 150 ml boiling water.
   b. Store the solution in the presence of the extra crystals overnight and filter before use.
   c. In a 100 ml volumetric flask, add 6 g of cobalt nitrate and 0.8 ml of glacial acetic acid and dilute to volume with the saturated potassium sulfate solution .
   d. Store at room temperature.

2. Solution B:
   a. Prepare a saturated sodium sulfate solution by adding several grams of sodium sulfate to boiling water.
   b. Filter the solution next day and store at room temperature.

3. Cobalt reagent: Must make fresh each analysis. (Use 500 µl /Assay)
   Mix 1.35 volumes of triethanolamine, 10 volumes of solution A, and 7 volumes of solution B.
   Store at room temperature.

4. Indicator:
   A stock solution of indicator:
   a. Dissolve 400 mg of alpha nitroso-beta-naphthol in 96% (v/v) ethanol and diluted to 100 ml.
   b. To make 96% of ethanol: Mix 96 ml of absolute ethanol and 4 ml of DDW.

Working indicator:(Use 750 µl/Assay)

a. Dilute the stock indicator by a factor of 12.5 with 96% ethanol before use. (Indicator: ethanol = 1: 11.5)

5. Dole's Extraction Mixture (DEM): (use 250 µl/Assay)
   a. Mix:
      40 volumes of glass distilled isopropanol,
      10 volumes of glass distilled heptane, and
      1 volume 1N sulfuric acid
   b. For the 1N sulfuric acid: Add 14 ml of concentrated sulfuric acid to DDW, and diluted to the final volume to 500 ml. Store at room temperature.
   c. Store at refrigerator as an organic solvents.

6. Chloroform-heptane: (use 500 µl/Assay)
   a. Mix:
      5 volumes of glass distilled chloroform and
      1 volume of glass distilled heptane
   b. Store at refrigerator for organic solvents in the hallway.

7. Palmitic acid standard; 0.4 mEq/L (Palmitic acid; 256.4 g/mol)
   a. Dissolve 10.2 mg palmitic acid in DEM and diluted to 100 ml.
   b. Store at a freezer.

Assay Procedure

1. Prepare blank, standards and samples as following:

tube #	STD: Palmitic acid: 0.4 mEq/L (µl)	DEM (µl)	Water (µl)
Blank	0	250	50
Sample	0	250	50
STD 1	25 (0.01 µEq)	275	0
STD 2	75 (0.03 µEq)	225	0
STD 3	125 (0.05 µEq)	175	0
STD 4	175 (0.07 µEq)	125	0
STD 5	250 (0.1 µEq)	50	0

2. Add 250 µl of Dole's Extraction Mixture (DEM) and 50 ul of serum (or tissue homogenate) to 1.5 ml micro-centrifuge tubes, mix, and cool in an ice bath for 10 min.

3. Add 400 µl of heptane and 500 µl of water and vortex, develop a separation of phases.

4. Transfer 300 µl of the upper heptane phase to 2 ml micro-centrifuged tubes.

5. Add 500 µl of chloroform-heptane and 500 µl of cobalt reagent. Mix. The tubes were tightly capped, and placed in a shaker and vigorously mixed for 3 minutes.

6. Centrifuge tubes at 1500 X g for at least 15 min.

7. Transfer 600 µl of upper chlorofom-heptane phase to new micro-centrifuge tubes containing 750 µl of the working indicator. Be careful not to disturb the lower dark phase.

8. After a wait of 30 min measure absorbance of the samples and standards at 500 nm against the blank.

Calculations

Nonesterified fatty acids (mEq/L) = absorbance X inverse slope x dilution factor

APPENDIX C

MEAN NUTRIENT INTAKES: FACTORIAL ANALYSIS

TABLE A-1

Mean nutrient intakes in three groups: Effects of group, gender and their interaction[1]

