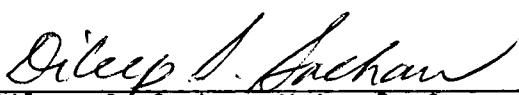
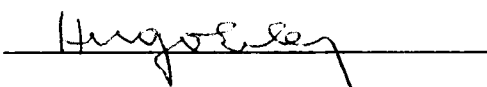
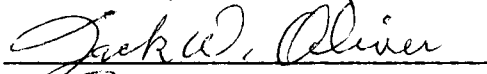


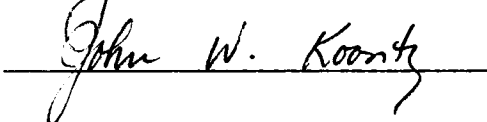
To the Graduate Council:

I am submitting herewith a dissertation written by Robert Berger entitled "Effects of L-Carnitine on Ethanol Metabolism." I have examined the final 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:



Accepted for the Council:


Vice Provost
and Dean of The Graduate School

EFFECTS OF L-CARNITINE ON ETHANOL METABOLISM

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Robert Berger

December 1987

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ABSTRACT

These studies were carried out to define the effects of L-carnitine on ethanol metabolism in Sprague-Dawley rats. In the first phase, it was established that 0.5% (w/w) L-carnitine supplementation for 3 days was optimum to attain blood-carnitine steady state concentrations. The urinary-carnitine profile was similar to that of blood-carnitine and the urinary-steady state concentrations of carnitine were attained after 5 days of supplementation which suggests that urinary-carnitines may be reasonable predictors of blood-carnitine steady state concentrations. The carnitine concentration of skeletal muscle was not affected by different levels of carnitine supplementation. It is concluded that 3 days of carnitine supplementation was adequate to attain blood-steady state concentrations of carnitine.

L-carnitine, like D,L-carnitine, attenuated ethanol metabolism. The effect of carnitine was not on the absorption of ethanol but on its metabolism, since portal blood-ethanol concentrations did not change significantly during the period when systemic blood-ethanol concentrations were significantly elevated. The effect of carnitine was quite specific because it could not be produced by choline.

Administration of carnitine via i.p. and i.v. routes for 3 days prior to ethanol administration retarded ethanol metabolism similarly to that seen following the oral administration of carnitine. These results further strengthen the argument that carnitine affects ethanol metabolism and not its absorption from the G.I. tract. Since the simultaneous

administration of carnitine with ethanol did not attenuate ethanol metabolism, it is proposed that carnitine may modulate cell structure or enzyme systems perhaps in some type of inductive manner.

The effect of carnitine on ethanol metabolism was influenced by the sex of the animal, since female rats were not affected to the same extent as were the male rats. This difference may be attributed to differences in hormonal activity, metabolic rates, volume of distribution, and adiposity of males and females.

Thus it is concluded that carnitine administration via any route prior to ethanol dosage retards ethanol metabolism in male rats significantly more so than in female rats.

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CHAPTER I

INTRODUCTION

Both the acute and chronic use of ethyl alcohol (ethanol) presents various metabolic derangements of the liver, including fatty infiltration (1, 2). It has been established that ethanol administration both in humans and in laboratory animals has consistently resulted in hyperlipidemia especially in the elevations of serum and hepatic triglycerides, that primarily is due to the decreased oxidation of fats (3). Because ethanol has an obligatory hepatic metabolism, in which about 98% of ingested ethanol is metabolized (4), hyperlipidemia and hepatic steatosis are features of the ethanol-induced alterations of fat metabolism (5, 6). These ethanol-induced changes are partly due to a reduced oxidation of fatty acids (6), and to an enhanced fatty acid biosynthesis from readily available acetate and NADPH in the ethanol compromised liver (4).

Carnitine (3-hydroxy-4-N-trimethyl-aminobutyrate) is known to enhance fat metabolism by facilitating fatty acid transport into the mitochondrial matrix where β -oxidation takes place (7, 8). The clinical and experimental use of carnitine has expanded in recent years (9). Carnitine has been used in both cardiac (10, 11) and muscle (11, 12) myopathies in man, in myopathic animal models (13, 14) and in isolated muscle preparations (15). Blood-carnitine concentrations have been replenished in hemodialyzed patients by both oral (16) and intravenous (17) carnitine administration. Intraperitoneal (18, 19) and oral (20)

carnitine administration has been found to be therapeutically beneficial against the toxic effects of hyperammonemia in both animals and humans.

In recent studies it was shown that D,L-carnitine significantly reduced the hepatic lipid content in the chronic alcoholic rat (21, 22). These authors provide a rationale for the conflicting view on adequacy of carnitine in chronic alcoholic cases (23, 24) under the guise of conditional carnitine deficiency.

Blood-ethanol concentrations were significantly elevated in carnitine supplemented rats over nonsupplemented rats under both acute and chronic alcoholic conditions (25). Further, it was reported that supplementary D,L-carnitine diet attenuated clearance of ethanol from the blood of rats after an oral dose of ethanol (26). Because the racemic mixture of carnitine had been used in all these studies, it was uncertain as to whether these effects were due to the L-isomer, or if the results were due to adverse effects of the D-carnitine moiety of the mixture. Thus it was deemed necessary to answer the following questions:

1. What would be the optimal dose and duration of L-carnitine supplementation necessary to reach steady state concentrations in the blood?
2. Once steady state blood-carnitine concentrations are attained, would a carnitine attenuate ethanol metabolism?
3. Would this effect be specific to carnitine or would other compounds like choline cause similar effects?

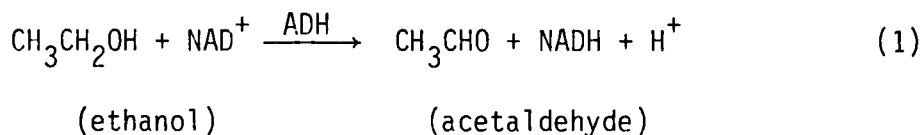
4. Could the carnitine effect be shown when administered parenterally as well as when administered enterally?
5. Would carnitine show this effect at different levels of ethanol administration?
6. In what sequence and for what duration must carnitine be administered to cause attenuation of ethanol metabolism?
7. Could the carnitine effect be the same in male and female rats?

CHAPTER II

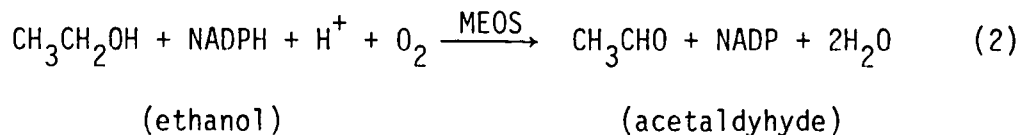
REVIEW OF LITERATURE

Ethanol Metabolism

The major pathway of ethanol metabolism involves the cytosolic enzyme, alcohol dehydrogenase (ADH), which catalyzes the conversion of ethanol to acetaldehyde with concomitant reduction of the cofactor NAD^+ to $\text{NADH} + \text{H}^+$ (27). The low K_m for ADH (1 mM) makes this enzyme very important for the in vivo metabolism of ethanol (27, 28).

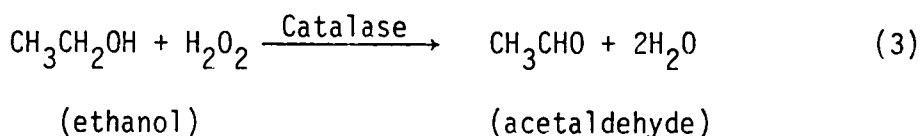


Because of the low K_m for ADH there is an increased NADH/NAD ratio (28). Ethanol also may be oxidized through an alternate pathway known as the hepatic Microsomal Ethanol Oxidizing System (MEOS), a component of the Mixed Function Oxidase System (MFOS), which is responsible for the metabolism of various drugs and environmental chemicals in the environment (29).

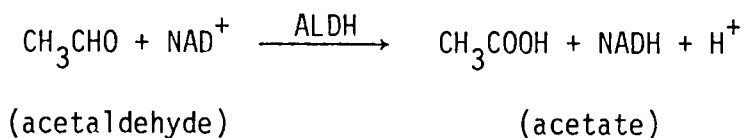


The MEOS pathway for ethanol oxidation is localized in the endoplasmic reticulum (29). Thus, when ethanol is administered in conjunction with a drug, the metabolism of either could be altered due to a synergistic

and/or antagonistic effect of one on the other, possibly by affecting a common metabolic pathway or receptor site (30, 31). There is a third pathway for ethanol oxidation, the catalase system found in the peroxisomes (32), physiologically which plays a minor role.



Under normal physiological conditions, the oxidation of ethanol proceeds primarily by the cytosolic ADH system (27). All three oxidative pathways produce the common metabolite, acetaldehyde. Acetaldehyde is then oxidized to acetate by aldehyde dehydrogenase (ALDH) primarily in the mitochondria (27, 28).



The metabolism of ethanol by ADH and acetaldehyde by ALDH generate reducing equivalents ($\text{NADH} + \text{H}^+$) that can then be used for mitochondrial ATP synthesis (27, 28).

Metabolic Effects of Ethanol

Through the electron transport process and oxidative phosphorylation, the energy gained during ethanol and acetaldehyde oxidation may be used for ATP production, for driving cellular processes, and for the biosynthesis of cellular compounds. In a study by Veech et al. (33), it

was found that within 15 minutes postadministration of an acute intra-peritoneal ethanol dose (2 g/kg body weight) to rats, both hepatic cell mitochondrial and cytosolic NAD/NADH ratios had decreased while ATP levels rose, illustrating an increase in phosphorylation potential. Ryle et al. (34) found that when fasted male rats were administered this same dose of ethanol (2 g/kg i.p.), that there was a 64% decrease in the cytosolic free NAD/NADH ratio, and a 41% decrease in the mitochondrial free NAD/NADH ratio, at 90 minutes postethanol injection. Thus, after acute ethanol administration, reducing equivalents are rapidly formed in the cytosol for transport into the mitochondria to drive cellular energy processes. Since NADH is formed in the cytoplasm, it must be shuttled into the mitochondria for ATP synthesis, with the major shuttle for this transport being the malate-aspartate system (35, 36). Although at various times, such as in fasting, shuttle intermediates may become depleted causing mitochondrial transport and NADH reoxidation to be rate limiting (37), the mitochondrial shuttle is capable of operating at rates in excess of those found during ethanol metabolism (35).

While small single doses of ethanol, whose metabolism involves the ADH pathway, provides energy by shuttling reducing equivalents into the mitochondria, chronic and/or large doses of ethanol involve the MEOS (27, 38) which is bound to the smooth endoplasmic reticulum (ER) of the hepatocyte (38). The MEOS has a higher K_m (8-10 mM) as compared to a K_m of 1 mM for ADH (27, 39), suggesting that this enzyme system is only active at high ethanol concentrations. The MEOS also differs from ADH in that its activity increases after chronic ethanol feeding due to its

induction in animals (40, 41) as well as in humans (31, 42). Thus, MEOS may be metabolically more important in alcoholics than in average consumers of alcohol. Shigeta et al. (38) found that deermice which lacked the ADH enzyme (ADH negative or ADH^{-}) metabolized ethanol at a rate almost 2/3 that of the ADH positive (ADH^{+}) strain. As the animals were chronically exposed to ethanol, both ADH^{+} and ADH^{-} mice exhibited faster rates of ethanol metabolism. Results of this study (38), as well as that of a later one (43), revealed that there are multiple forms of cytochrome P-450 in the rat and that chronic ethanol consumption induces a form that is distinct from those induced by other drugs or foreign compounds. The induction of MEOS activity in the microsomes of deermice, both in ADH^{+} and ADH^{-} strains, resulted in different morphological changes (38). Electron microscopy revealed that the smooth endoplasmic reticulum of the ADH^{-} hepatocytes was more abundant than those of the ADH^{+} strain. The most striking cellular changes were noted after chronic ethanol consumption in the ADH^{-} deermice, whose livers were packed with the proliferated membranes of the smooth ER. Results of these studies thus revealed that chronic ethanol intake induces its own metabolism by increasing MEOS activity, and that when ADH activity is either low or absent, the MEOS may exhibit a metabolic compensatory action.

A third difference between ADH and the MEOS is that NADPH serves as a cofactor for the latter, forming NADP, where ADH uses NAD, forming NADH. Thus, the ADH pathway results in an increase in energy, with reducing equivalents formed from $NAD \rightarrow NADH$ that are shuttled into the mitochondria either for immediate energy via coupling to oxidative

phosphorylation (44-48) or for the synthesis of fatty acids (46, 47, 49, 50) and α -glycerophosphate (46, 47) for triglyceride synthesis (46, 47, 49, 50). The MEOS system results in a loss of energy due to the depletion of $\text{NADPH} + \text{H}^+$ as well as to increased heat production (44, 45, 50).

Effects of Fasting on Ethanol Metabolism

Crabb et al. (51) observed that rats which were fasted exhibited ethanol elimination rates which were 40-50% slower than those of fed rats. Specific activities and kinetic constants were similar in both fed and fasted rats, which the hepatic ADH activity was 40% less with fasting. In an earlier study by Smith and Newman (52), the rate of ethanol oxidation was less in fasted rats compared to fed rats, despite the fact that ethanol increased cellular NADH/NAD ratios to a greater extent in the fasted animals. The results of this study indicated that the reoxidation of NADH to NAD could be an important factor in the rate of ethanol oxidation. In fasted animals, the normal NADH reoxidation mechanism may not be rapid enough to maintain the increased requirement of available NAD needed for the oxidation of both ethanol and acetaldehyde (52). In contrast to the study by Crabb et al. (51), where low dietary protein may be the limiting factor for ADH content, an earlier study (52) illustrated the limiting factor to be a decreased capacity for NADH-reoxidation. Ryle et al. (34), when administering ethanol (2 g/kg i.p.) to fasted rats, found that there was a 64% decrease in cytosolic NAD/NADH ratio and a 41% decrease in mitochondrial NAD/NADH ratio compared to fed animals 90 minutes postethanol injection. These

researchers suggested that ethanol oxidation by ADH may be partially regulated by the mitochondrial acetaldehyde concentration achieved during ethanol metabolism, as well as by NADH reoxidation rates. Finally, Hillbom (53) emphasized that ethanol metabolism is dependent on both the availability of NAD in the liver cytosol as well as the maximum activity of ADH. In both protein deficient and fasted rats, ethanol elimination rates were slower compared to those of control animals because: (1) protein deficiency caused a decreased ADH activity, and (2) fasted rats had less available NAD due to limited NADH reoxidation.

Nutrient-Ethanol Interactions

Ethanol:Vitamins

While the ethanol-induced malabsorption of water-soluble vitamins such as thiamine, folic acid, and vitamin B₁₂ has been demonstrated (27, 28) ethanol also is known to alter the metabolism of both water- and fat-soluble vitamins (27, 28). In the chronic alcoholic, water-soluble vitamins such as folic acid and pyridoxine (B₆) may be lost due to gastrointestinal mucosal irritation and/or alteration causing megaloblastic anemia (54). Thiamine malabsorption also is a common manifestation of the alcoholic and is associated with neurologic syndromes due to its deficiency (54). While water-soluble vitamins may be lost due to alcohol-induced gastrointestinal inflammation, fat-soluble vitamins also may be depleted due to both dietary insufficiency and steatorrhea (54). Ethanol intake, both acute and chronic, has been observed by Sorrel et al. (55) to cause cellular damage to rat hepatocytes. These

researchers found there was an increased release of Vitamins A and D in alcoholic rat liver perfusates caused by the severe cellular damage. It was also noted that the hepatic acetaldehyde production may promote Vitamin B₆ catabolism by the displacement of pyridoxal phosphate from its active site (55), thus promoting the vitamin to degrade further.

A chronic ethanol state enhances the microsomal metabolism of Vitamins A (56-58) and D (59, 60). Vitamins A and D are structurally related to steroids and may serve as substrates for the microsomes (56-60). When microsomal oxidation activation is induced, such as in chronic alcoholism, Vitamin A and D requirements will be increased due to increased degradation. In both humans (56) and experimental animals (57, 58) chronic ethanol ingestion decreases Vitamin A levels. Hepatic lesions in alcoholic rats were found at necropsy which were symptomatic of Vitamin A deficiency (61). Sato and Lieber (58) supported this by illustrating that retinol degradation was enhanced in livers of alcoholic rats. Recently it has been shown by Leo et al. (62) that both retinol and retinoic acid serve as substrates for microsomal oxidation which can be induced by either ethanol or drug administration (63).

Susick and Zannoni (64) found that ascorbic acid pretreatment for 14 days increased the metabolic rate of ethanol while toxicity and behavioral impairment were decreased after acute doses of ethanol in human male subjects. Ethanol clearance in this study was determined by both direct blood as well as by breathalyzer analysis, while the behavioral tests were measured by motor coordination, intellectual function, and color discrimination during blood-ethanol peak times. Although the

mean maximum blood-ethanol concentrations were essentially identical for both vitamin pretreatment (0.078 ± 0.01 gm/dl) and placebo (0.074 ± 0.01 gm/dl) groups, the former group had a significant increase (10%) in blood-ethanol clearance. While ethanol consumption impaired motor coordination and color discrimination in both groups, the vitamin pretreated group showed 20% less impairment for both parameters than the controls. This study was supported by an earlier investigation by Yunice and Lindeman (65) who illustrated that Vitamin C pretreated rats had a 50% lower mortality rate than untreated rats following a potentially lethal dose of ethanol. In a recent study, Susick et al. (66) found that high levels of ascorbate protected guinea pigs from hepatic damage associated with chronic alcohol intake. Although the catalase system is believed to play a physiologically minor role in ethanol oxidation (32, 67), ascorbate generates a peroxide that is used by this system which may increase catalase activity (64). Thus, the enhanced blood-ethanol clearance observed in man, as well as the protective effects of ascorbate in alcoholic animals may be due to: (1) the ability of ascorbate to supply peroxide and allow for a greater catalase contribution to ethanol oxidation, (2) the ability of this vitamin to increase fat transport out of the liver, and (3) the vitamin may have an important neurochemical role in the body.

Ethanol:Minerals

Minerals such as zinc, calcium and magnesium may be decreased in the plasma of alcoholics as the result of either a decreased dietary

intake of these nutrients or because of ethanol-induced renal losses. Calcium absorption may be impaired due to steatorrhea, while duodenal transport of calcium is inhibited in rats by ethanol ingestion (54).

Iron metabolism is important during both acute and chronic alcohol administration due to the high correlation of liver injury and excess iron (68). Acute alcohol administration may increase iron absorption, possibly due to the stimulation of gastric acid secretion which increases ferric iron solubility in the small intestine. Chronic alcohol intake causes both humans and animal to develop increased iron stores causing both hepatic and extrahepatic organ injury (68).

Ethanol:Protein and Amino Acids

The hepatic damage induced by ethanol includes both fat accumulation as well as hepatocyte enlargement (69), which may decrease protein and amino acid metabolism and disposition. Baraona et al. (70) illustrated that excess protein contributes to hepatocyte enlargement in the human alcoholic. Although hepatomegaly was considered to be caused by fat accumulation, it also was found that fat accounts for only half of the liver dry weight increase, while the other half was attributed to protein increase (70, 71). The same reason for hepatocyte enlargement may be due to MEOS induction. The MEOS is constituted of cytochrome P-450, NADPH, cytochrome-C-reductase, and phospholipids, which are also MFOS components. Because ethanol ingestion has been found to cause membrane proliferation of the smooth ER, there is an increased phospholipid and protein content of this cellular fraction, which is related to

the enlargement of the liver (72). Hepatomegaly may also contribute to the inhibition of protein synthesis in rats reported by Chapman et al. (73), as well as to the increased protein degradation reported by Kessler et al. (74) after chronic ethanol consumption in man. Mortinore and Mondon (75) found that chronic ethanol caused a depression in the metabolism of hepatic amino acids. Baraona et al. (76) reported that the concentrations of branched-chain amino acids (BCAA) increased both in the liver and blood after chronic ethanol administration in rats. Baraona et al. (70) also found that the increases in liver protein were partly due to the retention of albumin and transferrin. Mezey (77) supported the findings of Baraona et al. (70, 76) by revealing that during chronic liver injury, there is an increase in the concentrations of the BCAA's valine, leucine, and isoleucine. The oxidation of acetaldehyde which generates an enhanced mitochondrial NADH/NAD ratio, also has been reported to inhibit hepatic protein synthesis when studied on rat liver slices (78).

Protein deficiency also plays an important role in ethanol metabolism. Crabb et al. (51) presented results that low dietary protein may decrease the content of ADH. It was found that low protein diets caused rats to eliminate ethanol at rates which were 50% slower than rats on normal protein diets. Finally, Hillbom (53) found that in protein deficient rats, ethanol elimination rates were significantly slower than in control animals, possibly due to a reduced maximum activity (content) of ADH.

Ethanol:Fats

During acute or chronic administration of ethanol the enhanced NADH/NAD ratio results in an increased lactate/pyruvate ratio and hyperlactacidemia (79, 80). The enhanced reducing equivalents can be transferred into the manufacture of both fatty acids (46) and α -glycerophosphate (47) for the synthesis of triglycerides. These fatty acids which normally serve as a major hepatic energy source, are now supplanted by ethanol (3, 6), while a decreased rate of hepatic fatty acid oxidation, along with an accumulation of both dietary and endogenously synthesized fat, is observed (81, 82). The oxidation of acetaldehyde also generates an enhanced NADH/NAD ratio causing both an increased lactate/pyruvate ratio as well as a depressed oxidation of fats by hepatic mitochondria. The above results suggest that ethanol inhibits β -oxidation of fatty acids while increasing hepatic phospholipids and the esterification of free fatty acids and hepatic triglycerides (81, 82).

Fatty liver will develop in both humans and experimental animals during chronic ethanol administration, especially in the presence of large amounts of dietary fat (81). Lieber and DeCarli (71) noted a 40% increase in hepatic triglycerides in rats when a diet high in fat content (45%) was substituted for one low in fat (10%) while administering a 36% liquid-ethanol diet over a 2 month period. When the high-fat diet was replaced by a medium-fat diet (25%), hepatic triglycerides were reduced significantly. These researchers also found that when a diet consisting primarily of long-chain fatty acids (LCFA) was substituted

with one of medium-chain fatty acids (MCFA) in alcoholic rats, that the accumulation of both hepatic total lipids and triglycerides were reduced by about 65% (83).

Ethanol:Carbohydrates (Sugars)

Carbohydrates can contribute to the biosynthesis of hepatic fatty acid and triglycerides by supplying the carbon backbone, especially in chronic alcoholic conditions (27, 32). In chronic ethanol intake there is an increased lactate availability caused by an enhanced NADH/NAD ratio (27, 28, 32). This altered redox state may stimulate the production of collagen by the enhancement of hepatic collagen proline hydroxylase activity, as reported in studies with rats and baboons (84). Simultaneously, the enhanced redox ratio also may cause hypoglycemia due to the impairment of gluconeogenesis from amino acids (85).

Scholz and Nohl (86) found a stimulatory effect of fructose on ethanol oxidation in rats. This was due to the reduction of fructose or its metabolite, glyceraldehyde, to polyalcohol compounds. The reduction of these compounds caused a rapid reoxidation of NADH to NAD. This observation was supported by earlier in vitro studies using rat hepatocytes (87, 88) as well as by in vivo studies in human subjects (89, 90), who had increased ethanol elimination rates after fructose administration. Lundquist and Wolthers (89) and Brown et al. (90) concluded that the formation of glyceraldehyde by fructose may: (1) increase the reoxidation of NADH by glyceraldehyde formed from fructose and/or (2) induce a dissociation of the ADH-NADH complex, thus providing more available ADH.

Drug-Ethanol Interactions

The metabolism of most drugs, environmental chemicals, and toxic compounds primarily occurs in the enzyme system located in the smooth ER of the liver (91). The microsomes are the vesicles of the smooth ER after homogenization and centrifugation at 105,000 x g for 60 minutes (92). The scheme of the hepatic microsomal drug-metabolizing enzyme system is illustrated in Figure 1 (93). The main events of this system are briefly described as follows:

The ferric heme iron cytochrome P-450 (P-450, Fe^{3+}) reacts with the drug to form a drug- $[\text{Fe}^{2+}]$ complex. The drug-ferric complex is reduced to the ferrous heme iron by the transfer of an electron from NADPH by cytochrome P-450 reductase. The reduced complex (P-450, Fe^{2+}) reacts with oxygen to form an oxycytochrome P-450 complex (Drug- $[\text{Fe}^{2+}]\text{-O}_2$). The unstable intermediate further dissociates to form the oxidized drug, oxidized cytochrome P-450, and a molecule of water. The interactions of ethanol, drugs, and xenobiotics can affect both the absorption and metabolism of each (94, 95).