Variables	Placebo	%kcal	Supplement 1	%kcal	Supplement 2	%kcal	Statistical Significance		
							Group	Gender	GxG
Energy (kcal)									
0-d	1748 ± 88		1879 ± 185		2007 ± 278		NS	0.0001	NS
7-d	1840 ± 153		1925 ± 264		2223 ± 136		NS	0.0199	NS
14-d	1658 ± 164		1826 ± 386		2296 ± 235		NS	0.0014*	NS
21-d	1758 ± 335		2186 ± 383		2085 ± 262		NS	0.0007	NS
35-d	1857 ± 154		1974 ± 199		2122 ± 153		NS	NS	NS
Protein (g)									
0-d	61 ± 6.0	14 ± 0.9	71 ± 7.8	15 ± 1.5	75 ± 10	15 ± 1.6	NS	0.0340	NS
7-d	80 ± 13	17 ± 1.7	70 ± 8.0	15 ± 1.9	75 ± 9.3	13 ± 1.5	NS	0.0126	NS
14-d	59 ± 8.8	14 ± 0.9	72 ± 15	16 ± 1.0	78 ± 9.6	14 ± 1.1	NS	0.0012	NS
21-d	59 ± 10	14 ± 0.5	80 ± 13	15 ± 1.4	76 ± 13	15 ± 2.0	NS	0.0004	NS
35-d	70 ± 9.4	15 ± 1.8	69 ± 9.0	15 ± 1.8	78 ± 10	15 ± 1.9	NS	NS	NS
Carbohydrate (g)									
0-d	258 ± 22	59 ± 2.5	260 ± 30	55 ± 2.7	278 ± 38	56 ± 2.2	NS	0.0001	NS
7-d	239 ± 11	53 ± 3.6	271 ± 39	56 ± 3.2	322 ± 25	58 ± 2.9	NS	0.0451*	NS
14-d	224 ± 14	56 ± 4.1	261 ± 41	61 ± 3.5	307 ± 25	55 ± 4.8	NS	0.0278*	NS
21-d	226 ± 42	52 ± 2.5	307 ± 41	58 ± 3.3	280 ± 43	52 ± 4.2	NS	0.0017	NS
35-d	253 ± 28	55 ± 5.6	285 ± 34	57 ± 4.3	291 ± 33	54 ± 2.8	NS	NS	NS

[1]Values are expressed as mean ± SEM. GxG indicates a group and gender interaction. NS indicates no significant difference ($P > 0.05$) * indicates significant difference between the groups ($P < 0.05$), when comparisons were made only in female subjects.

TABLE A-1

Mean nutrient intakes in three groups: Effects of group, gender and their interaction[1] (Continued)

Variables	Placebo	%kcal	Supplement 1	%kcal	Supplement 2	%kcal	Statistical Significance		
							Group	Gender	GxG
Fat (g)									
0-d	50 ± 4.8	25 ± 1.6	64 ± 6.8	31 ± 2.5	67 ± 12	29 ± 1.8	NS	0.0074	NS
7-d	61 ± 8.7	30 ± 2.2	66 ± 13	30 ± 2.3	76 ± 7.5	31 ± 2.0	NS	NS	NS
14-d	55 ± 11	29 ± 3.7	56 ± 19	24 ± 3.8	87 ± 18	33 ± 3.5	NS	0.0046*	NS
21-d	69 ± 15	34 ± 2.2	69 ± 19	26 ± 2.8	78 ± 10	35 ± 2.7	NS	0.0064	NS
35-d	59 ± 9.5	28 ± 2.9	64 ± 11	29 ± 3.9	75 ± 6.7	32 ± 2.6	NS	NS	NS
Cholesterol (mg)									
0-d	142 ± 22		196 ± 42		170 ± 42		NS	NS	NS
7-d	202 ± 36		162 ± 28		212 ± 42		NS	NS	NS
14-d	138 ± 58		145 ± 48		256 ± 65		NS	NS	NS
21-d	177 ± 67		241 ± 83		222 ± 57		NS	0.0357	NS
35-d	163 ± 37		114 ± 27		199 ± 22		NS	NS	NS
Saturated fat (g)									
0-d	15 ± 1.9	7.5 ± 0.8	17 ± 1.1	8.5 ± 0.9	21 ± 4.7	9.0 ± 1.0	NS	NS	NS
7-d	20 ± 3.3	9.4 ± 1.0	22 ± 4.8	10 ± 1.5	23 ± 3.1	9.1 ± 0.8	NS	NS	NS
14-d	16 ± 2.6	8.2 ± 0.9	16 ± 5.5	7.0 ± 1.4	29 ± 7.7	11 ± 1.6	NS	0.0045*	NS
21-d	20 ± 4.9	9.8 ± 1.3	20 ± 5.2	7.7 ± 0.9	26 ± 5.2	11 ± 1.4	NS	0.0195	NS
35-d	18 ± 2.8	8.6 ± 1.3	20 ± 3.8	9.0 ± 1.5	22 ± 3.2	9.3 ± 1.1	NS	NS	NS
Monounsaturated fat (g)									
0-d	12 ± 1.4	6.3 ± 0.6	17 ± 1.9	8.4 ± 0.7	16 ± 1.5	7.6 ± 0.8	NS	NS	NS
7-d	15 ± 1.8	7.5 ± 0.6	12 ± 1.3	5.8 ± 1.0	18 ± 3.1	7.3 ± 1.2	NS	NS	NS
14-d	17 ± 4.4	9.2 ± 1.8	16 ± 7.4	6.0 ± 1.8	26 ± 2.3	11 ± 0.8	NS	0.0032*	NS
21-d	17 ± 5.6	8.0 ± 1.3	17 ± 8.0	6.1 ± 1.7	23 ± 3.0	10 ± 1.1	NS	0.0091	NS
35-d	14 ± 2.4	7.0 ± 1.0	19 ± 2.4	8.5 ± 0.7	22 ± 2.9	9.5 ± 1.1	NS	NS	NS