Effects of Ethanol on Drug Absorption

The administration of ethanol may affect the absorption of drugs by increasing or decreasing gastrointestinal motility, changing gastric emptying time, or changing the morphology of the intestinal villi. Finch et al. (96) noted that when low concentrations of ethanol and various sedatives were simultaneously ingested in man, that ethanol increased drug absorption by enhancing both gastric solubility of the

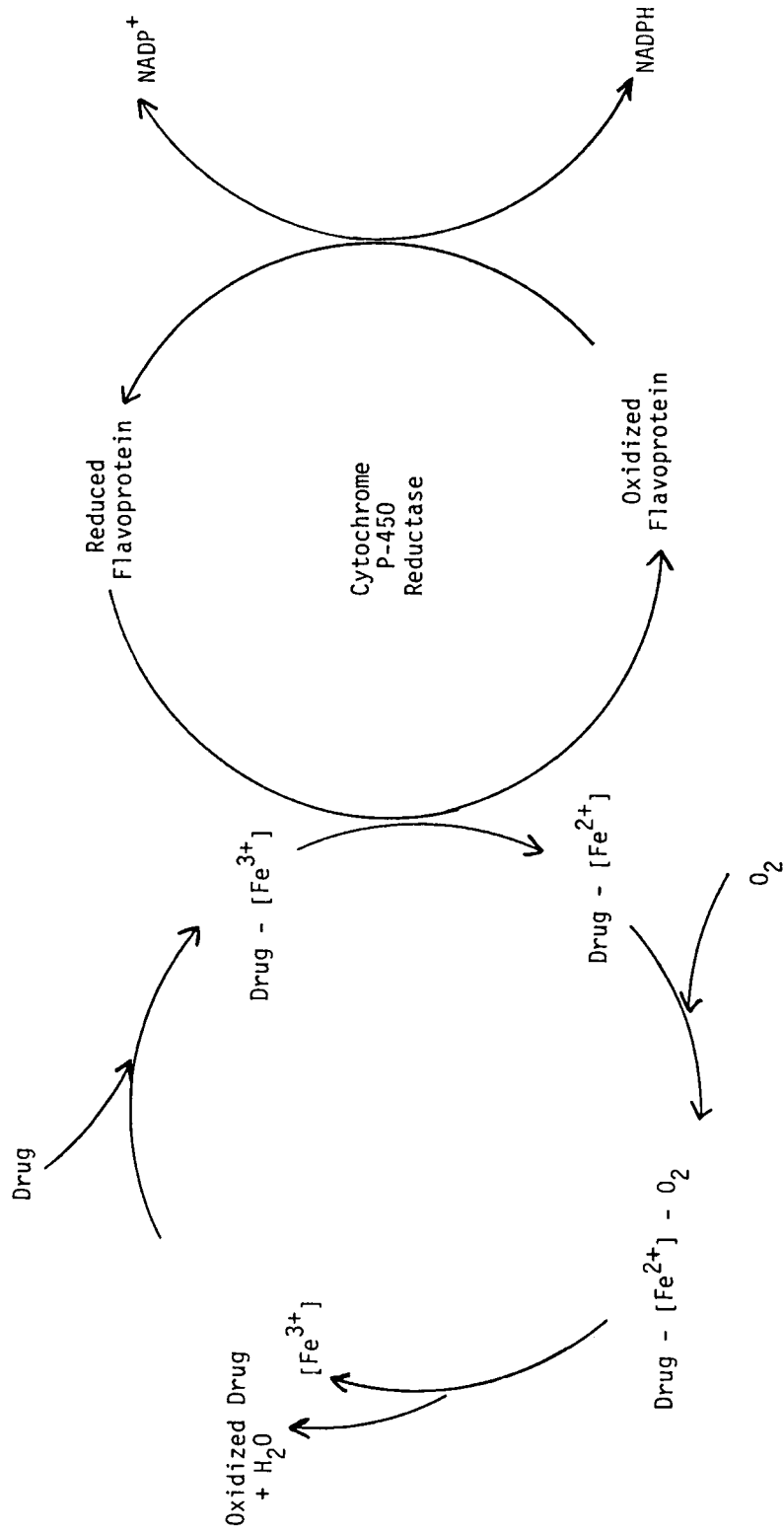


Figure 1. Proposed Scheme of the Hepatic Microsomal Drug-Metabolizing Enzyme System (93).

drug and gastrointestinal blood flow. When high concentrations of ethanol were administered, increased drug absorption was not observed due to the induced gastric irritation which may have delayed drug absorption due to pylorospasm. Cook (97) also found that high concentrations of ethanol (12%) irritated both gastric and duodenal mucosa in man causing slower gastric emptying, while Mezey (98) observed that very low ethanol concentrations (1-5%) stimulated both gastric and duodenal motility and absorption. Greenblatt et al. (99) demonstrated that the coadministration of a concentrated ethanol solution (28%) along with diazepam (Valium[®]), significantly slowed the rate of diazepam as well as ethanol absorption.

Ethanol may also modify gastrointestinal-hepatic membrane permeability (100), and thus affect tissue-drug concentrations. An altered membrane permeability will either increase or reduce the capacity of liver cells to extract the drug from the blood as it passes through the hepatic vessels. Also when high doses of ethanol are present in the stomach, the gastric pH will be lowered due to increased acid production and histamine release (101). Magnussen (102) found that the coadministration of a dilute ethanol solution and various barbiturates (weak acids), resulted in increased absorption of the barbiturates into the blood of rats.

Effects of Drugs on Ethanol Absorption

It is important to examine drug effects on the absorption of ethanol. Pirola (101) concluded that the anticholinergics, atropine and

Donnatol,[®] reduce the rate of ethanol absorption due to their effects of delaying stomach emptying. In contrast, nicotine and clofibrate, drugs which increase the rate of gastric emptying, increase ethanol absorption, due to their cholinergic activity when administered in small doses (101). Siegers et al. (103) found that when rats were simultaneously administered ethanol and caffeine via stomach gavage, the absorption of ethanol was inhibited by the presence of caffeine. Finally, it was demonstrated by Seitz et al. (104, 105) that cimetidine (Tagamet[®]) an H₂-receptor blocker, increased the ethanol absorption. Cimetidine blocks the action of histamine which in turn reduces gastrointestinal irritation by decreasing hydrochloric acid production. Due to the popular use of cimetidine, this finding may have direct implications for patients who take this drug while consuming alcoholic beverages.

Effects of Selected Drugs on Ethanol Metabolism

Because ethanol does not have a significant first pass hepatic metabolism (101) only a few drugs are known to inhibit the metabolism of ethanol (106). Sellers et al. (107) found that ethanol metabolism was attenuated by chloral hydrate in human subjects who were administered acute doses of ethanol. The inhibition is due to the presence of the active metabolite of chloral hydrate, trichloroethanol, which inhibited the activity of ADH by competing with ethanol for active binding sites on the enzyme. Mezey (28, 108) found that large doses of the phenothiazine sedative, chlorpromazine, also inhibited ADH action and sufficiently

attenuated the oxidation of ethanol. Instead of competitively inhibiting ADH as chloralhydrate did (107), chlorpromazine was bound to the ADH enzyme itself, blocking the binding site (108). By far the most commonly known drug used to inhibit the metabolism of ethanol is disulfiram (Antabuse®) (106). Disulfiram competes with the cofactor, NAD, for the active centers of the ALDH enzyme, thus reducing the oxidation rate of acetaldehyde (106). While the first step in ethanol oxidation is not affected, (acetaldehyde production via ADH), the second step, (acetate production via ALDH), is inhibited and a buildup of acetaldehyde is promoted. In patients who drink ethanol while simultaneously taking disulfiram, the "Antabuse®" reaction is provoked with ensuing symptoms of nausea, vomiting, vasodilation, and mental clouding, which are mostly attributed to the accumulation of high blood-acetaldehyde levels (106). Two other drugs which are widely used to inhibit ADH activity and block the metabolic effects of ethanol oxidation are pyrazole and 4-methylpyrazole (31, 109). The difference between pyrazole and 4-methylpyrazole is the effect on the content of the cytochrome P-450. Whereas pyrazole has no effect, 4-methylpyrazole treatment results in a two-fold increase in the content of cytochrome P-450 (109). When rats were treated with both compounds (109) pyrazole did not change the cytochrome P-450 enzyme activity or the activity of the cytochrome C reductase. By contrast, 4-methylpyrazole treatment increased both the content of cytochrome P-450 as well as the cytochrome C reductase activity. In support of this, Tolf et al. (110) observed that 4-methylpyrazole slowed ethanol metabolism in rats by forming a ternary complex with ADH and NAD, thus

occupying the ethanol binding site on the enzyme. The inhibitory power of the pyrazole derivatives on ADH activity was tested by observing the changes in fluorescence of the coenzyme (NAD) on its reduction with ethanol as substrate ($\text{NAD}^+ \rightarrow \text{NADH} + \text{H}^+$). In a series of pyrazoles tested, 4-methylpyrazole was found to be the most inhibitory for this reaction. Thus, because 4-methylpyrazole inhibits ADH activity, it may induce an increased activity of cytochrome C reductase as well as an increased content of cytochrome P-450. Conversely, pyrazole, although interfering with ADH activity due to its lower inhibitory action, may not induce the cytochrome C reductase activity and/or the cytochrome P-450 components.

There are various drugs and compounds that accelerate ethanol metabolism by either: (1) increasing MEOS activity, (2) increasing the liver weight, or (3) increasing the rate of NADH reoxidation and reversing the previously elevated NADH/NAD ratio (94). An enhanced metabolism of ethanol was reported in both man (111) and experimental animals (106, 108) previously exposed to phenobarbital for a number of weeks. In the animal studies (106, 108), there were reported increases in liver weights and well as hepatic blood and bile flow. Hawkins et al. (112) found that high doses of clofibrate also increased the rate of ethanol metabolism when administered to rats. Because one pharmacological action of clofibrate is to increase biliary excretion of neutral sterols (113), this causes an increase in both liver size and bile flow.

Effects of Ethanol on Drug Metabolism

Although the hepatic ADH system is the primary route for ethanol metabolism (93), chronic alcohol intake has induced pathways which are

ADH-independent; the most important being the MEOS (114, 115). Not only does the MFOS, of which the MEOS is an integral part (93), participate in the biotransformation of various drugs and toxic chemicals, but the activity of the enzymes present in this system can be induced by drugs and chemicals encountered in the environment (27, 28). The evolution of the drug metabolizing system in animals is probably related to exposure to toxic alkaloids in plants on which they had fed (59). Biotransformation is not equivalent to detoxification with respect to drugs and chemicals. In many instances it is the intermediates that are produced in or during biotransformation that are the actual toxins and not the parent compounds themselves (93). Thus, due to the increased interest as well as concern over ethanol-drug interactions, there continues to be debate as to the importance of the MEOS contribution to ethanol metabolism (114, 115).

Ethanol, whether administered acutely or chronically, has been found to affect drug metabolism by either inhibiting the microsomal drug metabolizing system or by inducing its activity (114). Various studies have proven that ethanol alters the half-life ($t_{1/2}$) of certain drugs. Cederbaum et al. (116) found that acute ethanol ingestion resulted in a reduced clearance of pentobarbital and meprobamate (Miltown®) from the blood of both human volunteers and rats. This study confirmed the earlier findings of Rubin et al. (117) and confirmed that the main effect of the presence of ethanol was the inhibition of drug metabolism mediated by the inhibition of certain hepatic microsomal drug-metabolizing enzymes. Because of these findings, this interrelationship

of enzyme inhibition may explain the heightened sensitivity of acutely inebriated individuals to these types of drugs (116, 117). Hayashi et al. (118) postulated that the inhibition of drug oxidation by acute ethanol doses may be due to the displacement of substrates from cytochrome P-450 as well as to the inhibited reduction of cytochrome P-450 by NADPH-cytochrome P-450 reductase. Thus, ethanol may interfere with microsomal drug metabolism directly by displacement of the drug from the enzyme, as well as indirectly, by affecting the supply of NADPH. Other investigations (119-121) have hypothesized that cytosolic NADH generation from ethanol oxidation by ADH may indirectly alter the mitochondrial redox state, and inhibit the Krebs cycle. Citrate cycle inhibition could in turn deplete the intermediates necessary for NADPH generation, which is essential for MFOS propagation.

Acute interactions of ethanol with drug metabolism have important practical consequences, especially in a society which places so much emphasis on drug and alcohol use. The metabolism of frequently used drugs such as methadone (122), chlorpromazine (123) and caffeine (124) were also attenuated in the presence of ethanol. The interaction of ethanol with narcotics is also very important due to their simultaneous use. Various epidemiological studies have suggested that alcohol potentiates the effects of morphine and increases the lethality of the drug (42). Acute ethanol administration also has been noted to affect the metabolism of various drug metabolites. Hoyumpa et al. (125) found that when ethanol was administered to dogs one hour prior to the intravenous administration of either chlordiazepoxide (Librium[®]), demoxepan, (a

Librium[®] metabolite), diazepam (valium[®]), or desmethyldiazepam, (a metabolite of both demoxepan and valium[®]), the ethanol had no effect on the systemic clearance of either Librium[®] or Valium[®] but reduced the clearance of the metabolites, which are responsible for the sedative effects. Thus, it was seen from this study that the clearance of drug metabolites was affected, rather than the parent compounds themselves, which in this case enhanced sedation. In a study by Banda and Quart (126) it was reported that the intraperitoneal administration of ethanol prior to an LD₅₀ dose of acetaminophen to mice increased the survival level to greater than 90% after 24 hours compared to mice given an LD₅₀ dose of acetaminophen without prior ethanol treatment. The survival level of the ethanol-treated mice was statistically indistinguishable from that level provided by the standard acetaminophen antidote, N-acetylcysteine. Acetaminophen is metabolized partially by the hepatic microsomal cytochrome P-450 monooxygenase system whose latter biotransformation yields an active metabolite which is highly hepatotoxic and causes the mortality during overdose (42). The results of this study illustrated that acute alcohol administration leads to a reduced amount of oxidized drug and may have an antidotal effect by reducing the generation of the toxic intermediates. This reduced generation of the toxic by-products may be caused by ethanol interfering with a common receptor site on the enzyme (126).

It is well known that the chronic administration of ethanol causes an increased capacity to oxidize ethanol (42, 94). This occurs due to

the adaptive activity of the non-ADH pathways, most prominent being the cytochrome P-450-dependent MEOS (42). Misra et al. (127) found that chronic ethanol intake increased the clearance of meprobamate from the blood in 4 human volunteers when tested 1 month after being placed on an alcoholic regime. Thus, here, the metabolism of meprobamate was accentuated by the ethanol-induced MEOS of these individuals which resulted in a greater drug tolerance. In separate studies with rats (127) it was also observed that pentobarbital-induced sleeping times were decreased due to the accelerated metabolism of this barbiturate after chronic ethanol treatments. Carulli et al. (128) observed a similar increase in tolbutamide clearance after chronic ethanol treatment, while Cushman et al. (129) reported this same effect on antipyrine in the alcoholic. Misra et al. (127) found that the chronic administration of ethanol to rats and humans caused proliferation of the smooth endoplasmic reticulum and an increase in microsomal protein content and increased cytochrome P-450 content, which resulted in an augmentation of drug metabolizing activity of the microsomal enzymes in vitro. Another in vitro study which documented the accelerated metabolism of drugs after chronic ethanol treatment was conducted by Rubin and Lieber (130) which found increased clearance of aniline and pentobarbital in rat liver slices.

The increases in microsomal enzyme activities also apply to the reactions which convert exogenous compounds to toxic metabolites. Acute administration of ethanol was found to interfere with the biotransformation of the toxic metabolites of acetaminophen (126), and it was also found that chronic ethanol consumption increased the biotransformation

rate of these metabolites and hence the hepatotoxicity of acetaminophen. In human case reports, large doses of acetaminophen have been found to produce fulminant hepatic failure, most notably in the chronic alcoholic (131). Sato et al. (132) found that rats chronically fed ethanol exhibited enhanced hepatic failure compared to those not fed ethanol when exposed to acetaminophen dosing. Morgan et al. (133) later found that liver slices of rabbits chronically exposed to ethanol, possessed a cytochrome P-450 system with elevated acetaminophen oxidizing activity. The intermediate toxic metabolite formed a conjugate with reduced glutathione which was significantly greater than that formed in rabbits not exposed to ethanol. Thus, it was concluded that the enhanced hepatotoxicity of acetaminophen observed after chronic ethanol exposure is at least partially due to the increased microsomal production of the toxic metabolites from an induced MFOS. Another hepatotoxic agent whose toxicity is increased during chronic ethanol exposure is carbon tetrachloride (CCl_4). Hasumura et al. (134) found that alcoholic rats had enhanced CCl_4 liver damage, hepatic-fat infiltration, and mortality rates compared to nonalcoholic animals after CCl_4 administration. Carbon tetrachloride exerts its toxicity after microsomal conversion to the harmful metabolites, which is enhanced in alcoholic rats due to MFOS induction and smooth ER proliferation (135).

Unlike the chronic administration of ethanol, an acute dose of ethanol stimulates both the adrenocortical (136) and sympatho-adrenomedullary (137) systems. The ethanol-stimulated catecholamine release causes the relinquishment of free fatty acids from adipose

stores and enhances the accumulation of hepatic triglycerides (138). Conversely, a chronic ethanol state enhances the microsomal metabolism of other steroids (59, 60, 139), including the increased degradation of testosterone (139).

Finally, because chronic ethanol administration induces the activity of the microsomal drug-metabolizing enzyme system, the activation of procarcinogenic and mutagenic compounds to their active harmful metabolites may be increased (140). In the rat, benzo (a) pyrene mutagenicity was enhanced following chronic ethanol administration, which was illustrated by the development of tumors as well as increased mortality in alcoholic animals (140). Garro et al. (140) reported that the activation of the procarcinogen, dimethylnitrosamine was increased in rats chronically administered ethanol for 2 months. Because digestive tract (141), lung and rectal cancers (142) were found to be prominent in human alcoholic clusters, chronic alcohol intake may induce mutagenicity/-carcinogenicity by increasing drug metabolite production both in humans as well as in experimental animal models.

Carnitine Interactions

Carnitine (3-hydroxy-4-N-trimethylaminobutyric acid) is also known as vitamin B_t for being water soluble and being essential for the growth of the meal worm, Tenebrio molitor. It has nearly ubiquitous distribution in nature and in the tissues of mammals (143). Both D- and L-carnitine are found in nature, however, only the L-isomer is functionally active. Carnitine is richly supplied by the foods of animal origin

especially milk and meats (144). With the exception of a few species of insects, most all other animals are able to biosynthesize carnitine from lysine and methionine (145). The full spectrum of its biosynthetic enzymes is present in the liver, kidney and brain of humans (146, 147), liver and kidneys of rabbit, rhesus monkey, hamster and cat, and liver of guinea pig, mouse, dog and rat (148). Besides organic diseases and general malnutrition, deficiency of specific nutrients (e.g., ascorbic acid, niacin, pyridoxine, iron, lysine and methionine) has been shown to compromise the endogenous biosynthesis of carnitine (149).

Carnitine concentrations are more than 10 fold higher in most tissues than in the plasma (50-60 μM) suggesting an active uptake process by the tissues (150). However, the turnover rate of carnitine varies a great deal from tissue to tissue. For example, the turnover times of carnitine in rat kidney, liver, heart, skeletal muscle and brain are 0.4, 1.3, 21, 105 and 220 hours, respectively (151). Tissue distribution and concentrations of carnitine are at least partially controlled by hormones such as insulin, glucagon, somatotropin, adrenocorticotrophic hormone, thyroid hormone and sex hormones (150).

The K_m for carnitine uptake by hepatocytes is about 5 mM and there is no difference in the relative uptake of D- and L-isomers of carnitine by the liver (150). However, the uptake systems of kidney, heart, skeletal muscle, epididymus and testes preferred L-carnitine over the D-isomer and therefore, are more stereospecific (150). The relative uptake (blood = 1) of D- and L-carnitine were 3.6 and 3.2 in liver, 6.6 and 8.1 in kidney, 8.6 and 19 in heart, 4.8 and 7.7 in skeletal muscle,

9 and 31 in caput epididymus, 9.5 and 54 in cauda epididymus, 1.1 and 2.4 in testes and 0.8 and 0.9 in brain tissues, respectively (150). The K_m for L-carnitine uptake is 60 μM in heart myocytes and the rate of L-carnitine uptake by heart and skeletal muscle was 1/1000 of that by the hepatocytes (151). The release of carnitine was slowest in the muscles and fastest in the kidney tissues (151). In kidney slices, the K_m and $V_{\max}$ were 166 μM and 14 nm/min/ml for D-carnitine and 90 μM and 22 nm/min/ml for L-carnitine (152). The in vivo studies revealed that the tubular reabsorption of L-carnitine was preferred; e.g., after 4 and 24 hours of administration 47 and 71% of D-carnitine and 10 and 16% of the L-carnitine were found in the urine (152). In a normal rat about 0.05% of the total body carnitine (1-2 μM /100 g b.w./day) is excreted in urine (153). The renal clearance of carnitine in animals is <50 ml/day (154) but the glomerular filtration rate is about 1.7 liters/day/100 g b.w. (155) suggesting that about 95% of carnitine in the glomerular filtrate is reabsorbed. The tubular reabsorption of L-carnitine is 99% in humans (150).

D-carnitine is eliminated from the body more readily than L-carnitine. Nevertheless, a significant portion of D-carnitine is retained in the tissues even after 24 hours (150). Negrao et al. (156) reported tissue carnitine and enzymes of fatty acid oxidation in D- or L-carnitine treated Sprague Dawley male rats of approximately 200-250 g body weight (2 month old). L-carnitine or D-carnitine was administered, i.p., (750 mg/Kg b.w.) 5 times per week, 1-2 hours before the exercise training program began. The D-carnitine depleted (50-70%) L-carnitine

concentrations of nonesterified carnitine in blood, heart, liver and skeletal muscle which was restored by L-carnitine supplementation except in the liver. The short-chain acylcarnitines but not the long-chain acylcarnitines also followed this pattern. This was contrary to the observations of Paulson and Shug (157) who were unable to reduce hepatic carnitine after 4 days of D-carnitine feeding. It has been shown that both D- and L-carnitine uncoupled the γ -butyrobetaine hydroxylase reaction (158). It was reported that 71% of D- and only 16% of L-carnitine was recovered in 24 hour urine following administration of the two isomers in the rat (159). It is possible that the overload of D-carnitine may be affecting tubular reabsorption of the L-carnitine.

It has been reported that L-carnitine was slightly more effective than the racemic mixture (3.72 vs. 2.78 nm/mg noncollagen protein) in raising hepatic carnitine contents after 32 days of dietary administration in rats (170). It was also found that 1% D-carnitine alone decreased liver, kidney, skeletal muscle, heart and serum L-carnitine concentrations by 35, 21, 17, 33, and 26%, respectively compared to those of the controls. This was suggested to be due to inhibitory effect of D-carnitine on L-carnitine transport (170). Urinary excretion of L-carnitine was increased 17 fold in D-carnitine supplemented rats suggesting an interference by the latter in tubular reabsorption of the former (170). Oral administration of L-carnitine (500 mg/Kg b.w.) significantly increased carnitine concentrations in serum, liver and kidney in addition to 75 fold increases in urine and feces after 2 hours of administration (174). These increases corresponded with significant

reductions in hepatic and cardiac triglyceride concentrations. Carnitine facilitates the transport of fatty acids via carnitine acyltransferase (160) into the mitochondria for beta-oxidation (24). Because decreased fatty acid oxidation is incriminated as a major causative mechanism for the ethanol-induced hepatic lipid accumulation (1-6), it is logical that carnitine, due to its lipotropic action, aids in the alleviation of fat accumulation in the ethanol-compromised liver (21, 22, 163).

Coadministration of 1% (w/w) D,L-carnitine with the liquid ethanol diet (36% of the calories as ethanol) for 8 weeks in male rats reduced hepatic lipid accumulation by almost 50% (21). Hepatic carnitine concentrations were not significantly different between the control (367 nm/mg DNA) and the ethanol fed (461 nm/mg DNA) group, however, the carnitine supplemented rats had significantly higher (595 nm/mg DNA) hepatic carnitine concentrations than either of the two groups. As much as a two fold increase in hepatic carnitine content (934 nm/g wet wt.) has been found in 1% D,L-carnitine supplemented (for 8 weeks) alcoholic rats (22).

In a study by Kondrup and Grunnet (24), it was found that the contents of both free and acetylcarnitine, (short-chain), were increased by 50% in rat livers after acute ethanol administration, whereas that of acylcarnitine, (long-chain), was decreased by 50%. Furthermore, the carnitine acylation ratio was decreased by 60%. From this study it was concluded that rats exposed to acute ethanol dosing acylated carnitine to a lesser degree than those not exposed to ethanol. Since carnitine

mediates the activation and transfer of acyl units (24), the degree of carnitine acylation appears to reflect the state of fat metabolism. Because the energy derived from ethanol oxidation serves as a primary fuel source (49, 50) may explain why long-chain fatty acid acylation was diminished 60%. It was also observed from this study that, under these conditions, fatty acid synthesis and esterification were enhanced (24). Lieber et al. (144) suggested that the ethanol-induced triglyceride accumulation may be due to the decrease in fatty acid oxidation because of decreased Krebs cycle activity. Rudman et al. (23) reported that alcoholics with advanced liver disease had significantly lower serum free and total carnitine concentrations than normals, which demonstrates a possible carnitine deficiency in these patients. Beneficial effects of supplementary D,L-carnitine in ethanol-fed rats on fatty acid oxidation was reported by Hosein and Bexton (162). In this study it was shown that D,L-carnitine (0.1 mg/g body weight) supplementation significantly lowered both hepatic and serum triglycerides.

It was suggested that although small changes in the acylation ratio of carnitine may not be discarded, the lipotropic effect of dietary carnitine in alcohol-induced hepatic steatosis may be caused by other mechanisms, such as an altered rate of ethanol oxidation and/or the elimination of acylcarnitines in the urine (21, 22). During the course of these studies (21, 22, 146), it was observed that rats fed a carnitine supplemented liquid-ethanol diet appeared more lethargic than those fed the liquid-ethanol diet alone. It was further found that blood-ethanol concentrations were significantly elevated in the carnitine

supplemented rats under both acute and chronic alcoholic conditions (25), which may be caused by a change in the rates of ethanol absorption, metabolism, excretion, or a combination of all three factors.