[1]Values are mean ± SEM. * indicates significant difference between the groups ($P < 0.05$), when comparisons were made only in female subjects.

TABLE A-1

Mean nutrient intakes in three groups: Effects of group, gender and their interaction[1] (Continued)

Variables	Placebo	%kcal	Supplement 1	%kcal	Supplement 2	%kcal	Statistical Significance		
							Group	Gender	GxG
Polyunsaturated fat (g)									
0-d*	7.2 ± 1.1	3.7 ± 0.5	18 ± 3.2	8.5 ± 1.5	13 ± 2.9	6.0 ± 0.7	NS	NS	NS
7-d	14 ± 3.8	6.5 ± 1.3	12 ± 4.6	5.1 ± 1.2	19 ± 3.0	7.6 ± 1.1	NS	NS	NS
14-d	10 ± 1.9	5.7 ± 0.9	11 ± 4.3	4.3 ± 1.2	16 ± 3.9	6.1 ± 1.2	NS	0.0143*	NS
21-d	16 ± 4.7	7.4 ± 1.5	10 ± 3.5	3.9 ± 0.6	14 ± 2.0	6.2 ± 1.0	NS	0.0470	NS
35-d	11 ± 2.4	4.9 ± 0.8	13 ± 5.4	5.8 ± 2.1	13 ± 2.5	5.5 ± 1.0	NS	NS	NS
Dietary fiber (g)									
0-d	10 ± 2.2		11 ± 2.5		13 ± 1.8		NS	NS	NS
7-d	10 ± 1.5		13 ± 2.5		13 ± 2.0		NS	NS	NS
14-d	10 ± 1.6		11 ± 1.9		15 ± 2.2		NS	NS	NS
21-d	10 ± 2.0		12 ± 2.8		12 ± 2.3		NS	NS	NS
35-d	12 ± 1.7		14 ± 2.7		13 ± 2.3		NS	NS	NS
Sugar (g)									
0-d	91 ± 15		69 ± 15		89 ± 22		NS	0.0076	NS
7-d	85 ± 10		112 ± 22		135 ± 28		NS	NS	NS
14-d	60 ± 14		77 ± 14		92 ± 29		NS	NS	NS
21-d	72 ± 18		100 ± 12		97 ± 19		NS	0.0100	NS
35-d	74 ± 14		89 ± 17		93 ± 22		NS	0.0461	NS
Vitamin A (µg RE)									
0-d	795 ± 159		744 ± 101		848 ± 132		NS	NS	NS
7-d	537 ± 81		483 ± 118		738 ± 139		NS	NS	NS
14-d	721 ± 157		621 ± 254		597 ± 116		NS	NS	NS
21-d	908 ± 209		888 ± 244		940 ± 346		NS	NS	NS
35-d	847 ± 451		1126 ± 253		1068 ± 570		NS	NS	NS

[1]Values are mean ± SEM. GxG indicates a group and gender interaction. NS indicates no significant difference ($P > 0.05$)

* indicates significant difference between the groups ($P < 0.05$), when comparisons were made only in female subjects.

TABLE A-1

Mean nutrient intakes in three groups: Effects of group, gender and their interaction[1] (Continued)

Variables	Placebo	%kcal	Supplement 1	%kcal	Supplement 2	%kcal	Statistical Significance		
							Group	Gender	GxG
β-carotene (μg)									
0-d	55 ± 34		164 ± 68		336 ± 107		NS	NS	NS
7-d	92 ± 31		161 ± 92		212 ± 141		NS	NS	NS
14-d	123 ± 50		224 ± 173		65 ± 20		NS	NS	NS
21-d	47 ± 28		132 ± 53		126 ± 48		NS	NS	NS
35-d	221 ± 80		225 ± 102		346 ± 215		NS	NS	NS
Thiamin (mg)									
0-d	1.1 ± 0.2		1.6 ± 0.2		1.7 ± 0.3		NS	NS	NS
7-d	1.3 ± 0.2		1.3 ± 0.3		1.6 ± 0.3		NS	NS	NS
14-d	1.2 ± 0.2		1.2 ± 0.3		2.3 ± 0.8		NS	NS	NS
21-d	1.6 ± 0.4		1.6 ± 0.3		1.2 ± 0.1		NS	NS	NS
35-d	1.2 ± 0.3		1.4 ± 0.3		2.5 ± 0.8		NS	NS	NS
Riboflavin (mg)									
0-d	1.4 ± 0.2		1.6 ± 0.2		1.7 ± 0.2		NS	NS	NS
7-d	1.4 ± 0.2		1.1 ± 0.3		1.8 ± 0.3		NS	NS	NS
14-d	1.3 ± 0.1		1.2 ± 0.4		1.5 ± 0.1		NS	NS	NS
21-d	1.7 ± 0.3		1.6 ± 0.3		1.5 ± 0.2		NS	NS	NS
35-d	1.4 ± 0.2		1.6 ± 0.2		1.7 ± 0.3		NS	NS	NS
Niacin (mg)									
0-d	16 ± 2.0		19 ± 3.0		21 ± 2.8		NS	0.0137	NS
7-d	27 ± 3.6		20 ± 2.2		20 ± 3.0		0.0226	0.0164*	NS
14-d	16 ± 1.9		17 ± 4.4		25 ± 4.3		NS	0.0044	NS
21-d	17 ± 2.2		19 ± 3.8		22 ± 4.1		NS	0.0052	NS
35-d	19 ± 5.1		19 ± 2.6		22 ± 2.9		NS	NS	NS

[1]Values are mean ± SEM. GxG indicates a group and gender interaction. NS indicates no significant difference ($P > 0.05$)

* indicates significant difference between the groups ($P < 0.05$), when comparisons were made only in female subjects.

TABLE A-1

Mean nutrient intakes in three groups: Effects of group, gender and their interaction[1] (Continued)

Variables	Placebo	Supplement 1	Supplement 2	Statistical Significance		
				Group	Gender	GxG
Vitamin B_6 : pyridoxine (mg)						
0-d	1.0 ± 0.2	1.4 ± 0.2	1.3 ± 0.2	NS	NS	NS
7-d	1.4 ± 0.2	1.1 ± 0.2	1.3 ± 0.2	NS	NS	NS
14-d	1.0 ± 0.2	1.0 ± 0.2	1.7 ± 0.3	NS	NS	NS
21-d	1.1 ± 0.1	1.1 ± 0.2	1.3 ± 0.1	NS	NS	NS
35-d	1.2 ± 0.3	1.5 ± 0.3	1.7 ± 0.4	NS	NS	NS
Vitamin B_{12} (µg)						
0-d	2.7 ± 0.7	2.9 ± 0.6	2.7 ± 0.6	NS	NS	NS
7-d	3.4 ± 0.7	2.7 ± 0.8	2.4 ± 0.5	NS	NS	NS
14-d	1.8 ± 0.3	2.5 ± 1.1	3.1 ± 1.0	NS	NS	NS
21-d	3.6 ± 0.9	2.6 ± 0.7	2.6 ± 0.5	NS	NS	NS
35-d	1.8 ± 0.4	2.3 ± 0.6	2.4 ± 0.9	NS	NS	NS
Vitamin C (mg)						
0-d	99 ± 15	79 ± 13	77 ± 16	NS	NS	NS
7-d	83 ± 17	114 ± 18	90 ± 18	NS	NS	NS
14-d	80 ± 31	58 ± 27	103 ± 25	NS	NS	NS
21-d	84 ± 21	94 ± 35	141 ± 56	NS	NS	NS
35-d	77 ± 18	109 ± 28	111 ± 38	NS	NS	NS
Vitamin D (µg)						
0-d	2.2 ± 0.9	3.1 ± 1.3	2.7 ± 1.0	NS	NS	NS
7-d	1.8 ± 0.7	1.1 ± 0.6	1.8 ± 0.9	NS	NS	NS
14-d	1.1 ± 0.4	2.5 ± 1.7	1.6 ± 0.5	NS	NS	NS
21-d	2.9 ± 0.9	3.5 ± 1.4	1.9 ± 0.4	NS	NS	NS
35-d	1.5 ± 0.5	2.4 ± 0.9	2.2 ± 1.3	NS	NS	NS