In order to shed some light on the mechanism of the carnitine-mediated elevation of blood-ethanol, a time course of ethanol appearance and disappearance in the blood of nonsupplemented (NS) and carnitine supplemented (CS) rats was deemed necessary (26). This was performed in two steps. First, it was established that the DL-carnitine supplement had to be fed for at least 5 days at 1.0% (w/w) in the diet to achieve a steady state blood concentration of carnitine (Figure 2). In the second phase, a single dose of ethanol (3 g absolute ethanol/kg body weight) was given to 5 NS and 5 CS (1.0%) rats fed their respective diets for 5 days. Serial samples of tail blood were analyzed for ethanol concentration from 0-8 hours postethanol administration. There were no significant differences in the concentration of blood-ethanol of NS and CS rats in the first 60 minutes. Following 60 minutes, CS rats exhibited significantly higher blood-ethanol concentrations than the NS rats up to 8 hours (Figure 3). It was concluded that carnitine must inhibit ethanol metabolism and not absorption. However, a number of questions still remained to be answered.

1. It could not be said that the effect was caused by L-carnitine and not only by the D-isomer.

2. The optimal dose and duration of L-carnitine supplementation was not established.

3. Whether other carnitine-like compounds, i.e., choline, could cause a similar effect.

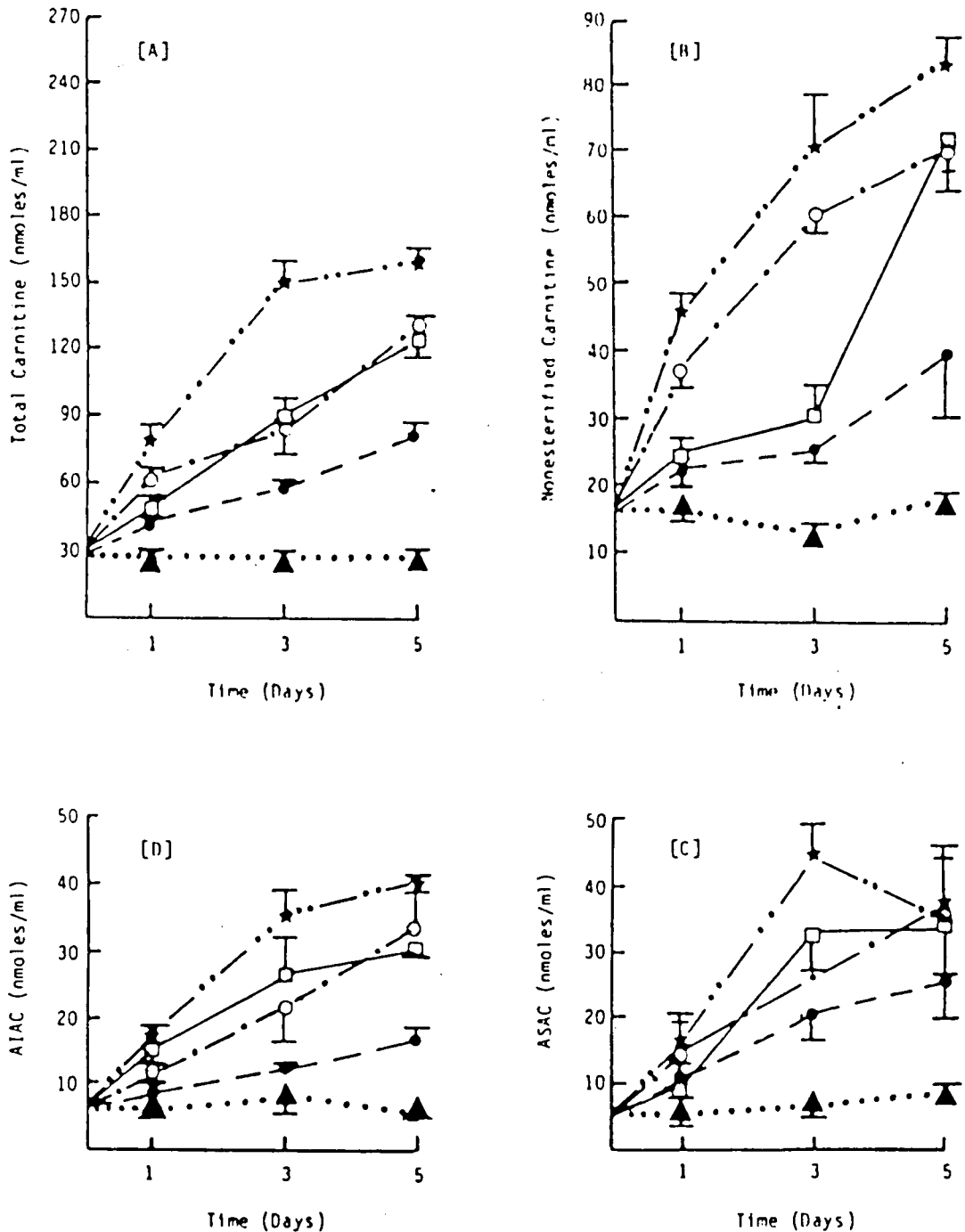


Figure 2. Changes in Blood-Total Carnitine (A), Nonesterified Carnitine (B), Acid Soluble Acylcarnitine (C), and Acid Insoluble Acylcarnitine (D), Following 1, 3, and 5 Days of Feeding Diet Supplemented with D,L-carnitine at 0% (▲), 0.25% (●), 0.50% (□), 0.75% (○) and 1.0% (★). Values shown are means $\pm$ SEM for 3 animals.

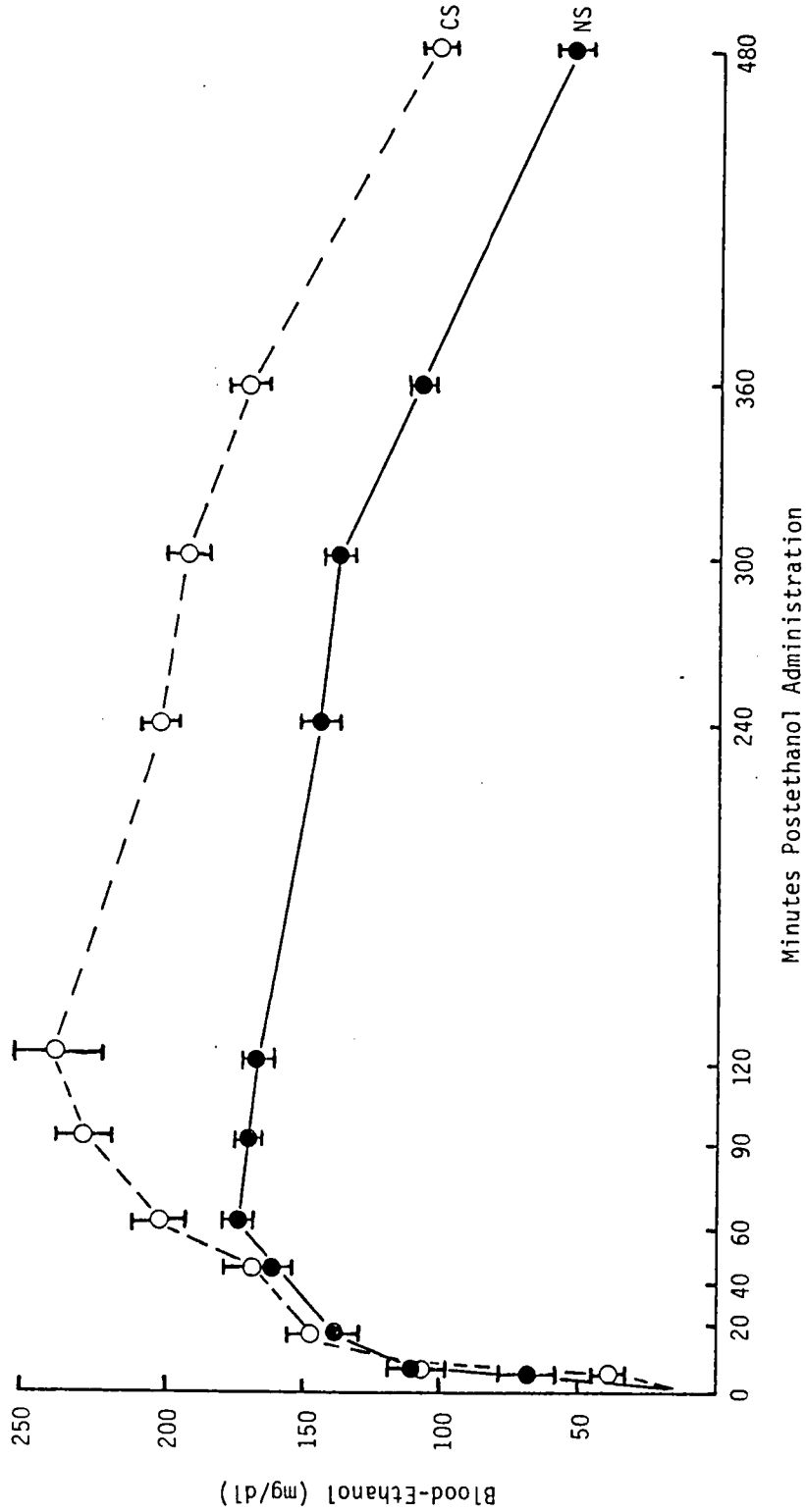


Figure 3. Time Course of Blood-Ethanol Concentration from 0-8 Hours After a Single Oral Dose of Ethanol in Rats Fed 1.0% Supplementary D,L-Carnitine for 5 Days. Each point represents the mean $\pm$ SEM for 5 animals. Slopes and correlations are as follows: CS; $y = (-18.9)x + 273.5$, $r = -0.96$, and NS; $y = (-15.9)x + 198.2$, $r = -0.96$. Slopes were determined by "Linear Regression."

4. Whether carnitine would be equally effective when ethanol is parenterally administered.

5. Whether carnitine would effect different doses of ethanol administration.

6. Whether carnitine would be effective when given only prior to ethanol administration or would it be equally effective following or simultaneous to ethanol administration.

7. Whether carnitine would be equally effective in male and female rats.

CHAPTER III

EXPERIMENTAL PROCEDURES

Animals and Housing

Animals used in these studies were mature male or female Sprague-Dawley rats (Taconic Inc., Germantown, NY) weighing between 250-600 g. All animals were individually housed in either 7" x 10" x 7" wire mesh, stainless steel cages or 7" x 10" x 7" wire mesh, stainless steel metabolic cages in the AAALAC approved animal facility located in the Department of Nutrition and Food Science in the College of Human Ecology. Animals in each experiment were kept in ventilated cubicles with glass sealing doors. The temperature in each cubicle was maintained at $72 \pm 2^{\circ}\text{C}$, relative humidity between 40-50% with a cycle of 12 hours light and 12 hours dark. All specifications of the animal facility comply with AAALAC regulations.

Diets

Diets consisted of either ground Purina Rodent Chow #5001 (Ralston Purina Co., St. Louis, MO) given as such (NS) or supplemented with L-carnitine (CS) at a single (0.5%, w/w) and varying levels (0.25-1.0%, w/w) or an NS or CS semisynthetic diet (Table 1). All animals were provided feed and water ad libitum.

Table 1
Composition of the Experimental Diet

Ingredient	Composition (%)
Casein	20.0
Sucrose	29.0
Cornstarch	31.0
Alphacel	6.0
Crisco	6.0
Wesson Oil	2.0
Vitamin mix ¹	2.0
Salt mix ² (with Se ³ & Zn ⁴)	4.0

¹Vitamin diet fortification mixture, ICN Pharmaceuticals Inc., Life Sciences Group, 26201 Miles Road Cleveland, Ohio 44128. Composition per kg of mixture: (in g) vitamin A concentrate (200,000 IU/g), 4.5; vitamin D concentrate (400,000 IU/g) 0.25; α -tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-amino benzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine hydrochloride, 1.0; thiamin hydrochloride, 1.0; calcium pantothenate, 3.0; (in mg) biotin, 20; folic acid, 90; vitamin B-12, 1.35.

²Hubbell, Mendel and Wakeman salt mixture, ICN Pharmaceuticals Inc., Life Sciences Group, 26201 Miles Road, Cleveland, Ohio 44128. Composition of mixture: calcium carbonate 54.3%; magnesium carbonate 2.5%; magnesium sulfate (7H₂O) 1.6%; sodium chloride 6.9%, potassium chloride 11.2%; potassium phosphate (monobasic) 21.2%; ferric phosphate 2.05%; potassium iodide 0.008%; manganese sulfate (H₂O) 0.035%; sodium fluoride 0.10%; aluminum potassium sulfate 0.017%; copper sulfate (5H₂O) 0.09%.

³NaSeO₄ @ 0.0003% of salt mix.

⁴ZnCO₃ @ 0.06% of salt mix.

Experimental Protocols

Experiment 1A. Level and Duration of Oral L-Carnitine Supplementation on Steady State Concentrations of Carnitine in the Blood and Urine.

The objective of this experiment was to determine the dose and duration of L-carnitine supplementation necessary to reach steady state concentrations of carnitine in the blood and urine of mature rats. In addition it was aimed to determine changes on portal and systemic blood-ethanol concentrations under these steady state concentrations of blood-carnitine.

Twenty male rats weighing between 250-350 g were divided into 5 groups of 4 animals and placed in metabolic cages. The animals of the control or NS group were given the chow diet as such while diets of the CS groups were supplemented with 0.25, 0.50, 0.75, and 1.0% (w/w) L-carnitine (Sigma tau, Rome, Italy) on a dry weight basis. The assigned diets were fed ad libitum to the animals of each group for 10 days (Table 2). On days 0, 1, 3, 5, 7, and 10, 20 μ l of tail vein blood and 24-hour urine samples were collected for carnitine determination. The procedure for tail vein blood and urine collection is described later in the chapter.

1B. Portal-Systemic Blood-Ethanol Concentrations in Carnitine Supplemented Rats

After 10 days of treatment, each animal was fitted with portal vein and posterior vena cava catheters for the purpose of collecting portal

Table 2
Experimental Design for Experiment 1A

Parameters	Groups				
	NS ¹	CS-0.25% ²	CS-0.50%	CS-0.75%	CS-1.0%
Number of animals	4	4	4	4	4
L-carnitine (% w/w)	0	0.25	0.50	0.75	1.0
Days of treatment	10	10	10	10	10

¹Nonsupplemented.

²L-carnitine supplemented.

and systemic blood. Serial portal and systemic blood samples were collected in 20 μ l capacity microhematocrit tubes (Clay Adams, Parsippany, N.J.) through the indwelling catheters up to 30 minutes postduodenal ethanol infusion described in 4B. Blood sample treatment is described later in the chapter. At this time, a 50-100 mg biopsy of medial quadriceps muscle was collected and frozen for carnitine determinations as also described later in the chapter.

Experiment 2. Specificity of the Carnitine Effect on Ethanol Metabolism

The objective of this study was to determine if the effect of carnitine on ethanol metabolism was carnitine specific or could it be produced by other lipotropes like choline (CHS).

Eighteen male rats weighing between 250-300 g were divided into 3 groups of 6 animals each. The semisynthetic diet (Table 1) was used in this study. The NS group was fed this diet as such while the CS and CHS groups were fed this diet after supplementing it with 0.5% (w/w) L-carnitine and 0.43% (w/w) choline chloride respectively, which amounted to equimolar (0.031 moles/kg of diet) supplementation of the diet by carnitine or choline. After feeding the diets for 1 week, all animals were administered a single oral dose of ethanol by way of a gastric gavage. A 10 cc syringe with an attached blunt-end, curved, stainless steel cannula was used for this purpose to ensure nontraumatizing esophageal-stomach entrance. The dose of ethanol for each animal was 3 g absolute ethanol/kg body weight in a 13% aqueous solution, similar to that used by Kondrup and Grunnet (24) (Table 3). Tail vein blood was

Table 3
Experimental Design for Experiment 2

Parameters	Groups		
	NS ¹	CHS ²	CS ³
Number of animals	6	6	6
L-carnitine (moles/kg diet)	0	0	0.031
Choline chloride (moles/kg diet)	0	0.031	0
Days of treatment	7	7	7
Oral ethanol dose (g/kg)	3	3	3

¹Nonsupplemented.

²Choline supplemented.

³L-carnitine supplemented.

collected immediately prior to and at various time intervals up to 8 hours after ethanol administration. After the 8-hour collection period an additional 20 μ l of tail blood was drawn for carnitine determination.

The animals were maintained on their respective diets for an additional 7 days, and on the 8th day they were placed in metabolic cages for 24-hour urine collections for carnitine determination as described later in the chapter.

Experiment 3A. Effects of Intraperitoneal Ethanol Administration on Its Metabolism in Carnitine Supplemented Rats

The objective of this study was to determine if the metabolism of parenterally administered ethanol would also be attenuated by carnitine.

Five male rats weighing between 450-500 g were used for this purpose. After 3 days of adjustment to ground chow, the rats were administered an i.p. ethanol dose (0.2 g/kg body weight in a 50% aqueous solution) and tail blood was collected immediately prior to and at various time intervals postethanol dosage. Three days later these animals were fed the 0.5% (w/w) CS ground chow for 3 days. On the 4th day they were administered an i.p. dose (0.2 g/kg) of ethanol. Tail blood was collected at the regular time intervals as earlier (Table 4) for blood-ethanol determination.

3B. Effects of Various Doses (i.p.) of Ethanol on Its Metabolism in Carnitine Supplemented Rats

The objective of this study was to determine if the carnitine-mediated attenuation of ethanol metabolism worked at various doses of ethanol.

Table 4
Experimental Design for Experiment 3A

Number of animals:	5
Days on unsupplemented diet:	3
I.P. Ethanol dose (g/kg):	0.2
Days on 0.5% (w/w) L-carnitine supplemented diet:	3
I.P. Ethanol dose (g/kg):	0.2

Ten male rats weighing between 450-500 g were divided into 2 groups of 5 animals (Table 5). Animals of the NS group were given the chow as such while those of the CS group were fed this diet after supplementing it with 0.5% (w/w) L-carnitine for 90 days. Ethanol was administered (i.p.) in doses of 0.05, 0.10, 0.20, 0.40, and 0.8 g/kg body weight as a 50% aqueous solution to NS and CS animals on days 91, 92, 93, 94, and 95, respectively. Serial tail blood samples were collected at various time intervals immediately prior to and up to 8 hours postethanol administration. Following the last collection on day 95, all animals were decapitated and livers were collected for immediate hepatic-ethanol determinations. Blood was also collected for triglyceride and carnitine determination as described later in the chapter.

Experiment 4. Effects of Route and Sequence of Carnitine and Ethanol Administration on Ethanol Metabolism

The purpose of these studies was to determine the duration and sequence of carnitine and ethanol administration via different routes on ethanol metabolism.

4A. Duration of Oral Carnitine Administration Prior to Ethanol Gavage

The purpose of this study was to determine how long in advance carnitine needed to be administered to bring about the attenuation of ethanol metabolism as seen by supplementing 0.5% L-carnitine in the diet for 3-5 days.

In this study 8 male rats (400-600 g) were divided into 2 groups of 4. The CS group was administered an oral gavage of L-carnitine

Table 5
Experimental Design for Experiment 3B

Parameters	Groups	
	NS ¹	CS ²
Number of animals	5	5
L-carnitine (% w/w)	0	0.5
Days of treatment	90	90
I.P. Ethanol doses (g/kg)		
Day 91	0.05	0.05
Day 92	0.10	0.10
Day 93	0.20	0.20
Day 94	0.40	0.40
Day 95	0.80	0.80

¹Nonsupplemented.

²L-carnitine supplemented.

(400 mg/kg body weight) dissolved in tap water (200 mg/ml). The dose was calculated for a 250 g rat which ate approximately 20 g feed/day. If this animal was supplemented with L-carnitine at 0.5% (w/w), it would consume 400 mg L-carnitine/kg body weight/day. The NS group was administered a placebo of tap water only. Twenty-four hours after gavage, all rats were administered an ethanol gavage (3 g/kg body weight in a 13% aqueous solution) and tail blood was collected immediately prior to and at various time intervals up to 8 hours postethanol dosage. Fifty-six hours following the initial carnitine dose, a second gavage of ethanol was administered, and tail blood was collected at various time intervals for 3 hours postethanol dosage (Table 6). All animals were decapitated and livers were rapidly removed for the immediate hepatic-ethanol determination.

4B. Duration of Intraperitoneal Carnitine Administration Simultaneously or Prior to I.P. Ethanol Dosage

The purpose of this study was to determine if parenteral administration of carnitine, simultaneously or prior to what, had the same effects as enterally administered carnitine had on ethanol metabolism.

Ten male rats weighing between 400-600 g were divided into 2 groups. On day 1, immediately following an i.p. ethanol dose (0.2 g/kg body weight in a 50% aqueous solution) the NS group was administered an i.p. placebo of normal saline while the CS group was administered L-carnitine (400 mg/kg body weight) dissolved in sterilized water (200 mg/ml). Serial samples of tail blood were collected immediately prior to and at various time intervals up to 8 hours postethanol dosage.

Table 6
Experimental Design for Experiment 4A

Parameters	NS ¹	CS ²
Number of animals	4	4
Treatment (oral gavage)	water	L-carnitine ³
Ethanol dose at 24 hours postcarnitine (g/kg)	3	3
Ethanol dose at 56 hours postcarnitine (g/kg)	3	3

¹Nonsupplemented.

²L-carnitine supplemented.

³400 mg/kg dissolved in tap water (200 mg/ml).

On day 2 both groups were again administered either saline or carnitine, but no ethanol. On day 3, saline or carnitine was administered again, followed immediately by an ethanol dose. Serial tail blood samples were collected up to 8 hours postethanol administration as before (Table 7).

4C. Intravenous Carnitine Administration Simultaneously or Prior to I.V. Ethanol Dosage

The purpose of this study was to determine if the attenuated metabolism of ethanol observed after both oral and i.p. carnitine supplementation could also be shown when carnitine was administered i.v., and if so, the duration of i.v. carnitine necessary to cause this effect.

For this study 8 male rats (400-600 g) were implanted with jugular catheters according to the procedure described later in the chapter. After recovery, 4 rats of the CS group were administered carnitine (400 mg/ml of sterile normal saline) intravenously at 400 mg/kg body weight, while the 4 rats of the NS group were administered i.v. normal saline. Immediately following carnitine administration, all animals were given absolute ethanol (0.2 g/kg in a 50% aqueous solution) i.v. Blood was collected from the catheters immediately prior to and at various time intervals up to 8 hours postethanol administration. Twenty-four hours preceding the initial carnitine and ethanol dosage this procedure was performed again (day 2). Finally, on day 3, the procedure was repeated once again with the only difference being that blood samples were collected only up to 6 hours postethanol dosage (Table 8).

Table 7
Experimental Design for Experiment 4B

Parameters	Groups	
	NS ¹	CS ²
Number of animals	5	5
Day 1 treatment (i.p.)	saline/ethanol ³	L-carnitine ⁴ /ethanol
Day 2 treatment (i.p.)	saline only	L-carnitine/only
Day 3 treatment (i.p.)	saline/ethanol	L-carnitine/ethanol

¹Nonsupplemented.

²L-carnitine supplemented.

³0.2 g/kg in a 30% aqueous solution.

⁴400 mg/kg dissolved in sterilized water (200 mg/ml).

Table 8
Experimental Design for Experiment 4C

Parameters	Groups	
	NS ¹	CS ²
Number of animals	4	4
Day 1 treatment (i.v.)	saline/ethanol ³	L-carnitine ⁴ /ethanol
Day 2 treatment (i.v.)	saline/ethanol	L-carnitine/ethanol
Day 3 treatment (i.v.)	saline/ethanol	L-carnitine/ethanol

¹Nonsupplemented.

²L-carnitine supplemented.

³0.2 g/kg in a 50% aqueous solution.

⁴400 mg/kg dissolved in sterilized normal saline (400 mg/ml).

The animals were decapitated and livers were removed for the immediate hepatic-ethanol determination.

Experiment 5. Sex Differences in Carnitine-Attenuated Ethanol Metabolism

The objective of this study was to compare the effect of carnitine on ethanol metabolism in male and in female rats.

Ten female rats weighing between 250-350 g and 10 male rats weighing between 350-450 g at the same age (60 days old) served as their own controls. After 5 days on the NS diet, all animals were administered an oral dose of ethanol (3 g/kg body weight in a 13% aqueous solution) by gastric gavage and tail blood was drawn immediately prior to and at various time intervals up to 8 hours postethanol gavage (Table 9). A 20 μ l sample of tail blood was collected for blood-carnitine determination. L-carnitine (0.5%, w/w) was then supplemented in the diet of all animals for 5 days. On the 6th day all animals were administered ethanol (3 g/kg) and blood was collected for 8 hours as before. Also a 20 μ l blood sample was collected from the tail vein for blood-carnitine determination just prior to ethanol administration. At the end of this period all animals were placed back on NS chow. Tail vein blood samples were drawn from all animals on 3, 9, and 18 days after being on the NS diet to determine if and when blood-carnitine levels returned to pre-CS concentrations.

Table 9
Experimental Design for Experiment 5

Parameters	Groups	
	Male	Female
Number of animals	10	10
Days on unsupplemented diet	5	5
Oral ethanol dose (g/kg)	3	3
Days on 0.5% (w/w) L-carnitine supplemented diet	5	5
Oral ethanol dose (g/kg)	3	3

Surgical Procedures

Sampling Tail Vein Blood

The dorsal tail vein was exposed by making an incision directly over the vein. The vein was then pricked and blood was collected in a 20 μ l capacity, heparinized Micro-hematocrit capillary tube (Clay Adams, Parsippany, N.J.). The contents of the tube were immediately delivered into a 1.5 ml capacity Microfuge tube (Walter Sarstedt, Inc., Princeton, N.J.) containing 980 μ l of cold normal saline. The total volume of 1 ml was then processed for the determination of blood-ethanol.