[1]Values are mean ± SEM. GxG indicates a group and gender interaction. NS indicates no significant difference ($P > 0.05$)

* indicates significant difference between the groups ($P < 0.05$), when comparisons were made only in female subjects.

TABLE A-1

Mean nutrient intakes in three groups: Effects of group, gender and their interaction[1] (Continued)

Variables	Placebo	Supplement 1	Supplement 2	Statistical Significance		
				Group	Gender	GxG
Vitamin E (mg α-TE)						
0-d*	3.7 ± 1.3	13 ± 2.9	5.4 ± 1.9	0.0179	NS	NS
7-d	12 ± 3.3	6.6 ± 1.5	9.6 ± 3.0	NS	NS	NS
14-d	7.7 ± 3.3	6.1 ± 2.0	11 ± 4.1	NS	NS	NS
21-d	14 ± 4.8	3.4 ± 1.2	8.4 ± 2.2	NS	NS	NS
35-d	5.5 ± 3.3	8.6 ± 2.5	4.3 ± 1.2	NS	NS	NS
α-Tocopherol (mg)						
0-d	2.4 ± 0.6	11 ± 4.6	3.2 ± 0.8	NS	NS	NS
7-d	4.3 ± 0.6	4.1 ± 0.9	6.1 ± 1.4	NS	NS	NS
14-d	3.9 ± 0.8	3.8 ± 1.2	3.8 ± 0.8	NS	NS	NS
21-d	4.3 ± 1.2	4.4 ± 1.6	4.0 ± 0.8	NS	NS	NS
35-d	3.4 ± 1.2	9.2 ± 5.7	4.0 ± 0.8	NS	NS	NS
Folate (μg)						
0-d	213 ± 45	214 ± 45	186 ± 39	NS	NS	NS
7-d	186 ± 40	204 ± 22	201 ± 28	NS	NS	NS
14-d	214 ± 44	170 ± 44	233 ± 44	NS	NS	NS
21-d	232 ± 37	241 ± 77	184 ± 33	NS	NS	NS
35-d	183 ± 28	242 ± 40	228 ± 57	NS	NS	NS
Pantothenic acid (mg)						
0-d*	2.0 ± 0.4	4.1 ± 0.7	2.4 ± 0.5	0.0408	NS	NS
7-d	2.8 ± 0.2	2.0 ± 0.3	2.5 ± 0.4	NS	NS	NS
14-d	2.0 ± 0.4	2.5 ± 0.7	3.5 ± 0.5	NS	NS	NS
21-d	2.7 ± 0.7	3.2 ± 1.0	2.8 ± 0.6	NS	NS	NS
35-d	1.9 ± 0.4	4.2 ± 0.7	3.3 ± 1.0	NS	NS	NS

[1]Values are mean ± SEM. GxG indicates a group and gender interaction. NS indicates no significant difference ($P > 0.05$)
* indicates significant difference between the groups ($P < 0.05$), when comparisons were made only in female subjects.