Cannulation of Portal Vein and Posterior Vena Cava

The surgical preparation of the animals used in Experiment 1B is described below.

After 10 days of treatment each animal was anesthetized by an intramuscular injection of a mixture of Ketaset[®] (Ketamine hydrochloride, Bristol Laboratories, Syracuse, N.Y.) and Acepromazine[®] (Ayerst Laboratories, New York, N.Y.) in a 5:1 ratio. The doses of Ketaset[®] (50 mg/kg) and Acepromazine[®] (10 mg/kg) were calculated on the basis of body weight. The hair was shaved from the chest, extending from the upper torso to the pelvic region. The abdominal wall was opened exposing the liver, stomach and duodenum, and the portal vein and posterior vena cava were catheterized. Catheters consisted of .02 in. i.d. x .037 in. o.d. Silastic[®] Medical-Grade Tubing (Dow Corning Corp., Midland, MI) with attached 27G $\frac{1}{2}$ " needle (Beckman Dickinson Co., Rutherford, N.J.). A dose of ethanol (3 g/kg in a 13% aqueous solution)

was infused into the duodenum via a peristaltic pump (Gilson Medical Electronics, Inc., Middletown, WI) at a rate of 1 ml/min. The infusion was accomplished by attaching an 1/8 in. i.d. x 3/16 in. o.d. Tygon® R-3603 Silastic tubing (Fisher Scientific Co., Pittsburg, Pa.) to the pump and a semiblunt 19G 1½" needle attached and placed through the stomach into the gastroduodenal opening. The needle was secured in place with 2-0 silk surgical suture to prevent back flow of duodenal fluid into the stomach. Serial portal and systemic blood samples were collected as described later in the chapter.

Cannulation of Jugular Vein (Cardiac Catheterization)

The surgical procedure used for cardiac catheterization such as used in Experiment 4C is described below.

Each animal was anesthetized by an intramuscular injection of Ketaset® and Acepromazine® in a 5:1 ratio (Ketaset®, 50 mg/kg; Acepromazine®, 10 mg/kg) calculated according to the body weight. When the animal was anesthetized, the hair was removed from about a 3 cm square area above the right jugular vein.

The surgical procedure was carried out under aseptic conditions and all instruments were sterilized by autoclave or Nolvasan® solution (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa). A small skin incision (approximately 1 cm) was made above the right jugular vein in an anterior direction starting from a point above the collar bone. By blunt dissection, approximately 5 mm of the right jugular vein was exposed. A folded piece of suture (2-0 silk) was passed under the exposed vein and the loop was cut to make two single strands of thread. The anterior end

of the exposed vein was then ligated and the tie was left uncut. A small oblique cut was then made with a pair of fine iris scissors while the vein itself was retracted. Medical grade Silastic[®] tubing (.02 in. i.d. x .037 in. o.d.) (Dow Corning Corp., Midland, MI) was cut to 150 mm and rinsed with heparinized saline (89 units/ml). The beveled end of the tubing was then inserted into the vein and passed 30-40 mm towards the heart. The tubing was then secured with the posterior strand of suture tied firmly around the vein, just below the point of insertion, and again with another suture around the vein, just forward of the collar bone. The ties were then treated and secured with 1 drop of Nexabond[®] liquid (Bionexus, Inc., Raleigh, N.C.). The tubing was then inserted by use of a 9 gauge trochar under the skin and passed subcutaneously to exit at a point on the back of the neck between the scapulae. A 1 cc syringe charged with heparinized saline was used to clear the tubing. After injecting about 0.2 ml of heparinized saline, the cannula was cut to a length of about 2 cm and plugged with a 21 gauge wire which had been cut to 2 mm in length. The surgical wound was then closed with 4-5 interrupted sutures (4-0 silk) and an antibacterial ointment was applied. The animal was allowed to recover for 48-72 hours. Direct cardiac blood samples were collected as described later in the chapter.

Sample Collection, Treatment and Storage

24-Hour Urine Collection for Carnitine Determination

The urine was collected in a 50 ml Pyrex[®] Erlenmeyer flask using Thymol as a preservative (1-5 crystals/dl urine) after placing animals

in metabolic cages. The volume of urine was measured and centrifuged (Model TJ-6, Beckman Instruments, Palo Alto, Ca.) for 10 minutes at 1500 x g at 4°C. After centrifugation, 1 ml of clear urine from each tube was transferred into 1.5 ml capacity Microfuge tubes. Samples were frozen at -70°C.

Portal and Systemic Blood Collection

Serial portal and systemic blood samples were collected about 15-30 seconds apart in 20 µl capacity microhematocrit capillary tubes through the indwelling catheters up to 30 minutes postethanol infusion. Each blood sample was delivered into 980 µl of ice cold normal saline contained in a microfuge tube and was analyzed for blood-ethanol concentration within 24 hours after collection.

Collection Procedure for Muscle Samples

Immediately following blood collection, a 50-100 mg biopsy of medial quadriceps muscle was collected and the animal was euthanized. The muscle tissue was frozen at -70°C.

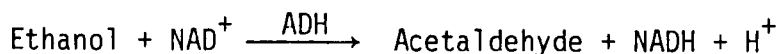
Collection Procedure for Blood and Liver

Following decapitation, blood was collected in Pyrex[®] conical test tubes, centrifuged at 1500 x g at 4°C for 10 minutes, and plasma was stored at -70°C. The liver was excised, weighed, and minced with a pair of surgical scissors. The liver was homogenized (Con-Torque Homogenizer, Eberback Corp., Ann Arbor, MI) in a ratio of 1 g tissue:2 ml cold normal saline. Aliquots of the homogenate were stored in 17 x 100 mm Polypropylene tubes (Fisher Scientific Co., Fair Lawn, N.J.) at -70°C.

Assay Procedures

Blood-Ethanol Determination

Blood-ethanol concentrations in each sample were determined by the modified procedure of Bernt and Gutmann (164). The assay is based on the principle that ethanol is oxidized by NAD^+ and Alcohol Dehydrogenase (ADH) to yield NADH and acetaldehyde.



The formation of NADH was measured spectrophotometrically by monitoring the increase in extinction at 340 nm. The amount of $\text{NADH} + \text{H}^+$ formed is proportional to the amount of ethanol present in a sample.

Reagents.

1. Sodium Pyrophosphate buffer (75 mM pyrophosphate buffer, pH 8.7, 75 mM semicarbazide, 21 mM glycine): 10 g $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$, 2.5 g semicarbazide-HCl and 0.5 g glycine were dissolved in 250 ml of glass distilled water (Corning Mega-Pure, Corning, N.Y.), adjusted to pH 8.7 with 4N potassium hydroxide and diluted to 300 ml with glass distilled water.

2. Nicotinamide-adenine dinucleotide (24 mM β -NAD): 50 mg of NAD (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 3.0 ml of glass distilled water.

3. Alcohol dehydrogenase (174 IU/mg at 30°C): 30 mg (5220 IU) of lyophilized enzyme (CalBiochem-Behring Corp., La Jolla, Ca.) was dissolved in 0.5 ml glass distilled water.

4. Ethanol stock standard (0.5% (v/v) absolute ethanol): 0.125 ml of absolute ethanol was diluted to 25 ml with glass distilled water. The working standard was prepared by diluting the stock standard 50-fold. One ml of the stock standard was then diluted to 50 ml with cold normal saline.

Procedure. A reaction mixture was prepared by mixing 2.38 ml of the sodium pyrophosphate buffer, 0.1 ml of NAD^+ , and 0.02 ml (208.8 units) of ADH, making a total volume of 2.5 ml of the mixture for each sample tested. Ethanol standards were prepared by diluting the working standard, using 0-100 μl , with cold normal saline. All standards and samples were centrifuged for 2 minutes at 1500 RPM in an Eppendorf Centrifuge, Model 5412 (Brinkman Instruments, Inc., Westbury, N.Y.). An 0.1 ml aliquot of supernatant from all standards and samples was added to 12 x 75 mm Pyrex test tubes followed by the addition of 2.5 ml of the reaction mixture. The contents of each tube were mixed, incubated for 25 minutes in a 37°C water bath, and after cooling to room temperature, the absorbance of each sample was determined by a Spectrophotometer (Model 34, Beckman Instruments, Fullerton, Ca.) at 340 nm. Ethanol concentrations in the blood were calculated as follows:

$$\text{Ethanol (mg/dl whole blood)} = \frac{\text{absorbance of sample}}{\text{inverse slope of standard curve}} \times 50.$$

50 = the appropriate factor to bring the blood volume used (0.02 ml) up to 1 ml.

Liver-Ethanol Determination

After the livers were removed, they were homogenized in cold normal saline (1 g tissue/2 ml). Thus, the homogenate represented 500 mg wet

weight tissue/ml. Where the blood-ethanol assay used normal saline to dilute the standards and sample, the liver-ethanol assay used 6% PCA. The remainder of the assay procedure for liver-ethanol determination was identical to that of blood-ethanol. Ethanol concentrations in the liver were calculated as follows:

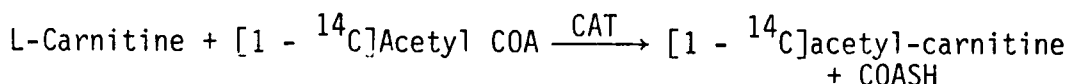
$$\text{Ethanol (mg/g tissue)} = \frac{\text{absorbance of sample}}{\text{inverse slope of standard curve}} \times 20$$

20 = the appropriate factor to bring the amount of tissue (0.1 ml used = 50 mg) up to 1 g.

A standard curve is shown in Figure 4.

Carnitine Determination

Blood, urinary and tissue carnitines were determined by the radioisotopic procedure of Cederblad and Lindstedt (165) as modified in our laboratory (22). Total carnitine in whole blood, urine and tissue was determined by measuring free carnitine, (NEC or nonesterified carnitine), acid soluble acyl carnitine, (ASAC or short chain carnitine), and acid insoluble acylcarnitine, (AIAC or long chain carnitine). The sum of these fractions was called total carnitine (TC). The radiometric method for carnitine determination is based on the following reaction:



The reaction is driven to the right upon the addition of sodium tetrathionate which binds the COASH molecule. The reaction mixture was passed over an anion exchange resin column which traps the unreacted

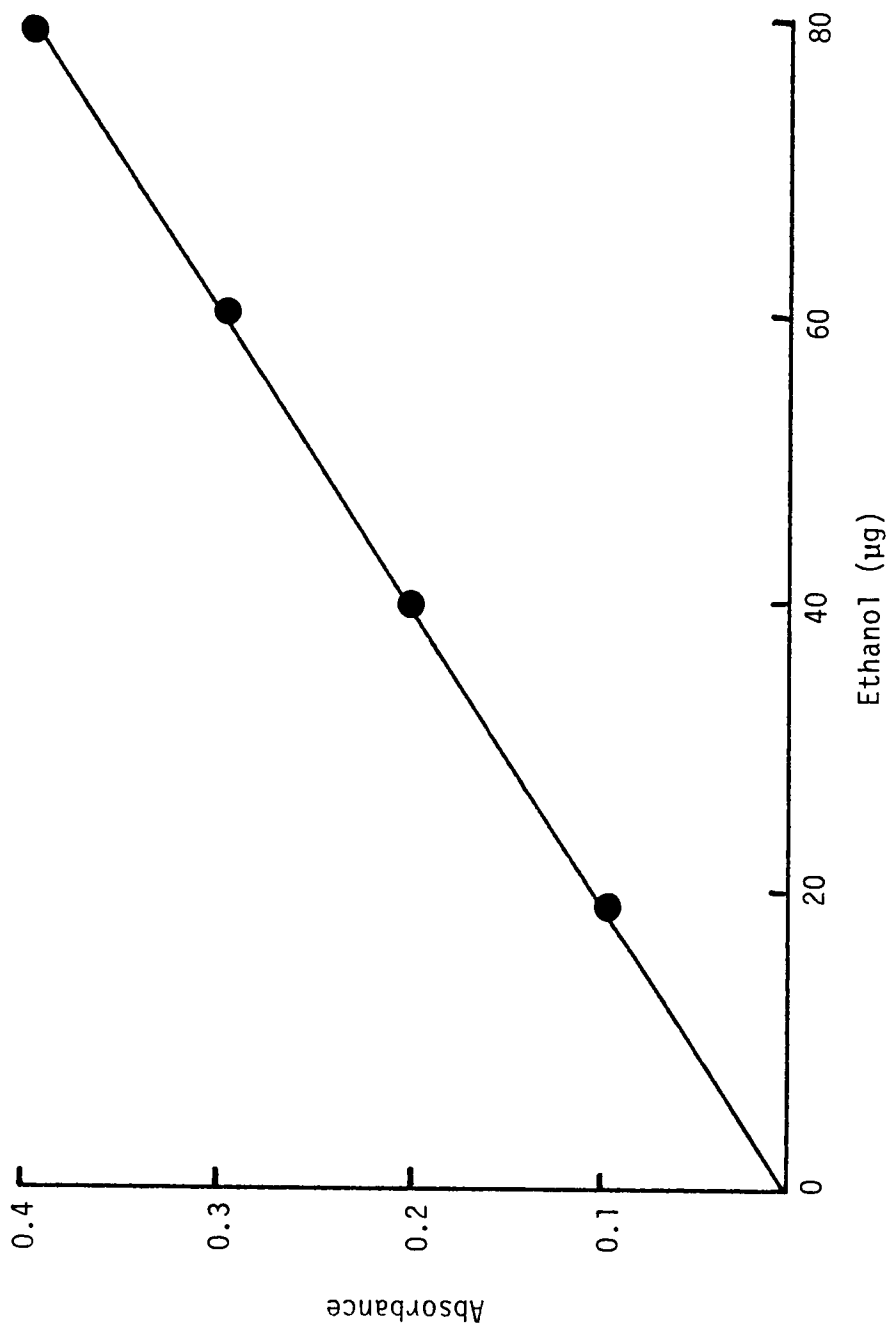


Figure 4. Standard Curve for Ethanol Obtained With Absolute Ethanol at 340 nm.

labeled acetyl COA, while allowing the labeled acetyl-carnitine to pass through the column. The eluate was mixed with scintillation solution and radioactivity was determined in a Beckman LS3801 scintillation counter. Blood carnitine was expressed in nmol/ml, urine in $\mu\text{mol}/24$ hours, and tissue carnitine in $\mu\text{moles/gm}$ wet wt of the tissue.

Reagents.

1. 0.5 mM Carnitine standard solution (stock): 9.88 mg L-carnitine HCl (Sigma Chemical Co., St. Louis, Mo.) was dissolved in cold glass distilled water and diluted to a volume of 100 ml in a volumetric flask. The stock standard was diluted 1:2 to give a concentration of 0.25 mM. This was the working standard.

2. 22.9 mM L-palmitoylcarnitine standard (stock): 1.0 ml of glass distilled water was added to a vial containing 10 ml of L-palmityl carnitine (Sigma Chemical Co., St. Louis, Mo.). The stock standard was diluted 1:100 to give a concentration of 0.229 mM. This was the working standard.

3. Carnitine standard mixture: L-carnitine and L-palmitoyl carnitine working standards were mixed together in equal volumes.

4. 0.5 N Potassium hydroxide: 28.05 g of KOH pellets was dissolved in glass distilled water and the total volume was brought to 1 liter.

5. 4 N Potassium hydroxide: 56.11 g of KOH pellets was dissolved in glass distilled water and diluted to a volume of 250 ml.

6. 0.6 M Perchloric acid (PCA): 51.26 ml of 70% PCA was added to glass distilled water and made to 1 liter.

7. 0.1 M Sodium tetrathionate: 0.6756 g of anhydrous sodium tetrathionate (J. T. Baker Chem. Co., Phillipsburg, N.J.) was dissolved in glass distilled water and made to a volume of 25 ml.

8. 0.1% Phenol red: 100 mg of phenol red (Sigma Chemical Co., St. Louis, Mo.) was dissolved in absolute ethanol and diluted to 100 ml. (Phenol red tubes were made by adding 1 drop of phenol red to 12 x 75 mm Pyrex glass tubes and allowed to dry.

9. Radioactive acetyl Coenzyme A, (50 mci/mmol): 50 μ ci of [1 - ^{14} C] Acetyl Coenzyme A (Amerstam Corp., Chicago, Ill.) was dissolved in 300 ml of cold glass distilled water. Aliquots of 5.0 ml were dispensed into plastic vials and frozen at -70°C .

10. 1 M potassium bicarbonate: 1 g potassium bicarbonate (Certified ASC, Fisher Scientific Co., Fair Lawn, N.J.) was dissolved in glass distilled water and diluted to 10 ml. The solution was stored at room temperature and restored weekly.

11. 0.1 M acetic anhydride: 0.5 ml of acetic anhydride (Fisher Scientific Co., Fair Lawn, N.J.) was added to 4.95 ml of cold glass distilled water, mixed, and used immediately for the acetyl CoA solution.

12. 0.1 mM acetyl coenzyme A: 10 mg of acetyl coenzyme A (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 0.5 ml of cold glass distilled water. To this, 100 μ l of 1 M potassium bicarbonate was added. Then, 200 μ l of 0.1 M acetic anhydride was added and the volume brought up to 80 ml with glass distilled water. After mixing thoroughly, the solution was dispensed into 5 ml plastic tubes and frozen at -70°C .

13. Carnitine acetyltransferase (CAT): carnitine acetyl transferase from pigeon breast muscle (Sigma Chemical Co., St. Louis, Mo.) was diluted with glass distilled water to 50 units/ml.

14. 1 M [3-(4-morpholino)propanesulfonic acid] MOPS: 20.92 g of MOPS (Sigma Chemical Co., St. Louis, Mo.) was dissolved in approximately 80 ml of glass distilled water. The pH was adjusted to 7.4 with 4N potassium hydroxide, and the volume was brought up to 100 ml with glass distilled water.

15. PCA/MOPS-I: 20.9 g of MOPS was dissolved in 50 ml of 0.6 M PCA and diluted to a volume of 100 ml with glass distilled water. This solution was stored at 4°C.

16. PCA/MOPS-II: 20.9 g of MOPS was dissolved in 20 ml of 0.6 M PCA and diluted to a volume of 100 ml with glass distilled water. This solution was stored at 4°C.

17. 0.1 M ethylene glycol-bis (beta-amino-ethylether) N,N-tetraacetic acid (EGTA), pH 7: 1.902 g EGTA (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 30 ml of glass distilled water and neutralized with 4 N potassium hydroxide to a pH of 7.0. The volume was then made to 50 ml with glass distilled water.

18. 0.1 mM [1 - ^{14}C] acetyl coenzyme A solution: 2 volumes of [1 - ^{14}C] acetyl COA and 1 volume of 0.1 mM acetyl COA were mixed and kept on ice.

19. Reagent mixture for one assay: 1 M MOPS buffer, pH 7.4, 120 μl ; 0.1 M EGTA, pH 7.0, 20 μl ; 0.1 M sodium tetrathionate, 20 μl ; 0.1 mM [1 - ^{14}C]-acetyl COA solution, 200 μl ; glass distilled water, 40 μl ; to a total volume of 400 μl .

20. 8% bovine serum albumin (BSA): 4 g of bovine serum albumin (Fatty acid poor, Fraction V, ICN Pharmaceuticals Inc., Life Sci. Group, Cleveland, OH) was dusted into about 20 ml of glass distilled water, gently stirred, and diluted to a volume of 50 ml.

21. Scintillation fluid: Ready-Solv[®]CP purchased from Beckman Instruments was used.

Procedure. Carnitine standards were prepared as shown in Table 10. For each blood sample tested, 20 μ l of whole blood was added to 200 μ l of PCA contained in a 12 x 75 mm Pyrex glass test tube. Since 400 μ l was desired for the final volume, 180 μ l of glass distilled water was added. All standards and samples were vortexed and centrifuged (Model TJ-6, Beckman Instruments, Palo Alto, Ca.) at 1500 x g for 10 minutes at 4°C.

The urine was prepared for assaying by adding 20 μ l of urine diluted 1:10 with water to 200 μ l of PCA, 80 μ l of glass distilled water and 100 μ l of 8% BSA to bring the total volume to 400 μ l. Each tube was vortexed and centrifuged at 1500 x g for 10 minutes at 4°C.

Muscle samples for carnitine analysis were prepared by weighing out 100 mg tissue and placing it into a glass homogenizing vessel containing 3 ml of 0.6 M PCA. A Polytron[®] tissue homogenizer was used to homogenize the muscle, and the homogenate was then transferred to a graduated conical test tube and rinsed with about 2 ml of 3% PCA. After bringing the final volume up to 5 ml, the homogenate was vortexed and 50 μ l were transferred to a 12 x 75 mm Pyrex test tube. To this 200 μ l of PCA and 150 μ l of glass distilled water were added to bring the total volume to

Table 10
Standard Mixtures of Carnitine

0.25 mM Carnitine		0.23 mM L-Palmityl Carnitine		GDW ¹ (μ l)	8% BSA ² (μ l)	6% PCA ³ (μ l)	Total (μ l)	Total Carnitine (nm)
(μ l)	(nm)	(μ l)	(nm)					
0	0	0	0	100	100	200	400	0
5	1.25	5	1.15	90				2.40
10	2.50	10	2.30	80				4.80
15	3.75	15	3.45	70				7.20
20	5.00	20	4.60	60				9.60
30	7.50	30	6.90	40				14.40

¹Glass distilled water.

²Bovine serum albumin.

³Perchloric acid.

400 μ l. Each tube was vortexed and centrifuged at 1500 x g for 10 minutes at 4°C.

For all standards as well as blood, urine, and muscle samples, the supernatant was immediately separated from the pellet. Aliquots of 150 μ l and 100 μ l were transferred to 12 x 75 mm phenol red tubes that had been labeled NEC and ASAC, respectively. The tubes containing the pellets were completely drained off of the supernatant liquid and labeled AIAC.

Nonesterified Carnitine Determination

After transferring 150 μ l of the PCA-supernatant to the phenol red test tube, 35 μ l of 1 M potassium bicarbonate was added and the contents were vortexed. The contents turned a golden yellow color indicating a slightly acidic pH. The mix was then placed in ice for 30 minutes.

Acid Soluble Acylcarnitine Determination

After transferring 100 μ l of the PCA-supernatant to the phenol red test tube, 75 μ l of 0.5 N potassium hydroxide was added. After vortexing, the contents turned a purple-red color indicating an alkaline pH which was required for hydrolysis. The tube was incubated for 30 minutes in a 37°C hot water bath, shaking gently at about 100 shakes per minute in order to complete hydrolytic action. At the end of the 30 minutes, the contents of the tube was neutralized with 30 μ l of PCA/MOPS-II. The mix was vortexed and kept on ice for 30 minutes.

Acid Insoluble Acylcarnitine Determination

After the remaining supernatant in the tube was drained, the pellet was washed twice with 200 μ l of 0.6 M PCA and inverted to drain. A

single drop of phenol red indicator was added to the tube and then 200 μ l of 0.5 N potassium hydroxide was added to take up the pellet containing only the acid insoluble fraction. The tube was vigorously vortexed for several minutes until the pellet was completely dissolved in the potassium hydroxide. The contents were purple-red in color indicating an alkaline pH. The tube was then hydrolyzed for 60 minutes at 65°C in a shaking incubator water bath (100 SPM). After hydrolysis it was neutralized with 100 μ l of PCA/MOPS-I, followed by vortexing and placed in an ice bath for 30 minutes.

Assay for Carnitine

The NEC, ASAC, and AIAC tubes were all centrifuged for 10 minutes at 1500 x g at 4°C. A 100 μ l aliquot of each of the three fractions was transferred to 1.5 ml capacity microfuge tubes and 400 μ l of reagent mixture was then added. This was followed by the addition of 20 μ l of carnitine acetyltransferase (CAT). The tubes were capped, gently mixed by tapping, and placed in a shaking incubator water bath at 37°C for 30 minutes. At the end of the incubation time, 200 μ l of incubation mixture was transferred onto a mini column. Mini columns were prepared by using 5-3/4" Pasteur pipettes (Fisher Scientific Co., Fair Lawn, N.J.) and stuffing the area above the tips with a small ball of glass wool. Anion Exchange Resin, AG I-X8, 200-400 mesh, Cl-form (Bio-Rad Laboratories, Richmond, Ca.) was added up to the 9 cm mark measured from the tip of the pipette. After the sample was added to the column and absorbed, the column was rinsed down with 2 aliquots of glass distilled water containing 500 μ l each into a plastic mini scintillation vial

(Beckman Instruments, Palo Alto, Ca.). To each scintillation vial, 5 ml of scintillation fluid was added and the vial was capped and mixed by swirling. All vials were then placed in an LS-3801 table top scintillation counter (Beckman Instruments, Irvine, Ca.) and radioactivity determinations were done.

After the counts per minute (CPM) were recorded for all 3 sets (NEC, ASAC, and AIAC) of tubes, the blank in each set was subtracted from each standard and sample in that set. A standard curve was then plotted for each set. For each of the 3 fractions, to calculate either the nmoles carnitine/ml blood, μ moles carnitine/24 hours, or the μ moles carnitine/g of muscle tissue, the following equations were utilized:

$$\text{Blood: } \text{I.S.} \times 50 \times \text{CPM} = \frac{\text{nmoles carnitine}}{\text{ml blood}}$$

$$\begin{aligned} \text{Urine: } \text{I.S.} \times 50 \times \text{CP} \times \text{D.F.} \times \text{ml excreted in 24 hours} \\ = \frac{\mu\text{moles carnitine}}{24 \text{ hours}} \end{aligned}$$

$$\text{Muscle: } \text{I.S.} \times 10 \times \text{CPM} = \frac{\mu\text{moles carnitine}}{\text{g tissue}}$$

where: I.S. = inverse slope,

50 = the appropriate factor to bring the volume of carnitine up to 1 ml ($20 \mu\text{l} \times 50 = 1 \text{ ml}$),

D.F. = dilution factor for urine,

10 = the appropriate factor to bring the amount of carnitine up to 1 g ($50 \mu\text{l}$ represents $100 \text{ mg} \times 10 = 1 \text{ g}$), and

CPM = counts per minute.

A standard curve is shown in Figure 5.

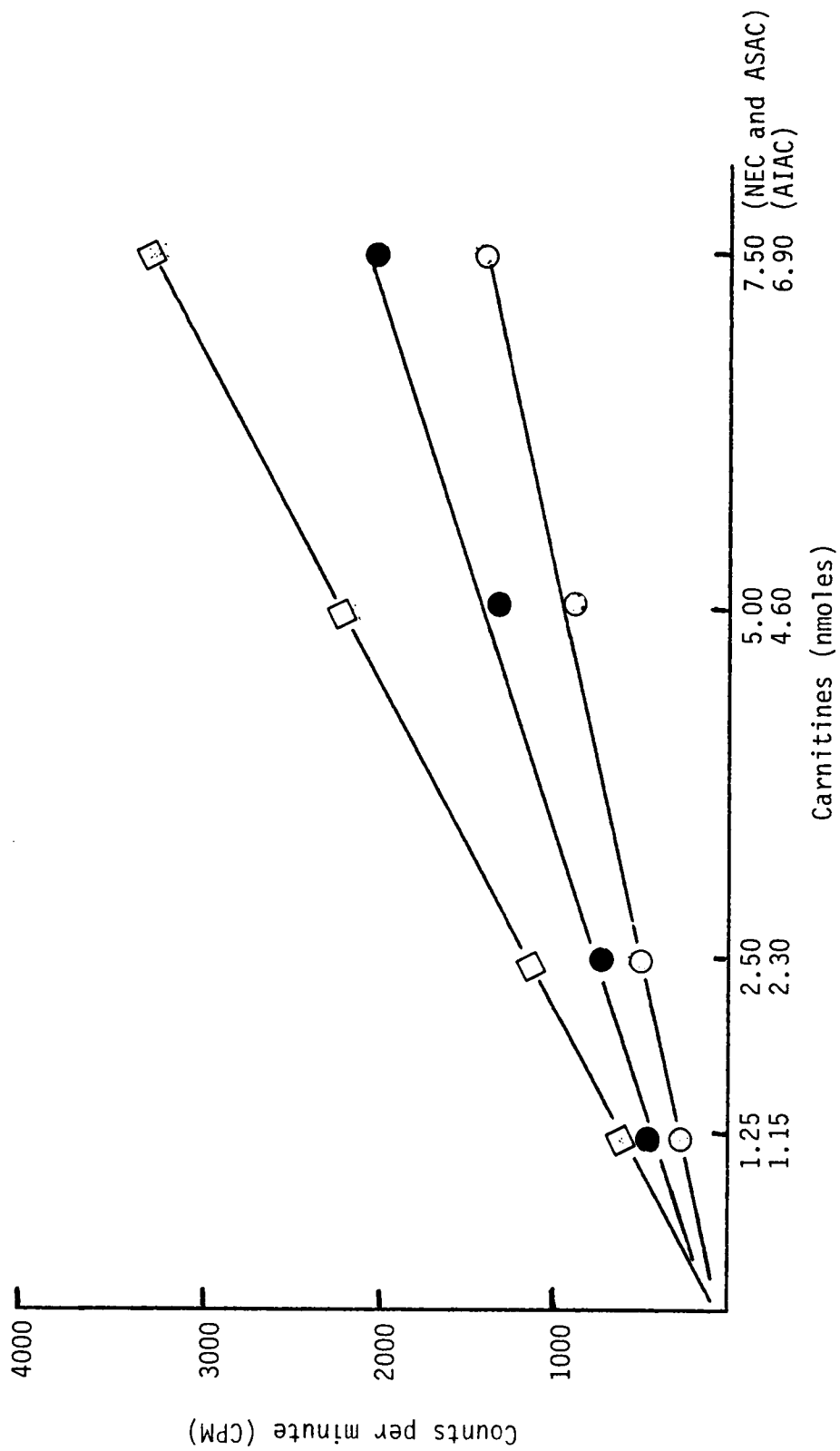


Figure 5. Standard Curves for Nonesterified Carnitine (NEC, -●-), Acid Soluble Acylcarnitine (ASAC, -○-), and Acid Insoluble Acylcarnitine (AIAC, -□-) Using Carnitine and Palmitoylcarnitine in an LS-3801 Scintillation Counter.

Triglyceride Determination in Plasma

The contents of the triglyceride in plasma were measured by the method of Giegel et al. (166). The principle behind this procedure is based on the fact that lipids are partitioned between water/isopropanol and nonane, with triglyceride extracted into the nonane. Glycerol is liberated by hydrolysis from triglyceride by sodium hydroxide and then oxidized to formaldehyde by sodium periodate. The formaldehyde reacts with 2,4-pentanedione to form 3,5-diacetyl-1,3-dihydrolutidine which is the colored compound measured.

Reagents.

1. Extraction reagent: n-nonane/isopropanol (2.0/3.5 by volume).
2. Triolein standard: triolein (Sigma Chemical Co., St. Louis, Mo.) 100 mg/dl in extraction reagent (1.13 mM/l).
3. Dilute sulfuric acid, 40 mM/l.
4. Transesterifying reagent: sodium hydroxide in isopropanol (100 mM/l).
5. Oxidizing reagent: sodium periodate, 18 mM/l in 2.0 M/l acetic acid.
6. Color buffer: ammonium acetate (6.0 M/l, pH 6.0 at 25°C).
7. Working color reagent: 4.0 ml of acetylacetone was added to 100 ml of the color buffer; the solution was mixed and allowed to stand for at least 15 minutes before use.

Procedure. The sample (0.2 ml) was extracted with a mixture of 5.0 ml extraction reagent in a 16 x 100 mm screw-capped test tube and

1.0 ml of dilute sulfuric acid was added to the tube. A blank and three standards were made by using 0, 0.1, 0.2, 0.4, and 0.6 ml of standard, respectively, in place of the volume of extraction reagent. Tubes were then centrifuged at 1500 x g at 4°C for 10 minutes, where a clear upper phase was recovered. An 0.5 ml aliquot of each upper phase extraction was mixed with 0.5 ml of transesterifying reagent and allowed to stand for 5 minutes at room temperature. Then 0.5 ml of the oxidizing reagent was added to each tube, and the mixture was allowed to stand for 5 minutes at room temperature. Once the two-phase system was developed, 6 ml of working color reagent was added to each tube, mixed, and all tubes were incubated at 60°C for 10 minutes for color development. After cooling to room temperature, the absorbance was measured at 415 nm against the reagent blank. The following equation was utilized:

$$\text{Triglycerides (mg/dl)} = \text{Au/As} \times \text{Cs} \times \text{D.F.}$$

where: Au = absorbance of unknown.

As = absorbance of standard.

Cs = concentration of standard.

D.F. = appropriate dilution factor.

Triglyceride Determination in the Liver

After the liver of each animal was removed, its weight was recorded and it was homogenized in a 1:2 dilution with normal saline. A blank and four standards were made by using 0, 0.1, 0.2, 0.4, and 0.6 ml of the triolein standard, respectively, to glass-stoppered conical test tubes. The solvent was then evaporated under a stream of nitrogen

(N-Evap analytical evaporator, Shrewbury, Ma.). Added was 1.0 ml of 40 mM sulfuric acid, 0.2 ml of glass distilled water, and 5.0 ml of the chloroform-methanol extraction reagent to these tubes. After this a 0.2 ml aliquot of homogenate, (representing 100 mg tissue) 1.0 ml of 40 mM sulfuric acid and 5 ml of the chloroform-methanol extraction reagent was added to one conical test tube per sample. The contents of the blank, standards, and samples were vortexed and centrifuged at $1500 \times g$ at 4°C for 10 minutes. After the tubes were removed from the centrifuge, three phases were present. From each tube, 0.5 ml of the chloroform phase was transferred to new conical screw cap test tubes and the chloroform was then evaporated by nitrogen steam. Once all test tubes were dry, 0.5 ml of N-nonane was added to each tube. Then 0.5 ml of transesterifying reagent was added, tubes were vortexed and allowed to stand for 5-10 minutes at room temperature. To each tube, 0.5 ml of oxidizing reagent was added, vortexed, and allowed to stand for 2-5 minutes at room temperature. This was adequate time for a two-phase system to develop. Six ml of working color reagent was then added to each tube, vortexed, and all tubes were incubated in a water bath at $56-60^{\circ}\text{C}$ for 10 minutes. After this time, all tubes were removed from the water bath and allowed to cool to room temperature. Absorbance was then measured at 415 nm against the reagent blank where the following equation was added:

$$\text{Triglyceride (mg/g wet wet liver)} = \text{Au/As} \times \text{CS} \times 10$$

where: Au = absorbance of unknown.

AS = absorbance of standard.

CS = concentration of standard.

10 = dilution factor of homogenate bringing it up to 1 gm total (originally 100 mg/sample).

A standard curve is shown in Figure 6.

Total Lipid Determination in the Liver

Total lipid contents were measured by the phosphoric acid-vanillin reaction method (167). In this method, the specimen is heated with concentrated sulfuric acid. After this, vanillin and phosphoric acid are added to yield a pink colored product. The principle is that unsaturated lipids react with vanillin or a derivative of vanillin under the influence of acid catalysis to yield the pink colored product. One possible explanation for this reaction is that the unsaturated bonds in a lipid oxidize to ketones first and when treated with acid, the ketones condense with vanillin to a derivative compound which absorbs visible light.

Reagents.

1. Sulfuric acid (AR).
2. Phosphoric acid-vanillin reagent: 1 g of vanillin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 160 ml of glass distilled water in a 500 ml volumetric flask. Concentrated phosphoric acid was then added to the mark and mixed thoroughly.
3. Stock standard: 1 g of olive oil was mixed in a small volume of chloroform in a 100 ml volumetric flask. The mixture was then diluted to the mark with chloroform and mixed thoroughly. The solution was stoppered and stored in a freezer at -20°C.

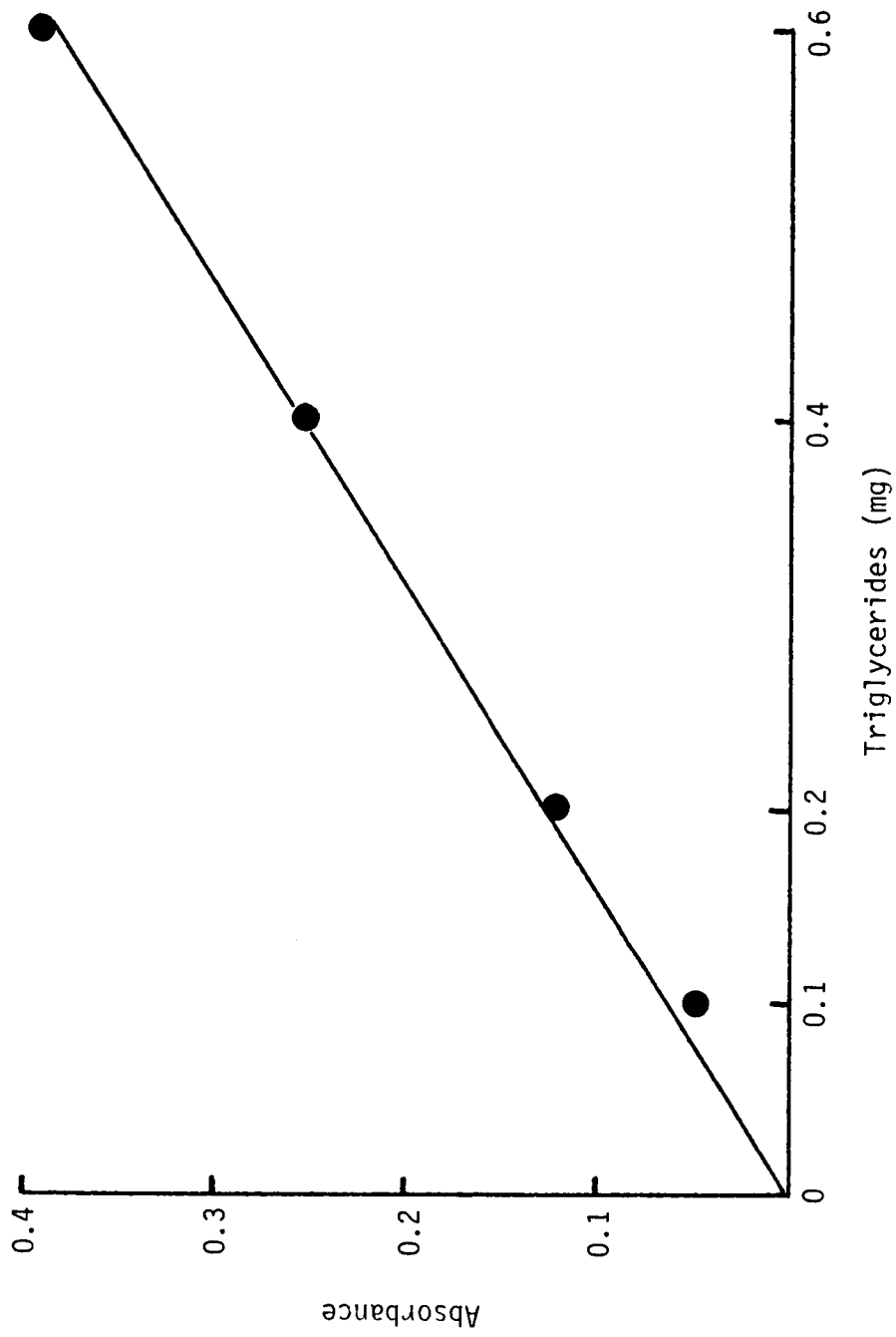


Figure 6. Standard Curve for Triglyceride Obtained With Triolein at 415 nm.

4. Working standard: 1 ml of the stock solution was transferred to a 10 ml volumetric flask, diluted to the mark with chloroform and mixed thoroughly. The solution was stoppered and refrigerated.

Procedure. The homogenate (0.2 ml, representing 100 mg/tissue) was transferred to a 16 x 150 mm test tube, while 0.2 ml of water was transferred to the blank tube. For the standards, 0.05, 0.1, and 0.2 ml of working standard were transferred into other tubes. The chloroform in the standards was evaporated off with a stream of nitrogen and 0.2 ml glass distilled water was added to them. Five ml of concentrated sulfuric acid was added to all tubes and vortexed. The tubes were then stoppered and heated at 100°C in a heating block (Precision, GCA Corp.) for 10 minutes. The tubes were allowed to cool to room temperature, and then 200 μ l of the contents of each tube were transferred to 12 x 75 mm test tubes. Three ml of the phosphoric acid-vanillin reagent was added to each tube and vortexed. All tubes were placed in the dark for 60 minutes after which time absorbances were read at 520 nm in a Beckman Spectrophotometer. The following equation was used:

$$\text{Total lipids (mg/g)} = \text{Abs.} \times \text{Slope} \times \text{D.F.}$$

where Abs. = absorption at 520 nm.

D.F. = Dilution factor (since 0.2 ml homogenate represented 100 mg of tissue, D.F. = 10 to make 1 g).

A standard curve is shown in Figure 7.

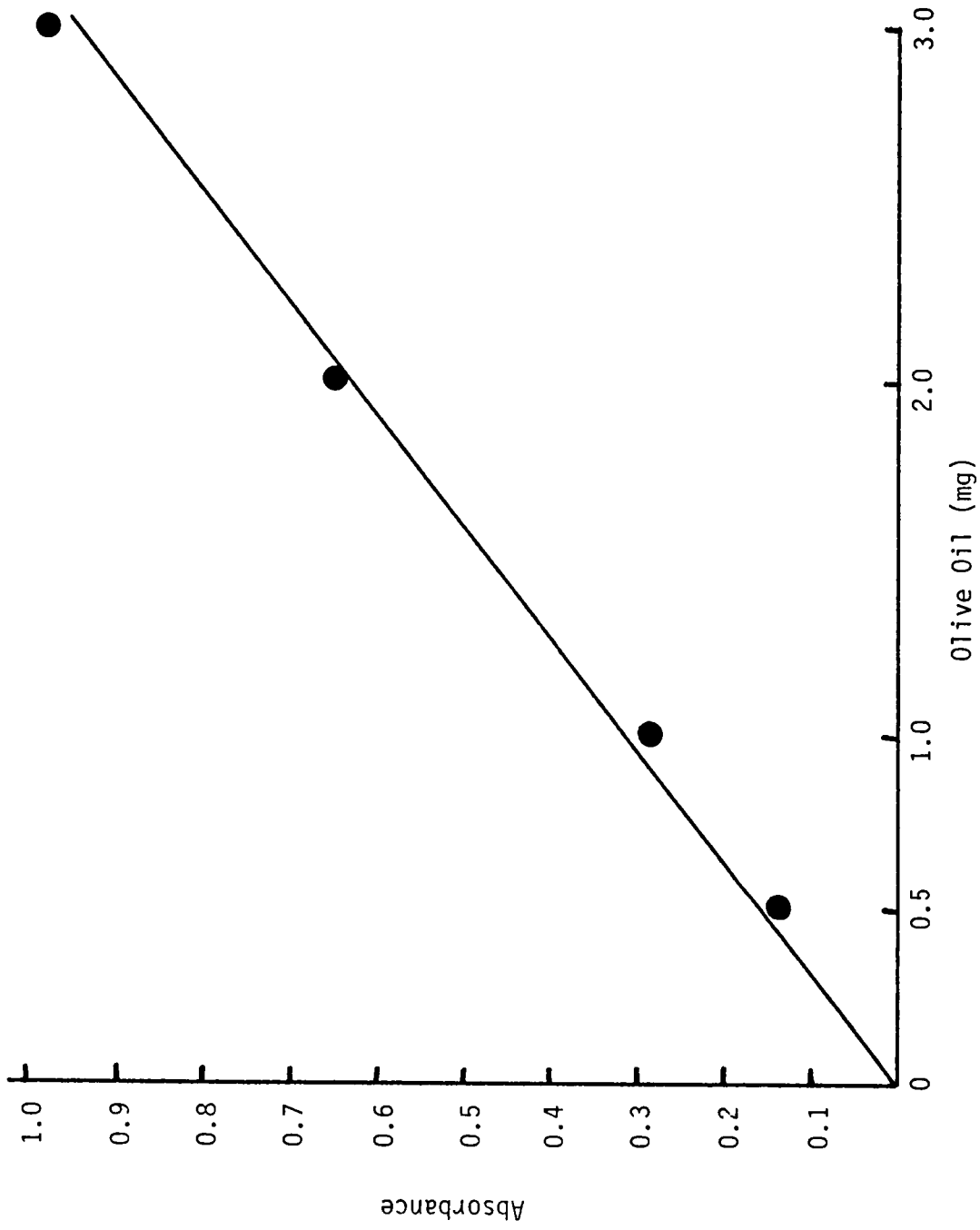


Figure 7. Standard Curve for Total Lipids Obtained with Olive Oil at 520 nm.

Statistics

All data were expressed as mean $\pm$ SEM.¹ The data were analyzed as follows: when 2 treatment groups were analyzed and compared, the students' t test (51) was utilized. When more than 2 treatment groups were analyzed and compared, the Analysis of Variance, and when significant, the Duncan's New Multiple Range Test (52) was used. "Linear Regression" was used to determine the rates of ethanol disappearance from the blood where lines were extrapolated to zero time after blood-ethanol peak concentrations were reached in order to evaluate hypothetical zero time values. The Pearson's "r" was utilized to correlate all lines (51, 52). For all data analyzed, the minimal level of statistical significance accepted was $p \leq 0.05$.

¹Standard error of mean.

CHAPTER IV

RESULTS

Experiment 1A. Level and Duration of Oral L-Carnitine Supplementation on Steady State Concentrations of Carnitine in the Blood and Urine

The effects of different levels (0, 0.25, 0.50, 0.75 and 1.0%) of L-carnitine on body weights and urinary outputs are presented in Table 11. The weight gains over the 10-day period were not significantly different among the groups. Conversely, the 24-hour urinary output was significantly higher in the CS-0.75% and CS-1.0% group compared to the NS and the CS-0.25% group. However, the urinary output of the CS-0.5% group was not significantly different from that of the other groups.

Changes in concentration of the blood-carnitine fractions in relation to the duration and levels of L-carnitine supplementation for all groups are presented in Table 12 and Figure 8. The concentrations of all carnitine fractions in the NS group remained unchanged during the 10-day period. However, the concentrations of all carnitine fractions excluding the AIAC fraction were significantly elevated after 1 day of feeding 0.25, 0.50, 0.75 or 1.0% supplementary L-carnitine. Peak concentrations were reached after 3 days of feeding 0.50% supplementary L-carnitine and higher doses did not significantly elevate the concentrations of the carnitine fractions in the blood.

Table 11
The Effects of Supplementary L-Carnitine Levels on Body Weights
and Urinary Outputs of Male Rats

Parameter	Groups				
	NS ¹	CS ² -0.25%	CS-0.50%	CS-0.75%	CS-1.0%
Number of Animals	4	4	4	4	4
L-Carnitine Supplementation (% w/w)	0	.25	.50	.75	1.0
Duration of Treatment (days)	10	10	10	10	10
Initial BWT (G) ³	300.3 ± 18.7 ^a	295.8 ± 13.8 ^a	297.3 ± 14.9 ^a	301.0 ± 15.0 ^a	298.3 ± 13.0 ^a
Weight Gain (G) ³	20.3 ± 2.4 ^a	20.0 ± 0.6 ^a	18.0 ± 3.4 ^a	14.0 ± 3.2 ^a	16.5 ± 2.5 ^a
Average Urinary Output (ml/24 hrs) ³	11.9 ± 0.3 ^a	12.0 ± 0.6 ^a	12.9 ± 0.7 ^{ab}	13.7 ± 0.5 ^b	14.3 ± 0.4 ^b

¹NS = Nonsupplemented group.

²CS = L-carnitine supplemented group.

³The values are mean ± SEM. Values in a row bearing the same superscript letter are not statistically different ($p \geq 0.05$).