TABLE A-1

Mean nutrient intakes in three groups: Effects of group, gender and their interaction[1] (Continued)

Variables	Placebo	Supplement 1	Supplement 2	Statistical Significance		
				Group	Gender	GxG
Biotin (μg)						
0-d	4.3 ± 1.8	7.6 ± 2.5	7.8 ± 2.7	NS	NS	NS
7-d	7.2 ± 2.2	5.1 ± 1.7	8.9 ± 2.5	NS	NS	NS
14-d	6.5 ± 1.9	4.7 ± 1.8	14 ± 4.4	NS	NS	NS
21-d	12 ± 3.2	12 ± 4.9	9.0 ± 3.1	NS	NS	NS
35-d	4.4 ± 1.5	5.7 ± 1.6	6.5 ± 2.7	NS	NS	NS
Vitamin K (μg)						
0-d	237 ± 104	138 ± 61	137 ± 62	NS	NS	NS
7-d	240 ± 68	122 ± 21	138 ± 64	NS	NS	NS
14-d	178 ± 44	148 ± 42	118 ± 50	NS	NS	NS
21-d	234 ± 48	227 ± 59	143 ± 43	NS	NS	NS
35-d	161 ± 61	171 ± 42	114 ± 54	NS	NS	NS
Sodium (mg)						
0-d*	2909 ± 397	2729 ± 212	3877 ± 253	0.0444	NS	NS
7-d	2627 ± 269	2873 ± 481	3437 ± 468	NS	0.0481	NS
14-d	2646 ± 227	2893 ± 563	3075 ± 314	NS	0.0017	NS
21-d	2579 ± 504	3415 ± 672	3058 ± 410	NS	0.0090	NS
35-d	2978 ± 238	3690 ± 545	3010 ± 369	NS	0.0024	NS
Potassium (mg)						
0-d	2044 ± 244	2110 ± 320	2402 ± 318	NS	NS	NS
7-d	2388 ± 273	2158 ± 295	2594 ± 142	NS	NS	NS
14-d	1848 ± 197	2041 ± 466	2465 ± 215	NS	0.0013	NS
21-d	1989 ± 233	2594 ± 592	2468 ± 257	NS	0.0037	NS
35-d	2209 ± 138	2578 ± 358	2538 ± 323	NS	NS	NS

[1]Values are mean ± SEM. GxG indicates a group and gender interaction. NS indicates no significant difference ($P > 0.05$)

* indicates significant difference between the groups ($P < 0.05$), when comparisons were made only in female subjects.

TABLE A-1

Mean nutrient intakes in three groups: Effects of group, gender and their interaction[1] (Continued)

Variables	Placebo	Supplement 1	Supplement 2	Statistical Significance		
				Group	Gender	GxG
Iron (mg)						
0-d	10 ± 1.1	12 ± 2.6	13 ± 1.7	NS	NS	NS
7-d	12 ± 1.5	12 ± 2.1	14 ± 1.3	NS	0.0052	NS
14-d	11 ± 1.3	11 ± 3.0	15 ± 2.1	NS	0.0292*	NS
21-d	16 ± 2.5	14 ± 3.8	11 ± 1.2	NS	0.0038	NS
35-d	14 ± 3.4	14 ± 1.9	14 ± 1.1	NS	NS	NS
Calcium (mg)						
0-d	664 ± 68	779 ± 151	888 ± 119	NS	0.0047	NS
7-d	601 ± 109	656 ± 202	856 ± 103	NS	0.0067	NS
14-d	545 ± 81	701 ± 214	779 ± 114	NS	0.0125	NS
21-d	750 ± 152	831 ± 198	788 ± 104	NS	0.0490	NS
35-d	766 ± 74	743 ± 86	873 ± 191	NS	NS	NS
Magnesium (mg)						
0-d	189 ± 26	254 ± 40	221 ± 28	NS	0.0207	NS
7-d	225 ± 27	195 ± 23	226 ± 20	NS	NS	NS
14-d	186 ± 21	208 ± 57	273 ± 42	NS	0.0154	NS
21-d	197 ± 25	251 ± 54	194 ± 28	NS	0.0005	NS
35-d	180 ± 25	274 ± 33	257 ± 45	NS	NS	NS
Phosphorus (mg)						
0-d	871 ± 117	1253 ± 142	1316 ± 189	NS	0.0011	NS
7-d	1106 ± 133	977 ± 182	1140 ± 159	NS	0.0022	NS
14-d	793 ± 78	932 ± 228	1098 ± 118	NS	0.0051	NS
21-d	1030 ± 166	1285 ± 288	1186 ± 189	NS	0.0007	NS
35-d	746 ± 112	1215 ± 161	1192 ± 147	NS	NS	NS

[1]Values are mean ± SEM. GxG indicates a group and gender interaction. NS indicates no significant difference ($P > 0.05$)

* indicates significant difference between the groups ($P < 0.05$), when comparisons were made only in female subjects.