Table 12
Blood-Carnitine Concentrations (nmoles/ml) After 1, 3, 5, 7, and 10 Days
of Feeding Varying Levels Supplementary L-Carnitine¹

Days on Supplementary L-Carnitine	Groups				
	NS (4) ²	CS-0.25% (4)	CS-0.50% (4)	CS-0.75% (4)	CS-1.0% (4)
Nonesterified Carnitine (NEC)					
Pretreatment	36.6 ± 6.6 ^{ad}	41.2 ± 2.3 ^{ad}	52.3 ± 2.6 ^{ae}	41.6 ± 1.6 ^{ad}	52.0 ± 3.1 ^{ae}
1	31.1 ± 7.2 ^{ad}	62.9 ± 5.8 ^{be}	62.3 ± 4.2 ^{be}	69.6 ± 4.6 ^{be}	74.9 ± 2.3 ^{be}
3	29.5 ± 6.3 ^{ad}	66.6 ± 5.4 ^{be}	76.4 ± 5.5 ^{be}	61.3 ± 7.4 ^{be}	78.1 ± 6.3 ^{bce}
5	33.2 ± 7.0 ^{ad}	57.5 ± 5.3 ^{be}	64.0 ± 3.4 ^{be}	63.5 ± 5.8 ^{be}	86.1 ± 8.6 ^{bf}
7	33.5 ± 6.9 ^{ad}	56.9 ± 3.1 ^{be}	71.1 ± 6.9 ^{be}	59.8 ± 6.1 ^{be}	90.5 ± 4.3 ^{cf}
10	31.3 ± 7.2 ^{ad}	57.6 ± 4.4 ^{be}	71.5 ± 4.4 ^{bfg}	61.9 ± 7.8 ^{bef}	80.8 ± 1.9 ^{bg}
Acid Soluble Acyl Carnitine (ASAC)					
Pretreatment	4.0 ± 0.8 ^{ad}	6.8 ± 0.7 ^{ae}	7.2 ± 2.3 ^{ade}	9.4 ± 4.2 ^{acde}	4.0 ± 0.8 ^{ad}
1	5.7 ± 2.4 ^{ad}	11.5 ± 6.7 ^{abde}	19.0 ± 5.1 ^{bce}	14.7 ± 1.4 ^{abce}	2.6 ± 0.6 ^{ad}
3	4.6 ± 2.6 ^{ad}	9.5 ± 5.4 ^{abde}	13.8 ± 4.4 ^{abcde}	20.6 ± 2.5 ^{be}	6.5 ± 0.4 ^{bd}
5	7.2 ± 1.9 ^{ade}	4.6 ± 0.6 ^{bd}	11.4 ± 5.5 ^{abcde}	13.8 ± 4.3 ^{abcde}	4.6 ± 1.3 ^{abd}
7	7.3 ± 0.9 ^{ade}	5.3 ± 0.7 ^{abd}	9.8 ± 1.6 ^{ace}	10.8 ± 3.2 ^{abcde}	---
10	5.8 ± 3.5 ^{ad}	---	13.5 ± 2.4 ^{abcd}	20.9 ± 8.5 ^{abcd}	12.3 ± 7.6 ^{abd}

Table 12 (continued)

Days on Supplementary L-Carnitine	Groups				
	NS (4)	CS-0.25% (4)	CS-0.50% (4)	CS-0.75% (4)	CS-1.0% (4)
Pretreatment		Acid Insoluble Acyl Carnitine (AIAC)			

1	3.9 ± 2.1 ^{ad}	2.8 ± 1.2 ^{ad}		3.3 ± 1.4 ^{ad}	3.4 ± 1.0 ^{ad}
3	3.5 ± 1.9 ^{ad}	3.3 ± 0.9 ^{ad}	2.8 ± 1.3 ^{ad}	2.4 ± 0.8 ^{ad}	2.8 ± 0.9 ^{ad}
5	2.5 ± 1.6 ^{ad}	3.5 ± 1.1 ^{ad}	4.4 ± 0.4 ^{ad}	2.5 ± 0.4 ^{ad}	2.5 ± 0.4 ^{ad}
7	3.5 ± 1.8 ^{ad}	4.0 ± 1.3 ^{ad}	5.0 ± 0.8 ^{ad}	3.0 ± 0.6 ^{ad}	3.0 ± 0.6 ^{ad}
10	6.7 ± 3.2 ^{ad}	3.1 ± 0.9 ^{ad}	4.9 ± 1.9 ^{ad}	2.4 ± 0.6 ^{ad}	2.4 ± 0.6 ^{ad}
	4.4 ± 1.8 ^{ad}	3.4 ± 1.0 ^{ad}	3.8 ± 0.4 ^{ad}	2.7 ± 0.5 ^{ad}	2.7 ± 0.5 ^{ad}
		Total Carnitine (TC)			
Pretreatment					
1	43.5 ± 3.8 ^{ad}	44.1 ± 2.8 ^{ad}	60.2 ± 3.8 ^{ae}	56.3 ± 3.7 ^{ae}	57.1 ± 4.4 ^{ae}
3	38.7 ± 3.6 ^{ad}	66.2 ± 6.0 ^{be}	83.4 ± 3.5 ^{bf}	82.3 ± 5.8 ^{bf}	79.6 ± 2.9 ^{bf}
5	36.7 ± 3.3 ^{ad}	70.0 ± 6.4 ^{be}	91.9 ± 7.2 ^{bf}	83.8 ± 9.1 ^{bef}	85.6 ± 5.2 ^{bcef}
7	42.1 ± 3.3 ^{ad}	61.5 ± 4.4 ^{be}	80.2 ± 7.5 ^{bf}	79.5 ± 7.3 ^{bf}	92.6 ± 8.3 ^{bdf}
10	42.4 ± 2.6 ^{ad}	60.1 ± 2.5 ^{be}	85.7 ± 9.6 ^{bfg}	74.1 ± 5.2 ^{bf}	94.6 ± 5.9 ^{cg}
	39.3 ± 5.1 ^{ad}	61.1 ± 3.7 ^{be}	87.3 ± 6.6 ^{bf}	85.1 ± 6.7 ^{bf}	87.5 ± 2.0 ^{cf}

¹Values are group means ± SEM. Values of a carnitine fraction in a column bearing the same superscript letter (a-c) and in a line (d-g) are not statistically different ($p \geq 0.05$).

²Number of animals per group.

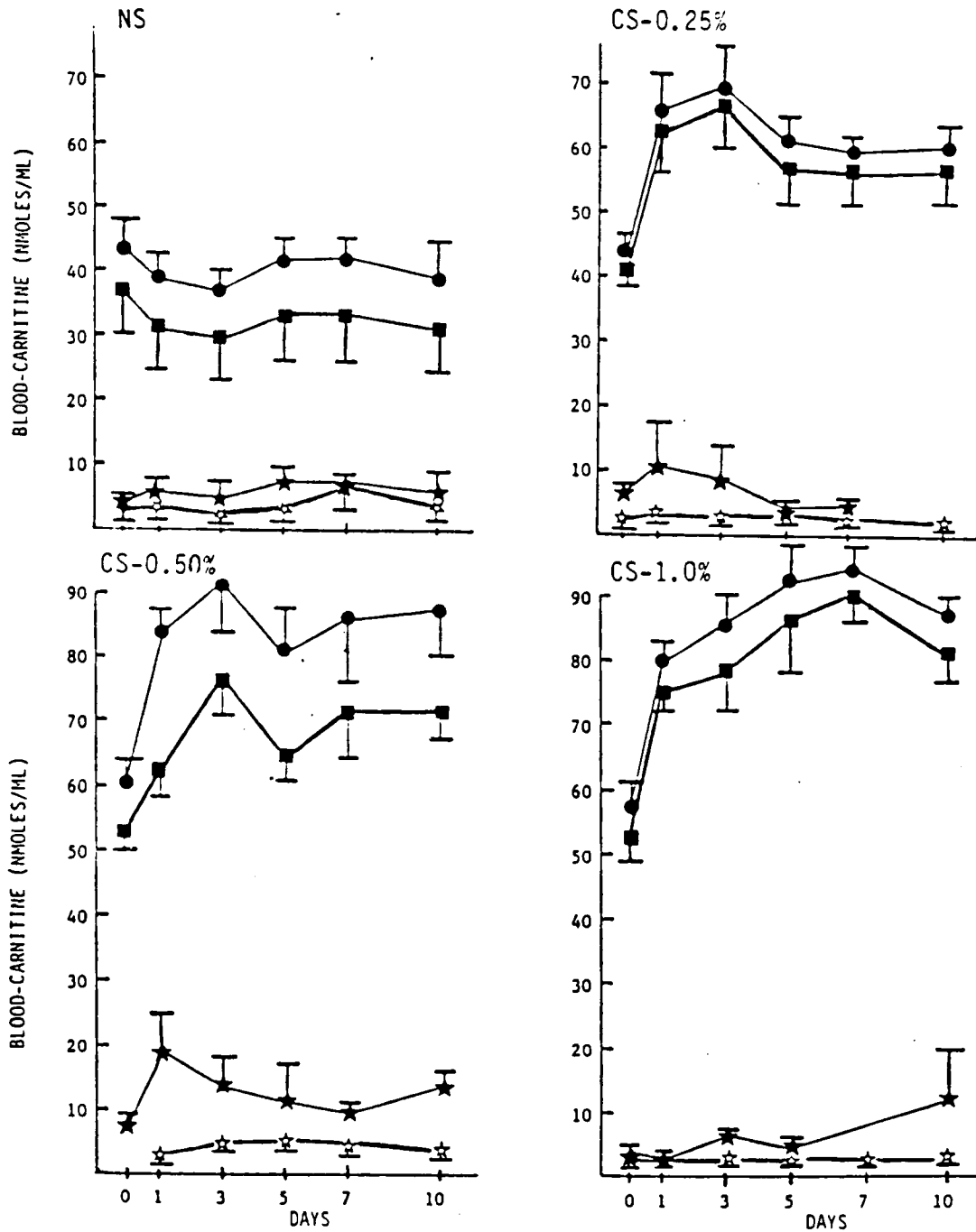


Figure 8. Time and Dose Dependent Changes in Blood Total Carnitine (●), Nonesterified Carnitine (■), Acid Soluble Acylcarnitine (★), and Acid Insoluble Acylcarnitine (☆) During 10 Days of Feeding Varying Levels of Supplementary L-Carnitine.

Table 13 and Figure 9 illustrate the concentrations of carnitine excreted in the urine in relation to the duration and levels of supplementation. The amounts of NEC, ASAC, AIAC and TC fractions in the NS group varied little during the 10-day period; the amount of ASAC excreted was higher than the amount of AIAC. Accordingly, in all CS groups the levels of carnitine fractions excreted over the 24-hour period were significantly elevated after 1 day and rose gradually thereafter. The peak excretion was reached after 5 days of supplementation at the 0.5% level. It is also illustrated that the CS groups excreted significantly higher amounts of AIAC than the NS group, and that the urinary levels of AIAC were higher than those of ASAC in the urine of the CS animals.

Since 0.50% L-carnitine for 3 days appeared to be the optimal dose and duration needed for attaining blood-carnitine steady state concentrations (Table 12 and Figure 8), the simultaneous changes in blood- and urinary-carnitine fractions of the CS-0.5% group during the 10-day period are shown in Table 14 and Figure 10. While blood concentrations of NEC, ASAC, AIAC and TC reached their peaks after 3 days and were elevated 46, 92, 47 and 53% over day 0, respectively; the urinary concentrations of NEC, ASAC, AIAC and TC did not reach their peaks until 5 days, where increases of 2,741, 1,379, 2,045, 2,488% over day 0, respectively (Table 14) were observed. These peak levels of blood- and urinary-carnitine fractions were maintained for the duration of treatment (Figure 10). At the initial rise, the AIAC concentration increased almost 20-fold in the urine while the concentration of ASAC also

Table 13

Urinary-Carnitine Concentrations ($\mu\text{moles}/24 \text{ hrs}$) After 1, 3, 5, 7, and 10 Days
of Feeding Varying Levels Supplementary L-Carnitine

Days on Supplementary L-Carnitine	Groups				
	NS (4) ²	CS-0.25% (4)	CS-0.50% (4)	CS-0.75% (4)	CS-1.0% (4)
Nonesterified Carnitine (NEC)					
Pretreatment	4.50 $\pm$ 0.22 ^{ad}	5.23 $\pm$ 0.80 ^{ad}	4.98 $\pm$ 0.41 ^{ad}	3.87 $\pm$ 0.56 ^{ad}	4.27 $\pm$ 0.67 ^{ad}
1	4.44 $\pm$ 0.37 ^{ad}	6.81 $\pm$ 8.0 ^{be}	9.62 $\pm$ 9.1 ^{bf}	80.2 $\pm$ 12.6 ^{bef}	100.7 $\pm$ 9.3 ^{bf}
3	5.14 $\pm$ 0.51 ^{ad}	73.6 $\pm$ 4.0 ^{be}	124.2 $\pm$ 12.7 ^{bef}	130.1 $\pm$ 14.4 ^{cef}	121.3 $\pm$ 20.1 ^{bef}
5	5.22 $\pm$ 0.33 ^{ad}	86.0 $\pm$ 7.8 ^{be}	141.5 $\pm$ 11.3 ^{cf}	133.5 $\pm$ 11.4 ^{cf}	127.6 $\pm$ 21.5 ^{bef}
7	4.70 $\pm$ 0.29 ^{ad}	87.3 $\pm$ 15.1 ^{be}	123.4 $\pm$ 11.2 ^{bef}	111.9 $\pm$ 11.7 ^{cef}	130.1 $\pm$ 17.9 ^{bef}
10	4.77 $\pm$ 0.43 ^{ad}	92.2 $\pm$ 11.8 ^{be}	148.3 $\pm$ 14.7 ^{cf}	158.2 $\pm$ 12.5 ^{cf}	140.6 $\pm$ 11.4 ^{cf}
Acid Soluble Acyl Carnitine (ASAC)					
Pretreatment	0.75 $\pm$ 0.14 ^{ad}	0.61 $\pm$ 0.18 ^{ad}	0.73 $\pm$ 0.13 ^{ad}	0.40 $\pm$ 0.10 ^{ad}	0.54 $\pm$ 0.11 ^{ad}
1	0.88 $\pm$ 0.08 ^{ad}	10.0 $\pm$ 1.5 ^{be}	13.0 $\pm$ 5.1 ^{bce}	2.4 $\pm$ 0.5 ^{bd}	11.4 $\pm$ 2.1 ^{be}
3	0.91 $\pm$ 0.05 ^{ad}	7.8 $\pm$ 2.3 ^{bce}	18.0 $\pm$ 0.9 ^{bf}	5.9 $\pm$ 1.6 ^{be}	9.7 $\pm$ 2.1 ^{be}
5	0.87 $\pm$ 0.10 ^{ad}	7.9 $\pm$ 1.4 ^{bce}	---	5.7 $\pm$ 2.7 ^{be}	12.3 $\pm$ 3.7 ^{bf}
7	0.68 $\pm$ 0.10 ^{ad}	8.3 $\pm$ 0.9 ^{be}	---	5.8 $\pm$ 1.7 ^{be}	17.8 $\pm$ 4.5 ^{bf}
10	0.97 $\pm$ 0.11 ^{ad}	4.6 $\pm$ 1.6 ^{ce}	10.8 $\pm$ 3.3 ^{cf}	4.5 $\pm$ 1.3 ^{be}	16.4 $\pm$ 5.0 ^{bf}

Table 13 (continued)

Days on Supplementary L-Carnitine	Groups				
	NS (4) ²	CS-0.25% (4)	CS-0.50% (4)	CS-0.75% (4)	CS-1.0% (4)
		Acid Insoluble Acyl Carnitine (AIAC)			
Pretreatment	0.74 ± .13 ^{ad}	0.75 ± .17 ^{ad}	0.89 ± .03 ^{ad}	0.49 ± .18 ^{ad}	0.79 ± .09 ^{ad}
1	0.56 ± .08 ^{ad}	12.8 ± 1.3 ^{be}	15.4 ± 1.6 ^{be}	15.9 ± 1.0 ^{be}	15.6 ± 3.5 ^{be}
3	0.48 ± .04 ^{ad}	15.2 ± 3.4 ^{be}	15.5 ± 0.8 ^{be}	21.1 ± 4.0 ^{be}	18.2 ± 2.1 ^{be}
5	0.54 ± .02 ^{ad}	15.5 ± 1.8 ^{be}	18.2 ± 1.1 ^{be}	18.4 ± 2.3 ^{be}	18.6 ± 2.3 ^{be}
7	0.47 ± .03 ^{ad}	13.0 ± 1.1 ^{be}	19.5 ± 2.0 ^{bef}	15.6 ± 2.0 ^{bef}	19.2 ± 2.3 ^{bef}
10	0.49 ± .05 ^{ad}	15.8 ± 0.5 ^{be}	19.7 ± 2.3 ^{bef}	22.1 ± 2.9 ^{bef}	19.3 ± 2.7 ^{bef}
		Total Carnitine (TC)			
Pretreatment	5.63 ± .23 ^{ad}	61.2 ± 0.84 ^{ad}	6.47 ± 0.30 ^{ad}	4.96 ± 0.51 ^{ad}	5.48 ± 0.77 ^{ad}
1	5.62 ± .48 ^{ad}	91.7 ± 9.5 ^{be}	124.7 ± 13.2 ^{bef}	98.5 ± 12.7 ^{bef}	127.6 ± 13.6 ^{bef}
3	6.08 ± .32 ^{ad}	93.8 ± 5.4 ^{be}	141.7 ± 11.5 ^{bef}	157.1 ± 19.6 ^{cf}	146.7 ± 22.1 ^{bef}
5	6.35 ± .46 ^{ad}	95.2 ± 4.5 ^{be}	161.0 ± 12.9 ^{cf}	156.2 ± 16.9 ^{cf}	155.5 ± 26.2 ^{bef}
7	5.63 ± .38 ^{ad}	113.2 ± 11.6 ^{be}	147.8 ± 10.2 ^{bef}	133.3 ± 12.7 ^{bcef}	167.1 ± 20.1 ^{bef}
10	6.00 ± .47 ^{ad}	111.4 ± 12.6 ^{be}	178.9 ± 17.5 ^{cf}	183.7 ± 14.1 ^{cf}	172.3 ± 15.9 ^{cf}

¹Values are group means ± SEM. Values of a carnitine fraction in a column bearing the same superscript letter (a-c) and in a line (d-g) are not statistically different ($p \geq 0.05$).

²Number of animals per group.

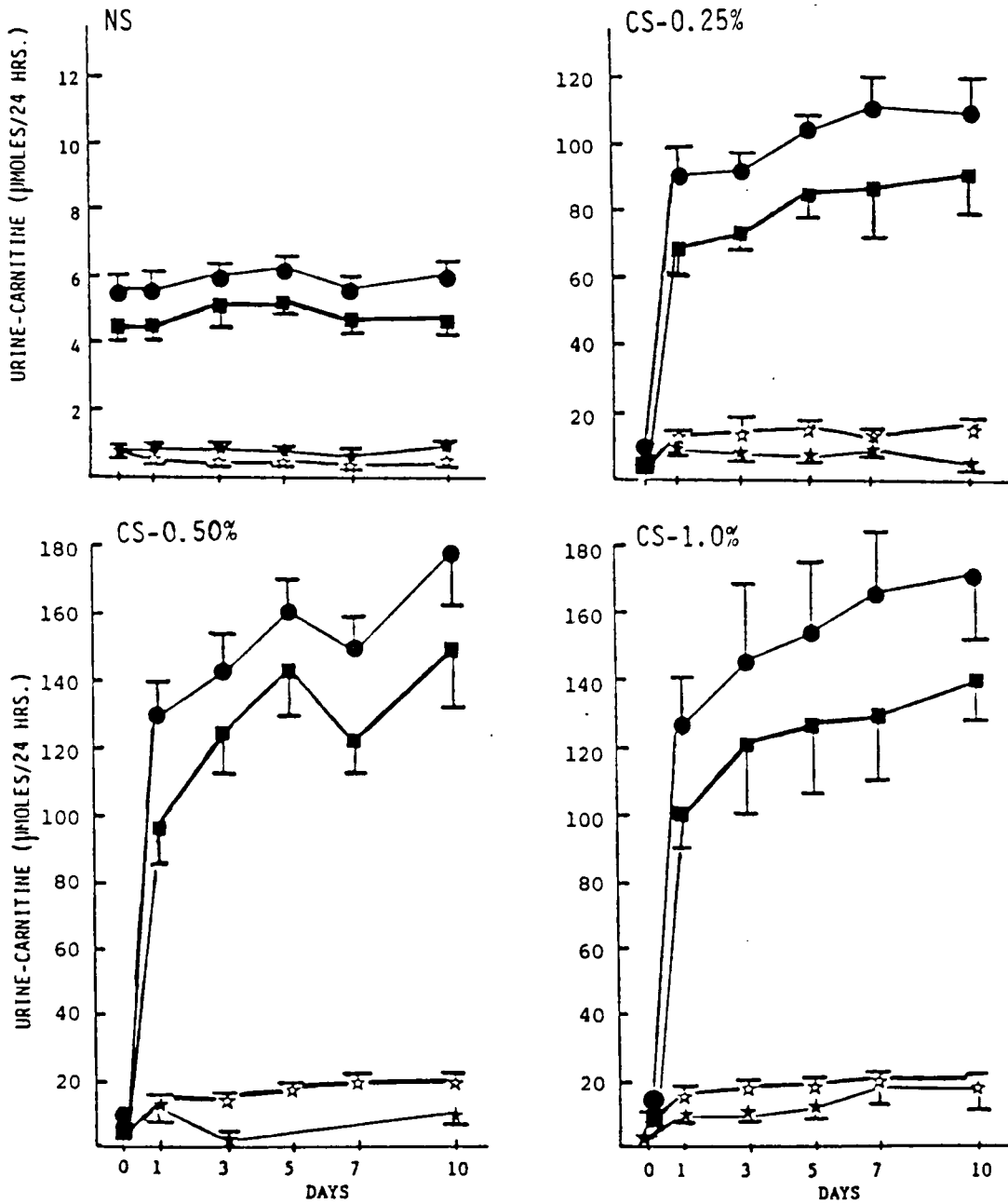


Figure 9. Time and Dose Dependent Changes in Urinary Total Carnitine (●), Nonesterified Carnitine (■), Acid Soluble Acylcarnitine (★), and Acid Insoluble Acylcarnitine (☆) During 10 Days of Feeding Varying Levels of Supplementary L-Carnitine.

Table 14

Increases (%) in All Blood- and Urinary-Carnitine Fractions After Steady State (Peak) Concentrations Were Reached After Oral 0.5% L-Carnitine Supplementation in Rats¹

	NEC ²	ASAC ³	AIAC ⁴	TC ⁵
Blood-Carnitine After 3 days of supplementation	46 ± 4.9 ^a	92 ± 8.3 ^a	47 ± 5.2 ^a	53 ± 6.7 ^a
Urinary-Carnitine After 5 days of supplementation	2,741 ± 90.1 ^b	1,379 ± 50.2 ^b	2,045 ± 72.3 ^b	2,488 ± 82.3 ^b

¹There were 4 rats in each group. All animals were supplemented with 0.5% L-carnitine.

²Nonesterified carnitine.

³Acid soluble acylcarnitine.

⁴Acid insoluble acylcarnitine.

⁵Total carnitine.

⁶The values are group means ± SEM and those in a column bearing the same superscript letter (a-b) are not statistically different (p ≥ 0.05).

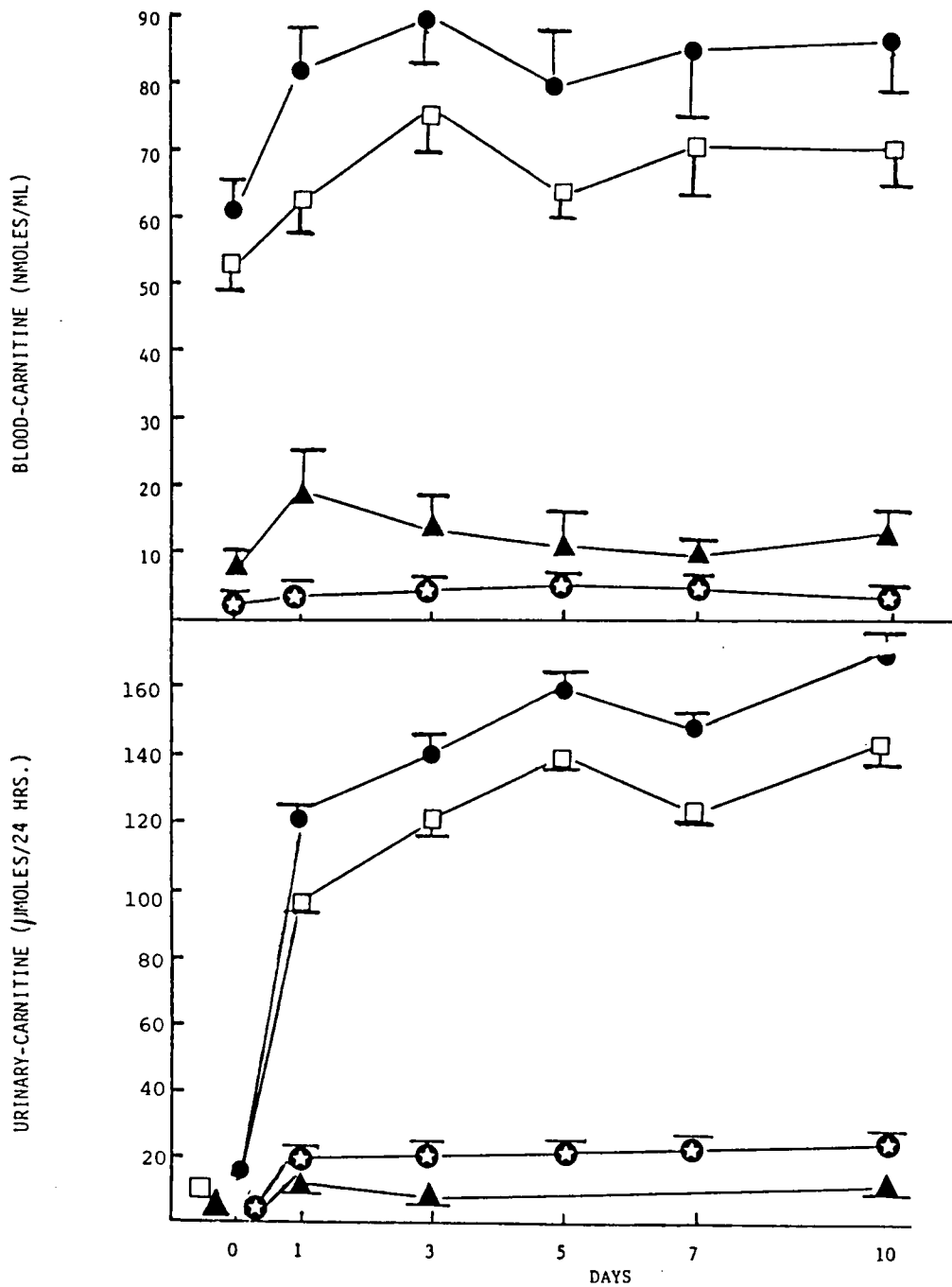


Figure 10. Relationship Between Changes in Total Carnitine (●), Non-esterified (□), Acid Soluble Acylcarnitine (▲), and Acid Insoluble Acylcarnitine (⊛) of Blood and Urine at 0.5% L-Carnitine Supplementation at 0, 1, 3, 5, 7, and 10 Days.

increased about 15-fold (Figure 10). Unlike blood- and urinary compartments, the carnitine concentration in the tissues, represented by quadriceps muscle, was not significantly affected by the supplementary doses of L-carnitine at the end of the 10-day period (Table 15 and Figure 11).