TABLE A-1

Mean nutrient intakes in three groups: Effects of group, gender and their interaction[1] (Continued)

Variables	Placebo	Supplement 1	Supplement 2	Statistical Significance		
				Group	Gender	GxG
Zinc (mg)						
0-d	6.0 ± 0.8	8.6 ± 1.3	7.7 ± 1.1	NS	NS	NS
7-d	7.9 ± 1.4	6.0 ± 1.2	7.4 ± 1.2	NS	NS	NS
14-d	6.6 ± 0.8	6.2 ± 2.1	8.3 ± 0.9	NS	0.0136*	NS
21-d	9.5 ± 3.9	7.2 ± 1.7	13 ± 5.4	NS	NS	NS
35-d	5.4 ± 1.0	7.9 ± 1.2	8.0 ± 1.9	NS	NS	NS
Copper (mg)						
0-d	0.7 ± 0.1	1.4 ± 0.4	0.9 ± 0.1	NS	NS	NS
7-d	1.3 ± 0.4	0.8 ± 0.1	0.9 ± 0.04	NS	NS	NS
14-d	0.7 ± 0.1	1.0 ± 0.3	1.2 ± 0.2	NS	0.0251	NS
21-d	1.1 ± 0.3	1.0 ± 0.2	0.9 ± 0.1	NS	NS	NS
35-d	0.8 ± 0.2	1.3 ± 0.1	1.1 ± 0.2	NS	NS	NS
Selenium (µg)						
0-d	30 ± 7.9	92 ± 25	42 ± 14	NS	NS	NS
7-d	51 ± 11	58 ± 15	33 ± 11	NS	NS	NS
14-d*	23 ± 7.3	35 ± 7.4	79 ± 20	0.0265	NS	NS
21-d	23 ± 6.0	56 ± 17	53 ± 11	NS	NS	NS
35-d	51 ± 23	119 ± 49	80 ± 24	NS	NS	NS
Chromium (µg)						
0-d	42 ± 16	47 ± 9.8	33 ± 13	NS	NS	NS
7-d	54 ± 7.6	70 ± 15	33 ± 11	NS	0.0452	NS
14-d	33 ± 10	36 ± 14	92 ± 31	NS	NS	NS
21-d	77 ± 19	81 ± 19	50 ± 8.3	NS	NS	NS
35-d	37 ± 16	33 ± 9.5	37 ± 15	NS	NS	NS

[1]Values are mean ± SEM. GxG indicates a group and gender interaction. NS indicates no significant difference ($P > 0.05$)

* indicates significant difference between the groups ($P < 0.05$), when comparisons were made only in female subjects.

TABLE A-2

Mean caffeine intakes in three groups: Effects of group, gender and their interaction[1]

Variables	Placebo	Supplement 1	Supplement 2	Statistical Significance		
				Group	Gender	GxG
Caffeine (mg)						
0-d	234 ± 46	167 ± 55	196 ± 64	NS	0.0173	NS
7-d	256 ± 38	174 ± 46	262 ± 58	NS	NS	NS
14-d	269 ± 67	226 ± 67	236 ± 66	NS	NS	NS
21-d	309 ± 69	297 ± 93	259 ± 73	NS	NS	NS
35-d	268 ± 97	231 ± 71	223 ± 68	NS	NS	NS

[1]Values are expressed as mean ± SEM. GxG indicates a group and gender interaction.
NS indicates no significant difference ($P > 0.05$)

APPENDIX D

MEAN MACRO-NUTRIENT INTAKES PER 1000 KCAL AND KG OF BODY WEIGHT

TABLE A-3

Mean macro-nutrient intakes per 1000 kcal and kg of body weight of participants in three groups[1]