Experiment 1B. Portal-Systemic Blood-Ethanol Concentrations in Carnitine Supplemented Rats

Portal and systemic blood ethanol concentrations following a duodenal infusion of ethanol are shown in Table 16. Although portal blood-ethanol concentrations did not differ statistically between the NS and CS groups over the 30-minute period, systemic blood-ethanol concentrations were significantly elevated in all CS groups compared to the NS group. However, the systemic blood-ethanol concentrations of the CS groups did not differ significantly among themselves, (except for the CS-0.75 and CS-1.0% animals at 30 minutes) and remained significantly higher than in the NS group at all times. In both NS and CS groups, systemic blood-ethanol concentrations peaked at 20 minutes and declined at 30 minutes at the rates inversely related to the levels of carnitine supplementation. This decrease in systemic blood-ethanol concentration between 20 and 30 minutes postethanol infusion diminished with increasing L-carnitine supplementation; NS (-41.8%) < CS-0.25% (-28.0%) < CS-0.50% (-14.4%) < CS-0.75% (-8.7%) < CS-1.0% (+3.2%).

Experiment 2. Specificity of the Carnitine Effect on Ethanol Metabolism

The effects of 0.5% L-carnitine and 0.43% choline supplementation on ethanol clearance in rats after an acute oral dose of ethanol are

Table 15
Muscle-Carnitine Concentrations (μ moles/G) After 10 Days of Feeding
Varying Levels of Supplementary L-Carnitine¹

Muscle-Carnitine Fractions	Groups			
	NS(4) ²	CS-0.25%(4)	CS-0.50%(4)	CS-0.75%(4) CS-1.0%(4)
Nonesterified Carnitine (NEC)	1.12 $\pm$ 0.03 ^a	1.25 $\pm$ 0.04 ^a	1.19 $\pm$ 0.11 ^a	1.22 $\pm$ 0.12 ^a 1.08 $\pm$ 0.13 ^a
Acid-Soluble Acyl Carnitine (ASAC)	0.65 $\pm$ 0.05 ^{ab}	0.60 $\pm$ 0.07 ^{ab}	0.47 $\pm$ 0.12 ^a	0.57 $\pm$ 0.21 ^{ab} 0.78 $\pm$ 0.05 ^b
Acid-Insoluble Acyl Carnitine (AIAC)	0.21 $\pm$ 0.01 ^a	0.22 $\pm$ 0.02 ^a	0.24 $\pm$ 0.08 ^a	0.23 $\pm$ 0.08 ^a 0.25 $\pm$ 0.10 ^a
Total Carnitine (TC)	2.05 $\pm$ 0.05 ^a	2.06 $\pm$ 0.22 ^a	1.75 $\pm$ 0.15 ^a	1.91 $\pm$ 0.28 ^a 2.09 $\pm$ 0.25 ^a

¹The values are group mean $\pm$ SEM. Those in a line bearing the same superscript letter are not statistically different ($p \geq 0.05$).

²Number of animals per group.

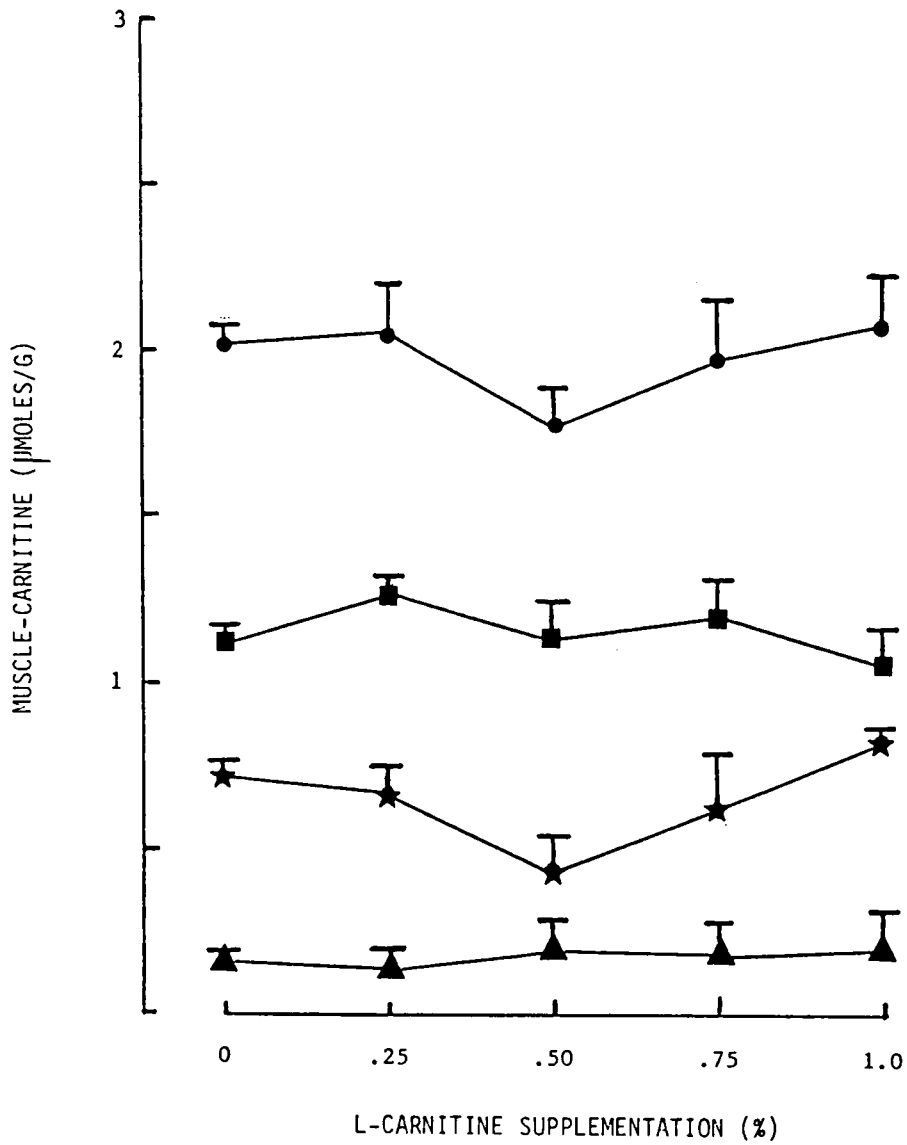


Figure 11. Dose Dependent Changes in Muscle Total Carnitine (●), Nonesterified Carnitine (■), Acid Soluble Acylcarnitine (★), and Acid Insoluble Carnitine (▲) After 10 Days of Feeding Varying Levels of Supplementary L-Carnitine.

Table 16

Blood-Ethanol Concentrations in Portal and Systemic Blood of Rats After Varying Levels of L-Carnitine Supplementation Following a Duodenal Infusion of Ethanol¹

Minutes Postethanol Infusion	Blood-Ethanol Concentrations (mg/dl) ²			
	NS	CS-0.25%	CS-0.50%	CS-1.0%
			<u>Portal</u>	
5	591.3 ± 33.8 ^a	613.0 ± 42.1 ^a	325.0 ± 36.7 ^b	668.0 ± 185.0 ^{ab}
10	874.8 ± 73.9 ^a	953.0 ± 91.0 ^a	608.8 ± 54.6 ^b	654.0 ± 184.0 ^{ab}
15	578.0 ± 77.2 ^a	664.0 ± 56.0 ^a	461.8 ± 36.7 ^a	690.7 ± 203.0 ^a
20	495.0 ± 99.1 ^a	487.5 ± 55.9 ^a	342.5 ± 36.3 ^a	692.0 ± 188.7 ^a
30	342.5 ± 51.5 ^a	532.5 ± 52.3 ^a	379.5 ± 56.3 ^a	522.3 ± 128.9 ^a
			<u>Systemic</u>	
5	115.0 ± 25.6 ^a	255.8 ± 43.8 ^b	210.3 ± 14.2 ^b	201.3 ± 29.8 ^b
10	187.5 ± 12.3 ^a	308.3 ± 40.6 ^b	258.5 ± 10.7 ^b	312.0 ± 27.3 ^b
15	218.5 ± 12.3 ^a	332.0 ± 25.2 ^b	275.8 ± 19.5 ^b	313.0 ± 20.0 ^b
20	239.0 ± 27.4 ^a	337.0 ± 26.3 ^b	294.5 ± 36.0 ^b	309.0 ± 40.0 ^b
30	168.5 ± 29.6 ^a	263.3 ± 3.3 ^b	257.5 ± 22.8 ^b	284.3 ± 23.1 ^{bc}

¹There were 4 rats in each group. All animals were fed ground Purina Rodent Chow #5001 with or without various supplementary levels for 10 days prior to ethanol infusion.

²The values are group means ± SEM and those in a line bearing the same superscript letter are not statistically different ($p \geq 0.05$).

shown in Table 17 and Figure 12. There were significant differences between all 3 groups at 20 minutes postethanol administration. The CS rats showed a blood-ethanol concentration of 128.1 mg/dl, while NS rats were 88.1 mg/dl, and CHS rats were 65.1 mg/dl at 20 minutes. At 40 minutes and beyond, there were no significant differences in blood-ethanol concentrations between the NS and CHS animals up to 480 minutes. However, at 20 minutes and beyond, the CS rats maintained significantly higher blood-ethanol concentrations than the NS and CHS groups. At 480 minutes postethanol administration, the differences in blood-ethanol concentrations between the 3 groups were not statistically significant.

The effects of carnitine and choline treatments on body weights, urinary outputs, blood and urinary-carnitine are presented in Table 18. The weight gains over the 7-day period were not significantly different among the 3 groups. Conversely, the 24-hour urinary output was significantly lower in the CS than the NS or CHS groups which were not significantly different from each other. After the 7-day treatment period the blood-NEC concentration was significantly higher in CS (49.64 nm/ml) than in NS (22.82 nm/ml) or CHS (25.95 nm/ml) groups. Also all urinary carnitine fractions were significantly higher in CS than in NS or CHS animals, with the NEC of the CS group being (70.31 μ m/24 hrs) about 300 times that of the NS and CHS groups (0.25 and 0.30 μ m/24 hrs, respectively), the ASAC of the CS group being (25.14 μ m/24 hrs) between 250-500 times that of the NS and CHS groups (0.06 and 0.13 μ m/24 hrs, respectively), the AIAC of the CS group being (6.77 μ m/24 hrs) about 70 times that of the NS and CHS groups (0.11 and

Table 17

Blood-Ethanol Concentrations at Various Times Postethanol
Administration in 0.5% L-Carnitine and 0.43% Choline
Supplemented Male Rats¹

Postethanol Administration (minutes)	Blood-Ethanol Concentrations (mg/dl) ²		
	NS (6) ³	CHS (4)	CS (6)
20	88.1 ± 8.0 ^a	65.1 ± 6.2 ^b	128.1 ± 10.4 ^c
40	100.4 ± 5.5 ^a	93.4 ± 12.8 ^a	151.7 ± 8.4 ^b
60	123.5 ± 11.5 ^a	108.6 ± 10.4 ^a	163.8 ± 9.4 ^b
90	126.9 ± 11.7 ^a	113.4 ± 13.4 ^a	179.2 ± 12.0 ^b
120	119.3 ± 6.7 ^a	122.9 ± 13.5 ^a	188.6 ± 11.2 ^b
240	96.6 ± 14.6 ^a	108.5 ± 13.5 ^a	156.7 ± 7.2 ^b
360	72.8 ± 18.5 ^a	94.5 ± 13.7 ^a	127.7 ± 11.2 ^b
480	57.1 ± 19.8 ^a	51.1 ± 11.7 ^a	85.8 ± 18.2 ^a

¹All animals were fed ground Purina Rodent Chow #5001 with or without 0.5% supplementary L-carnitine (CS) or 0.43% choline (CHS) for 7 days prior to ethanol administration.

²The values are group means ± SEM and those in a line bearing the same superscript letter are not statistically different ($p \geq 0.05$).

³Each group contains the number of rats specified in parentheses.

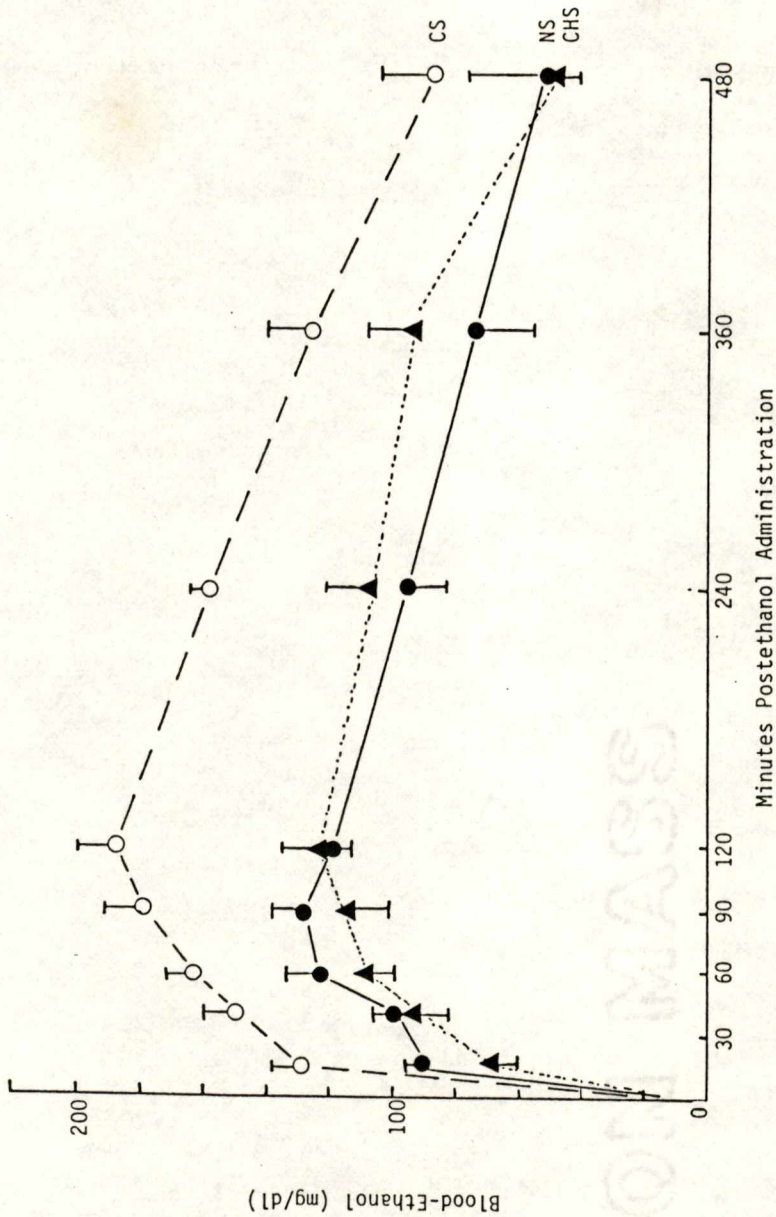


Figure 12. Time Course of Blood-Ethanol Concentrations from 0-8 Hours After a Single Oral Dose of Ethanol in Rats Fed 0.5% L-Carnitine (CS) or 0.43% Choline (CHS) for 7 Days. Each point represents the mean $\pm$ SEM for 6 animals in the NS and CS groups and 4 animals in the CHS group. Slopes and correlations are as follows: CS; $y = (-16.8)x + 224.1$; $r = -1.00$, NS; $y = (-10.9)x + 141.2$, $r = -1.00$ and CHS; $y = (-11.4)x + 151.6$, $r = -0.92$. Slopes were determined by Linear Regression analysis after blood-ethanol peak concentrations were reached. The slope for the CS group is significantly greater than those of the NS and CHS groups ($p \leq 0.05$) which are not different from each other ($p \geq 0.05$).

Table 18

Effects of 0.5% Oral L-Carnitine and 0.43% Choline Supplementation on Weight Gain, Urinary Output, and Blood- and Urinary-Carnitines in Male Rats

Parameters	Groups		
	NS	CHS	CS
Weight Gain (g)	42.4 ± 11.2 ^a	37.0 ± 4.8 ^a	33.5 ± 3.2 ^a
Urinary Output (ml/24 hrs)	10.9 ± 1.1 ^a	9.9 ± 0.7 ^a	7.0 ± 0.8 ^b
Blood NEC ² (nm/ml)	22.8 ± 1.1 ^a	26.0 ± 2.3 ^a	49.6 ± 1.7 ^b
Urinary NEC (μm/24 hrs)	0.25 ± 0.04 ^a	0.3 ± 0.05 ^a	70.3 ± 3.8 ^b
Urinary ASAC ³ (μm/24 hrs)	0.06 ± 0.02 ^a	0.13 ± 0.01 ^a	25.1 ± 3.7 ^b
Urinary AIAC ⁴ (μm/24 hrs)	0.11 ± 0.01 ^a	0.11 ± 0.01 ^a	6.77 ± 0.9 ^b
Urinary TC ⁵ (μm/24 hrs)	0.42 ± 0.07 ^a	0.54 ± 0.07 ^a	102.1 ± 8.1 ^b

¹There were 6 animals in each group. All animals were fed a semisynthetic diet with or without supplementary L-carnitine CS choline chloride CHS for 7 days.

²Nonesterified carnitine.

³Acid-soluble acyl carnitine.

⁴Acid-insoluble acyl carnitine.

⁵Total carnitine.

⁶The values are group means ± SEM and those in a line bearing the same superscript letter (a-b) are not statistically different ($p \geq 0.05$).

0.11 $\mu\text{m}/\text{hrs}$, respectively) and the TC of the CS group being (102.1 $\mu\text{m}/24 \text{ hrs}$) about 200 times that of the NS and CHS groups (0.42 and 0.52, respectively). However, blood-NEC concentrations as well as all fractions of urinary carnitine were not statistically different between the NS and CHS animals.

Experiment 3A. Effects of Intraperitoneal Ethanol Administration on Its Metabolism in Carnitine Supplemented Rats

This study was designed to determine whether the effect of carnitine could also be shown when ethanol was administered intraperitoneally (i.p.). Thus, the effects of 0.5% (w/w) L-carnitine supplementation for 3 days on blood-ethanol concentrations in rats after an i.p. ethanol dose (0.2 g/kg) were examined. After monitoring ethanol concentrations in the blood from 0-480 minutes postethanol dose (Table 19 and Figure 13), it was found that there were no significant difference between the NS and CS groups at 5 and 10 minutes postethanol administration. However, beyond 10 minutes the differences were significant and sustained for up to 480 minutes with the CS group maintaining a higher blood-ethanol concentration than the NS group. While the ethanol elimination rates were higher for the NS than the CS animals (9.06 vs. 5.5 mg/dl/hr), both groups reached about the same blood-ethanol peaks (203.2 vs. 211.0 mg/dl).

Experiment 3B. Effects of Various Doses (I.P.) of Ethanol on Its Metabolism in Carnitine Supplemented Rats

This study was conducted in the same 5 animals as in Experiment 3A. These animals were fed the 0.5% L-carnitine diet for 90 days, and rats

Table 19

Blood-Ethanol Concentrations at Various Times Postethanol
Intraperitoneal Administration in Rats Fed 0.5%
Supplementary L-Carnitine for 3 Days¹

Postethanol Administration (minutes)	Blood-Ethanol Concentrations (mg/dl) ²	
	NS	CS
5	203.2 ± 5.6 ^a	211.0 ± 4.2 ^a
10	194.8 ± 3.5 ^a	207.0 ± 7.3 ^a
20	179.8 ± 8.1 ^a	208.0 ± 5.0 ^b
40	163.0 ± 6.3 ^a	208.8 ± 5.0 ^b
80	155.8 ± 7.2 ^a	199.8 ± 4.4 ^b
160	141.2 ± 5.8 ^a	196.2 ± 6.1 ^b
320	134.4 ± 6.6 ^a	182.8 ± 7.5 ^b
480	118.2 ± 1.6 ^a	174.2 ± 7.5 ^b

¹There were 5 rats in each group. All animals were fed with (CS) or without (NS) 0.5% supplementary L-Carnitine for 3 days prior to intraperitoneal ethanol injection.

²The values are group means ± SEM and those in a line bearing the same superscript letter are not statistically different ($p \geq 0.05$).

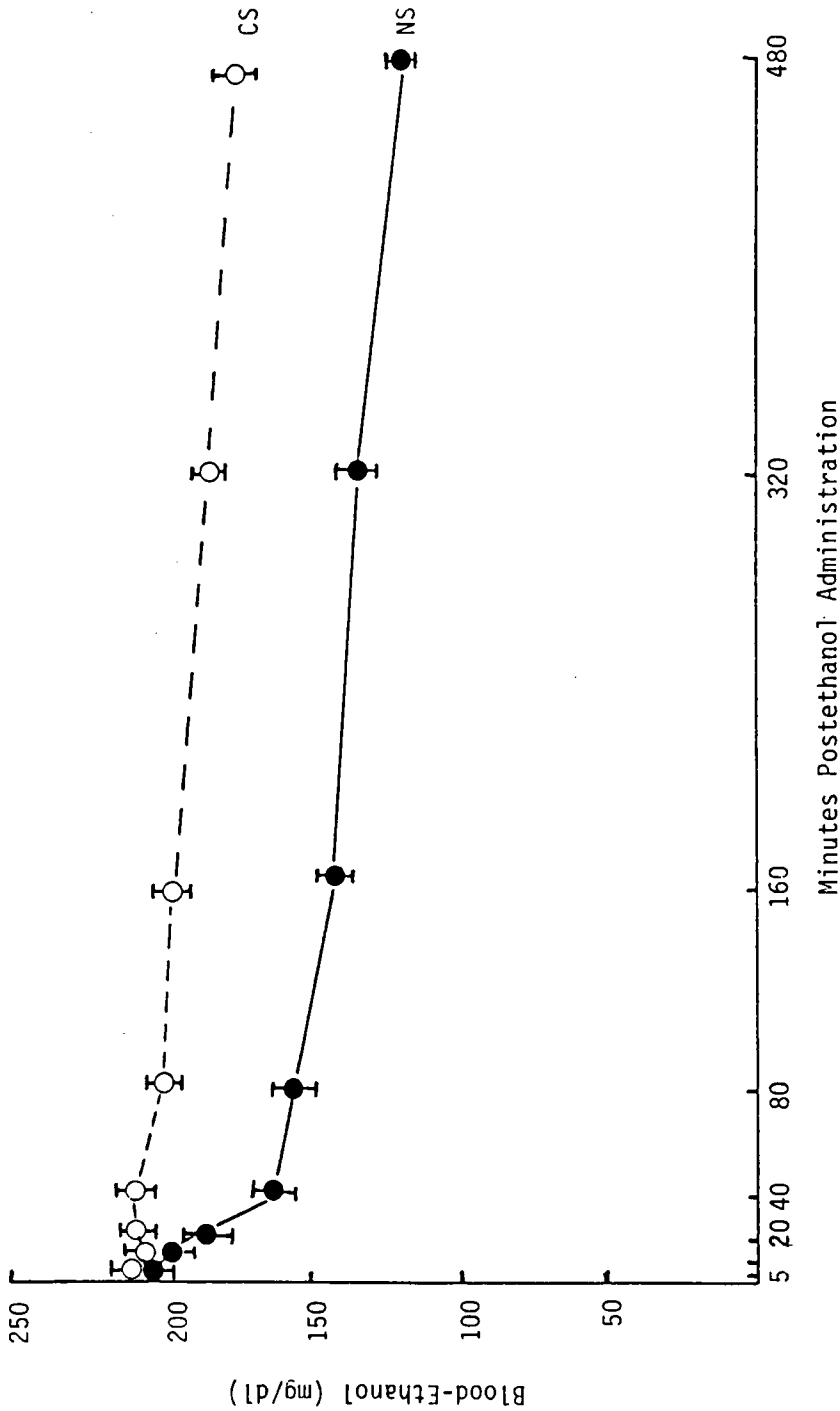


Figure 13. Time Course of Blood-Ethanol Concentration from 0-8 Hours After a Single Intraperitoneal Injection of Ethanol (0.2 g/kg) in Rats Fed 0.5% Supplementary L-Carnitine for 3 Days. Each point represents the mean $\pm$ SEM for 5 animals. Slopes and correlations are as follows: CS; $y = (-5.5)x + 206.6$, $r = -0.97$, and NS; $y = (-9.06)x + 182.4$, $r = -0.88$. Slopes were determined by Linear Regression analysis after blood-ethanol peak concentrations were reached. The slope for the CS group is significantly less than that of the NS group ($p \leq 0.05$).

from the same litter served as controls. It was designed to assess the effect of supplementary L-carnitine on ethanol metabolism at various ethanol doses (0.05, 0.1, 0.2, 0.4 and 0.8 g/kg). Table 20 and Figure 14 illustrate blood-ethanol concentrations of the NS and CS rats for 8 hours following i.p. administration of various doses of ethanol. There were no significant differences between the NS and CS groups at lower doses (0.05 and 0.1 g/kg) of i.p. ethanol administration, but the ethanol-clearance rates were 167% slower in the latter group at these doses. At the doses of 0.2, 0.4 and 0.8 g/kg the differences in blood-ethanol concentrations were significantly greater in the CS groups and similar to that seen in Experiment 3A. At these doses (0.05-0.8 g/kg) the blood-ethanol clearance rates in the CS rats were 167, 167, 73, 77 and 23%, respectively, slower than in the NS rats.

Blood and liver samples were collected from the 0.5% CS animals of this study. Serum nonesterified carnitine, serum triglyceride, liver triglyceride and total hepatic lipid levels are shown in Table 21. The concentrations of serum carnitine (NEC) were significantly higher in the CS (128.5 nm/ml) than in the NS (98.4 nm/ml) group. Although serum triglycerides were not significantly different, the CS group maintained a slightly lower level (34.4 mg/dl) than did the NS (43.4 mg/dl) rats. Liver triglycerides were significantly lower in the CS (17.7 mg/g) compared to the NS (26.7 mg/g) animals as were the total liver lipids (31.2 vs. 46.6 mg/g).