Variables	Placebo		Supplement 1		Supplement 2		Statistical Significance		
							Group	Gender	GxG
Protein	g/kcal	g/kg bw	g/kcal	g/kg bw	g/kcal	g/kg bw			
0-d	35 ± 2.3	0.92 ± 0.08	37 ± 3.7	0.96 ± 0.07	38 ± 4.0	1.02 ± 0.14	NS	NS	NS
7-d	43 ± 4.3	1.17 ± 0.16	39 ± 4.8	0.98 ± 0.10	34 ± 3.8	1.04 ± 0.13	NS	NS	NS
14-d	35 ± 2.3	0.86 ± 0.11	40 ± 2.5	0.95 ± 0.13	34 ± 2.9	1.04 ± 0.11	NS	0.0097(/bw)	NS
21-d	34 ± 1.1	0.91 ± 0.20	38 ± 3.4	1.08 ± 0.10	37 ± 5.0	1.03 ± 0.17	NS	0.0319(/bw)	NS
35-d	38 ± 4.5	1.02 ± 0.09	36 ± 4.5	0.97 ± 0.08	37 ± 4.8	1.06 ± 0.13	NS	NS	NS
Carbohydrate	g/kcal	g/kg bw	g/kcal	g/kg bw	g/kcal	g/kg bw			
0-d	146 ± 6.2	3.91 ± 0.46	138 ± 6.7	3.61 ± 0.28	139 ± 5.6	3.81 ± 0.54	NS	NS	NS
7-d	133 ± 8.2	3.65 ± 0.35	139 ± 8.0	3.77 ± 0.48	145 ± 7.1	4.55 ± 0.59	NS	NS	NS
14-d	140 ± 10	3.42 ± 0.38	151 ± 8.7	3.54 ± 0.38	138 ± 12	4.35 ± 0.60	NS	NS	NS
21-d	131 ± 6.3	3.52 ± 0.83	146 ± 8.2	4.26 ± 0.44	131 ± 11	3.83 ± 0.63	NS	NS	NS
35-d	137 ± 14	3.85 ± 0.55	143 ± 11	4.06 ± 0.46	134 ± 7.0	4.18 ± 0.67	NS	NS	NS
Fat	g/kcal	g/kg bw	g/kcal	g/kg bw	g/kcal	g/kg bw			
0-d	28 ± 1.8	0.77 ± 0.12	35 ± 2.8	0.90 ± 0.08	32 ± 2.0	0.89 ± 0.16	NS	NS	NS
7-d	33 ± 2.4	0.93 ± 0.17	34 ± 2.6	0.88 ± 0.10	34 ± 2.3	1.09 ± 0.18	NS	NS	NS
14-d	32 ± 4.1	0.80 ± 0.14	27 ± 4.2	0.71 ± 0.19	36 ± 3.8	1.14 ± 0.21	NS	0.0103(/bw)	NS
21-d	38 ± 2.4	1.05 ± 0.26	29 ± 3.1*	0.90 ± 0.19	39 ± 3.0	1.07 ± 0.14	NS	NS	NS
35-d	31 ± 3.2	0.84 ± 0.09	32 ± 4.2	0.89 ± 0.14	36 ± 2.9	1.08 ± 0.18	NS	NS	NS

[1]Values are expressed as mean ± SEM. GxG indicates a group and gender interaction. NS indicates no significant difference ($P > 0.05$) * indicates significant difference between the groups ($P < 0.05$), when compared with only female subjects.

VITA

Nobuko Hongu was born in Hyogo, Japan, on January 6, 1957. She attended Mukogawa Women's University and received the Bachelor of Arts in Physical Education with teaching certification in March, 1979. Following graduation, she came to the U.S., entered the Master's Program at the University of Akron, Ohio, and worked on the topic, physical activity and aging. Her Master thesis title was " The relation among age, physical activity level, and isokinetic knee joint-strength among the elderly."

In 1986, she returned to Japan and taught physical education classes at the Mukogawa Women's University and did research on exercise and vegetarian diet in young and old healthy Japanese men and women. After 5 years of teaching and researching at the University, she returned to the U.S., entered the dietetic program at Montclair State University, NJ, and had an internship at the University of Tennessee Medical Center. She passed the Registered Dietitian exam in April, 1996.

She started working at Dr. Sachan's Lab in the summer of 1996. Her research interest has been fat metabolism and nutrient-nutrient interactions. She has made presentations on her works at various scientific meetings and published four papers. She received a Ph.D degree in Human Ecology with a concentration in nutrition in May, 2002.