Table 20

Blood-Ethanol Concentrations at Various Doses of Ethanol
Administered Intraperitoneally in Rats Fed
0.5% Supplementary L-Carnitine¹

Postethanol Administration (minutes)	Ethanol Dose (g/kg)	Blood-Ethanol Concentrations (mg/dl) ²	
		NS	CS
	<u>0.05</u>		
10		200.4 ± 8.9 ^a	190.5 ± 9.3 ^a
30		169.9 ± 14.2 ^a	208.8 ± 17.0(4) ^a
60		160.2 ± 10.9 ^a	177.4 ± 16.7 ^a
120		148.4 ± 8.6(4) ^a	163.3 ± 19.5 ^a
240		101.1 ± 12.7 ^a	150.1 ± 18.8 ^b
480		0	125.7 ± 13.4
	<u>0.10</u>		
10		145.9 ± 18.1 ^a	99.4 ± 13.2 ^a
30		188.5 ± 13.1 ^a	131.8 ± 19.0 ^b
60		186.8 ± 8.4 ^a	150.9 ± 20.0 ^a
120		148.2 ± 5.8 ^a	139.5 ± 18.9 ^a
240		115.3 ± 16.8(4) ^a	127.4 ± 17.0 ^a
480		82.8 ± 14.4 ^a	111.0 ± 13.0 ^a
	<u>0.20</u>		
10		230.0 ± 10.2(4) ^a	237.3 ± 27.0 ^a
30		211.7 ± 13.2 ^a	255.8 ± 10.7 ^b
60		180.0 ± 25.1 ^a	240.1 ± 12.6 ^b
120		174.4 ± 13.0 ^a	213.5 ± 14.9 ^b
240		157.1 ± 10.1 ^a	197.5 ± 11.7 ^b
480		91.6 ± 11.7 ^a	181.7 ± 9.3 ^b

Table 20 (continued)

Postethanol Administration (minutes)	Ethanol Dose (g/kg)	Blood-Ethanol Concentrations (mg/dl) ²	
		NS	CS
	<u>0.40</u>		
10		161.3 ± 24.4 ^a	208.9 ± 21.9 ^a
30		196.7 ± 9.1 ^a	249.9 ± 22.5 ^b
60		222.1 ± 7.6(4) ^a	273.7 ± 21.0 ^b
120		224.3 ± 14.5(4) ^a	246.9 ± 10.5 ^a
240		199.6 ± 9.5 ^a	238.0 ± 10.3 ^b
480		158.5 ± 16.3 ^a	229.6 ± 10.9 ^b
	<u>0.80</u>		
10		238.0 ± 5.2 ^a	331.2 ± 16.2 ^b
30		243.8 ± 15.7(4) ^a	292.6 ± 23.0 ^a
60		244.7 ± 24.8 ^a	274.3 ± 13.7 ^a
120		230.1 ± 21.6 ^a	256.9 ± 6.6 ^a
240		193.8 ± 11.9 ^a	233.3 ± 8.5 ^b
480		144.6 ± 10.3 ^a	201.1 ± 3.4 ^b

¹There were 5 rats in each group unless otherwise indicated by number in parenthesis following a value. All animals were fed with (CS) or without (NS) 0.5% supplementary L-Carnitine for 90 days prior to series of ethanol administrations.

²The values are group means ± SEM and those in a line bearing the same superscript letter are not statistically different ($p \geq 0.05$).

Figure 14. Effects of 90-Day 0.5% Oral L-Carnitine Supplementation on Blood-Ethanol Concentration in Rats from 0-8 Hours Post-intraperitoneal Administration of Varying Doses of Ethanol. Each point represents the mean $\pm$ SEM for 5 animals. Slopes and correlations are as follows: A (0.8 g/kg): CS; $y = (-13.2)x + 301.3$, $r = -0.97$, and NS; $\bar{y} = (-16.2)x + 250.4$, $r = -1.00$. B (0.4 g/kg): CS; $y = (-5.4)x + 266.5$, $r = -0.86$ and NS; $\bar{y} = (-9.6)x + 237.6$, $r = -0.99$. C (0.2 g/kg): CS; $y = (-9.0)x + 245.7$, $r = -0.91$ and NS; $\bar{y} = (-15.6)x + 215.0$, $r = -0.97$. D (0.1 g/kg): CS; $y = (-5.4)x + 152.8$, $r = -0.98$ and NS; $\bar{y} = (-14.4)x + 188.9$, $r = -0.96$. E (0.05 g/kg): CS; $y = (-9.0)x + 193.7$, $r = -0.91$ and NS; $\bar{y} = (-24.0)x + 192.2$, $r = -0.99$. Slopes were determined by Linear Regression analysis after blood-ethanol peak concentrations were reached. The slopes for the CS groups were significantly less than those of the NS group ($p \leq 0.05$).

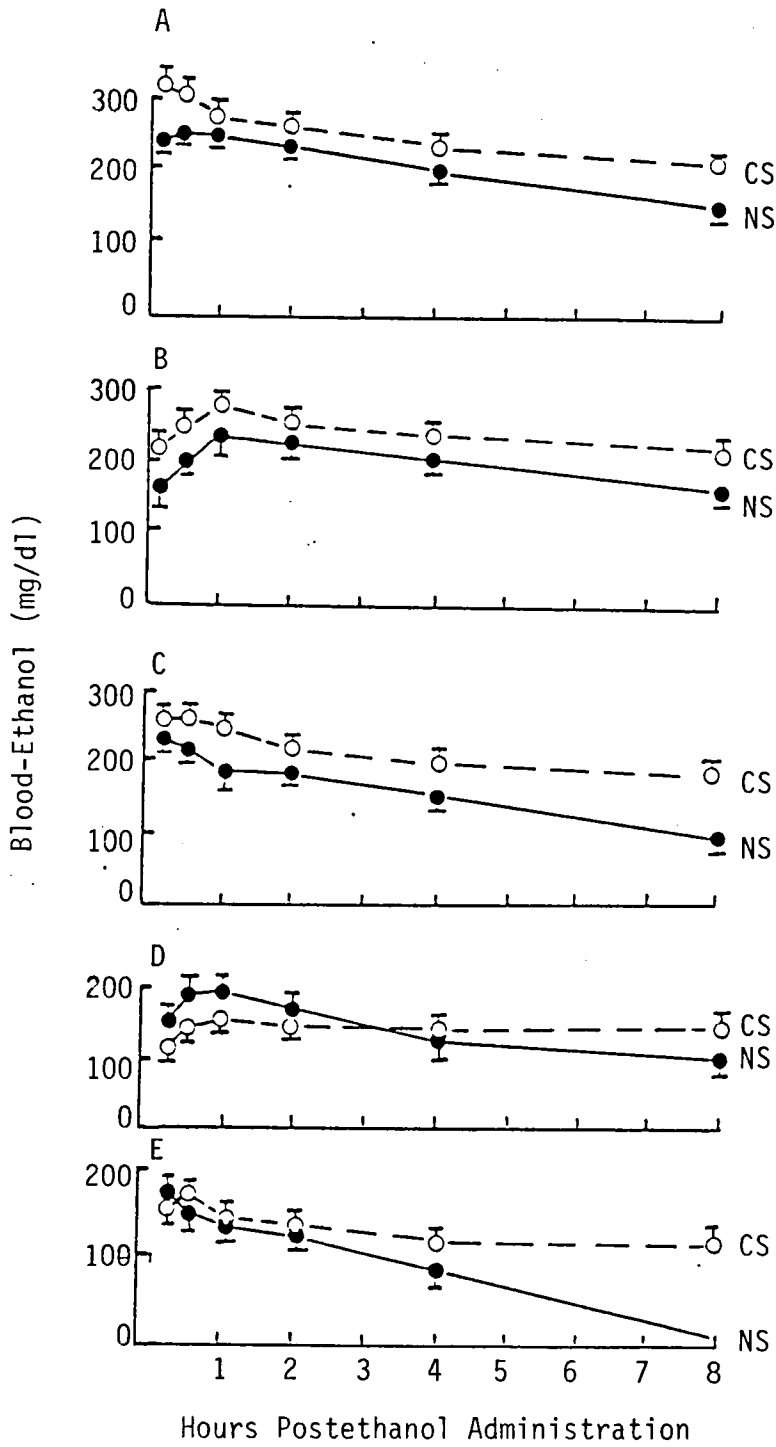


Figure 14 continued.

Table 21
Effects of 0.5% Supplementary L-Carnitine on Blood-Carnitine and Blood-
and Hepatic-Lipid Concentrations^{1,2}

Parameters	NS	CS
Serum Carnitine (nm/ml) (NEC)	98.4 ± 4.6 ^a	128.5 ± 8.2 ^b
Serum Triglycerides (mg/dl)	43.4 ± 4.9 ^a	34.4 ± 4.2 ^a
Liver Triglycerides (mg/g)	26.7 ± 1.1 ^a	17.7 ± 1.8 ^b
Liver Total Lipids (mg/g)	46.6 ± 0.7 ^a	31.2 ± 1.3 ^b

¹There were 5 animals in each group. All animals were fed with (CS) or without (NS) 0.5 % supplementary L-carnitine for 95 days.

²The values are group means ± SEM and those in a line bearing the same superscript letter are not statistically different ($p \geq 0.05$).

Experiment 4A. Duration of Oral Carnitine Administration
Prior to Ethanol Gavage

Table 22 and Figure 15A illustrate blood-ethanol concentrations following an oral gavage of carnitine 24 hours preceding an oral dose of ethanol. There were no significant differences in blood-ethanol concentrations between NS and CS rats except at 8 hours, where the CS group (210.5 mg/dl) maintained a higher concentration than the NS group (165.9 mg/dl). When a second dose of ethanol was administered to these rats 56 hours after they had been on carnitine, there were no statistically significant differences in blood-ethanol concentrations up to 3 hours (Table 22 and Figure 15B), even though the CS group maintained consistently higher blood-ethanol concentrations throughout this period. Table 23 shows that at 4 hours postethanol administration (60 hours after carnitine gavage) the NS animals showed significantly higher (92%) hepatic-ethanol concentrations than animals of the CS group.

Experiment 4B. Duration of Intraperitoneal Carnitine Administration Simultaneously Prior to I.P. Ethanol Gavage

The simultaneous i.p. administration of both ethanol and carnitine did not show significant differences in blood-ethanol concentrations between the NS and CS rats up to 8 hours postethanol administration (Table 24 and Figure 16A). However, when carnitine was administered i.p. for 3 days prior to another i.p. ethanol dose, there were significant differences at 4 and 6 hours postethanol administration with the CS group maintaining a higher blood-ethanol concentration than the NS animals (Table 24 and Figure 16B). At 4 hours, the NS and CS groups were

Table 22

Blood-Ethanol Concentrations at Various Times Postethanol
Administration at 24 and 56 Hours After L-Carnitine
Gavage¹

Postethanol Administration (minutes)	Hours Postcarnitine Gavage (400 mg/kg)	Blood-Ethanol Concentrations (mg/dl)	
		NS	CS
	<u>24</u>		
30		234.8 ± 27.2 ^a	257.0 ± 32.8 ^a
60		280.0 ± 21.4 ^a	275.5 ± 18.4 ^a
120		264.3 ± 21.1 ^a	267.3 ± 20.0 ^a
240		209.4 ± 17.2 ^a	229.8 ± 18.3 ^a
480		165.9 ± 14.7 ^a	210.5 ± 11.0 ^b
	<u>56</u>		
30		248.9 ± 24.3 ^a	246.9 ± 31.4 ^a
60		237.0 ± 22.1 ^a	283.5 ± 12.2 ^a
120		238.9 ± 35.0 ^a	290.5 ± 7.0 ^a
180		215.1 ± 31.7 ^a	279.5 ± 17.1 ^a

¹There were 4 rats in each group. The values are group means ± SEM and those in a line bearing the same superscript letter are not statistically different ($p \geq 0.05$).

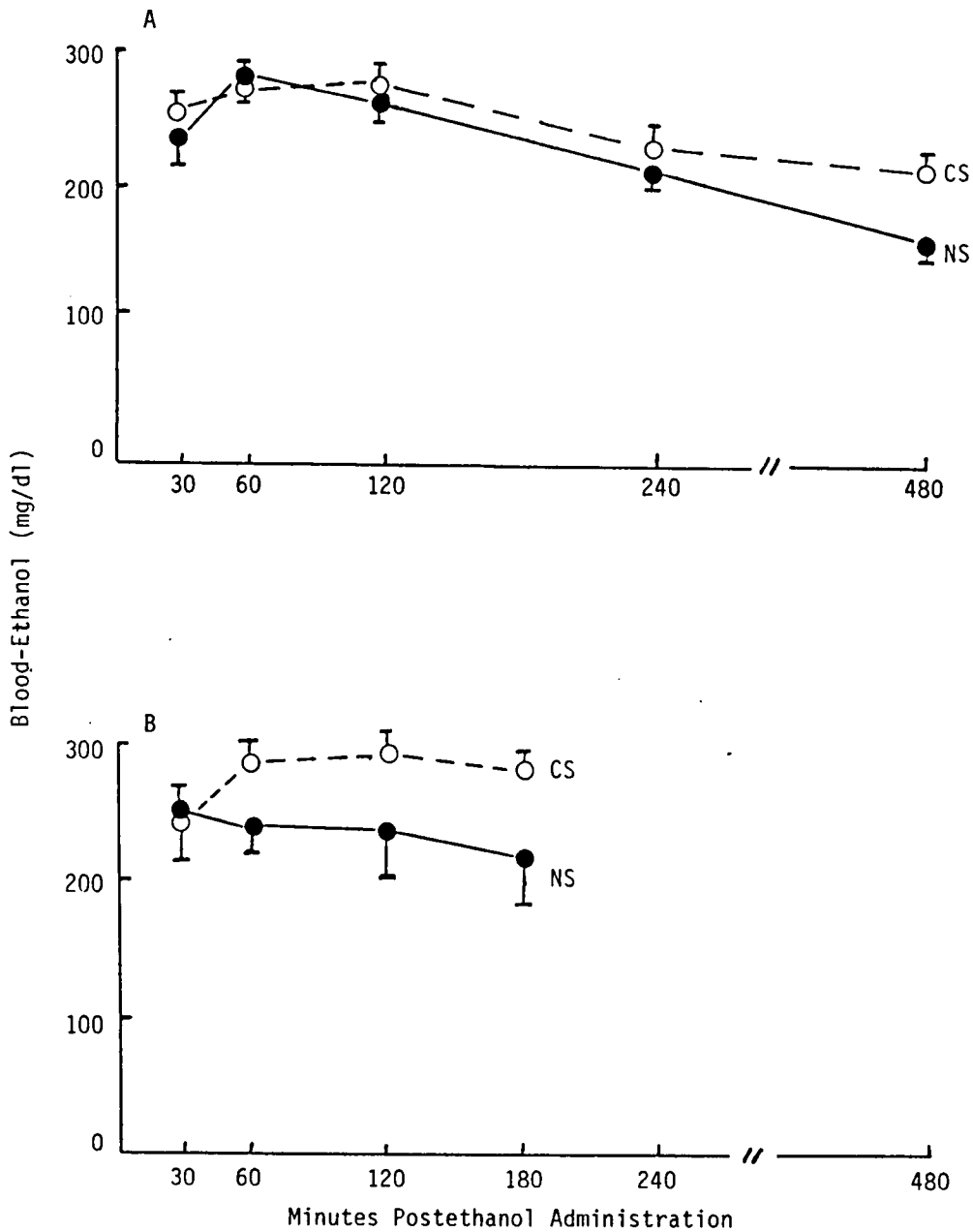


Figure 15. Blood-Ethanol Concentrations Up to 8 Hours Postethanol Dose After Carnitine Was Orally Administered 24- (A) and 56-Hours Prior to Ethanol Gavage (B). Each point represents the mean $\pm$ SEM for 4 animals. Slopes and correlations for (A) are as follows: CS; $y = (-9.5)x + 281.4$, $r = -0.96$ and NS; $y = (-16.5)x + 291.9$, $r = -0.98$. Slopes were determined by Linear Regression analysis after blood-ethanol peak concentrations were reached. The slope for the CS group is significantly less than that of the NS group ($p \leq 0.05$).

Table 23
Hepatic-Ethanol Concentrations in Rats 60 Hours After L-Carnitine Gavage¹

Postethanol Administration (hours)	L-Carnitine Dose (mg/kg)	Hepatic-Ethanol Concentrations (mg/g)	
		NS	CS
4	400	57.7 ± 6.3 ^a	30.0 ± 7.4 ^b

¹There were 4 rats in each group. The values are group means ± SEM and those in a line bearing the same superscript letter are not statistically different (p ≥ 0.05).

Table 24

Blood-Ethanol Concentrations at Various Times After Day 1
and Day 3 of Simultaneous Intraperitoneal¹ Administration
of L-Carnitine and Ethanol

Postethanol Administration (minutes)	Simultaneous Administration of L-Carnitine (400 mg/kg) and Ethanol (0.2 g/kg)	Blood-Ethanol Concentrations (mg/dl)	
		NS	CS
	<u>Day 1</u>		
30		156.5 ± 7.1 ^a	131.9 ± 2.1 ^a
60		134.8 ± 17.1 ^a	144.9 ± 12.6 ^a
120		127.1 ± 21.2 ^a	128.6 ± 15.0 ^a
240		106.6 ± 11.6 ^a	122.5 ± 10.7 ^a
480		100.5 ± 16.7 ^a	109.9 ± 8.3 ^a
	<u>Day 2</u>	L-Carnitine Administration Only	
	<u>Day 3</u>		
30		149.6 ± 20.7 ^a	141.5 ± 16.9 ^a
60		167.8 ± 11.8 ^a	159.9 ± 16.2 ^a
120		169.1 ± 11.6 ^a	177.8 ± 6.0 ^a
240		128.9 ± 17.8 ^a	168.5 ± 3.9 ^b
480		102.9 ± 6.5 ^a	151.8 ± 15.5 ^b

¹There were 4 rats in each group. The values are group means ± SEM and those in a line bearing the same superscript letter are not statistically different ($p \geq 0.05$).

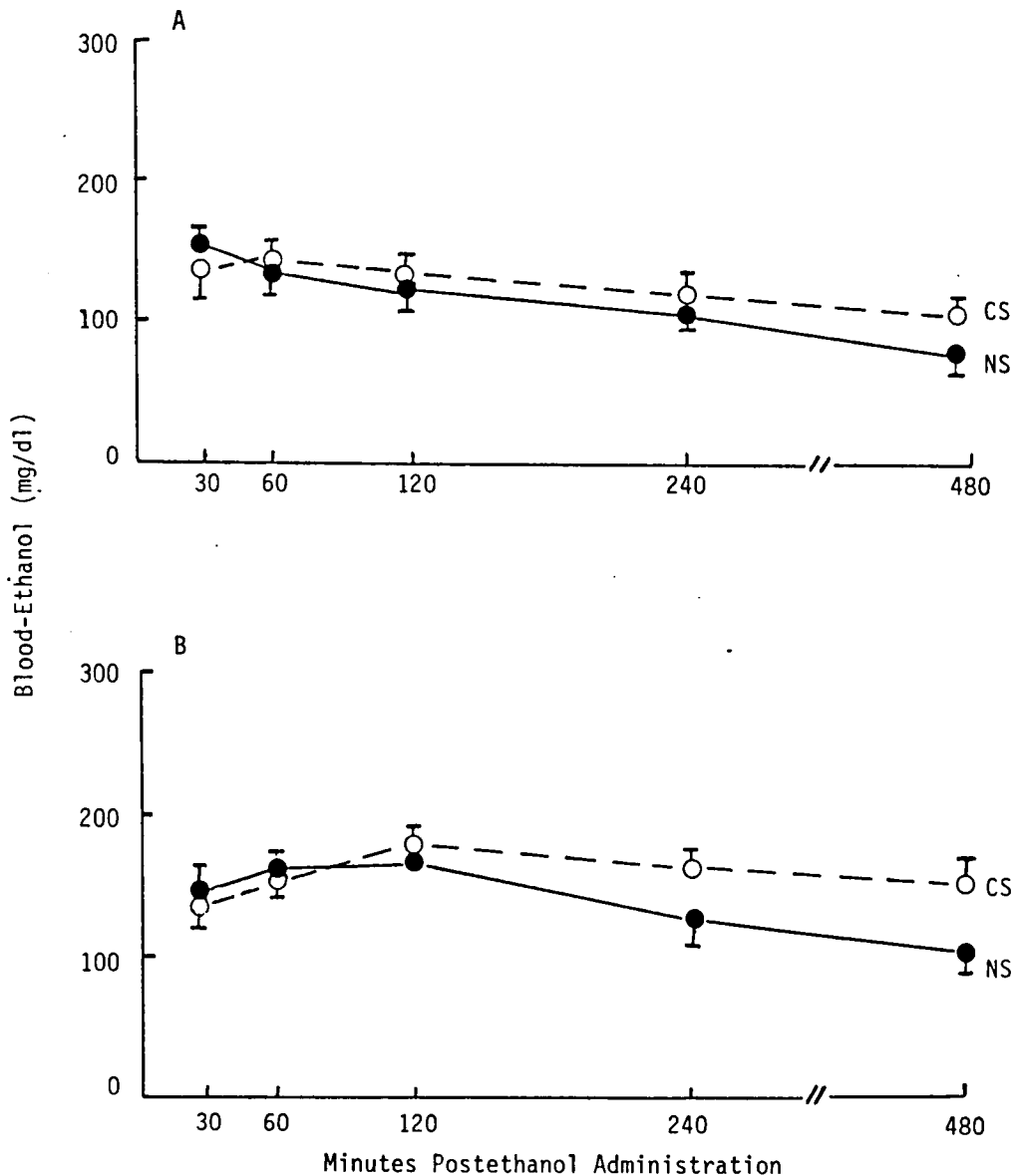


Figure 16. Blood-Ethanol Concentrations Up to 8 Hours Postethanol Dose After Acute (A) and 3-Day (B) Simultaneous Intraperitoneal Administration of L-Carnitine and Ethanol. Each point represents the mean $\pm$ SEM for 5 animals. Slopes and correlations are as follows: A: CS; $y = (-4.4)x + 142.9$, $r = -0.93$ and NS; $y = (-6.5)x + 145.2$, $r = -0.88$. B: CS; $y = (-4.3)x + 186.2$, $r = -1.00$ and NS; $y = (-10.4)x + 182.1$, $r = -0.95$. Slopes were determined by Linear Regression analysis after blood-ethanol peak concentrations were reached. In A, the slope differences between the groups are not significant ($p \geq 0.05$) when in B, the slope for the CS group is significantly less than that of the NS ($p \leq 0.05$).

128.9 and 168.5 mg/dl, respectively, while at 8 hours, the NS and CS groups were 102.9 and 151.8 mg/dl, respectively.

Experiment 4C. Intravenous Carnitine Administration Simultaneously or Prior to I.V. Ethanol Dosage

The blood-ethanol concentrations in rats after simultaneous intravenous (i.v.) ethanol and carnitine administration on 3 consecutive days is shown in Table 25 and Figure 17. Day 1, which represents the simultaneous administration of both substances with no prior exposure to supplementary carnitine, showed no significant differences in the blood-ethanol levels between the NS and CS animals up to 8 hours postethanol administration. The blood-ethanol concentrations at 8 hours were essentially identical for both groups with the NS group showing 168.3 mg/dl and the CS group showing 167.5 mg/dl. On the second day the simultaneous administration of ethanol and carnitine also did not show significant difference between the NS and CS animals except after 8 hours postethanol dosage, where the NS group showed 167.7 mg/dl and the CS group showed 220.8 mg/dl. Finally, on the third day the simultaneous administration of ethanol and carnitine showed a significant difference between the NS and CS groups. The CS group maintained higher blood-ethanol concentrations than the NS group at all times, and differences between the groups were statistically significant at 4 (266.8 and 305.2 mg/dl) and 8 hours (235.5 and 289.0 mg/dl) postethanol administration for the NS and CS groups, respectively. Table 26 shows that at 6 hours postethanol administration on the third day of carnitine and

Table 25

Blood-Ethanol Concentrations at Various Times After 1, 2,
and 3 Days of Simultaneous Intravenous L-Carnitine
and Ethanol Administration¹

Postethanol Administration (minutes)	Simultaneous Administration of L-Carnitine (400 mg/kg) and Ethanol (0.2 g/kg)	Blood-Ethanol Concentrations (mg/dl)	
		NS	CS
	<u>Day 1</u>		
30		246.0 ± 15.3 ^a	243.8 ± 15.7 ^a
60		252.1 ± 13.2 ^a	236.0 ± 9.9 ^a
120		247.5 ± 16.2 ^a	229.3 ± 4.8 ^a
240		218.1 ± 12.1 ^a	204.6 ± 11.0 ^a
480		168.3 ± 6.4 ^a	167.5 ± 7.6 ^a
	<u>Day 2</u>		
30		317.5 ± 8.7 ^a	277.0 ± 23.4 ^a
60		298.5 ± 12.6 ^a	263.5 ± 22.8 ^a
120		264.3 ± 12.1 ^a	259.0 ± 27.0 ^a
240		230.7 ± 9.2 ^a	244.7 ± 31.5 ^a
480		167.7 ± 2.8 ^a	220.8 ± 19.9 ^b
	<u>Day 3</u>		
30		327.2 ± 15.4 ^a	351.5 ± 23.4 ^a
60		301.5 ± 11.9 ^a	335.3 ± 19.7 ^a
120		287.3 ± 10.9 ^a	310.2 ± 2.9 ^a
240		266.8 ± 9.1 ^a	305.2 ± 5.4 ^b
360		235.5 ± 14.0 ^a	289.0 ± 10 ^b

¹There were 4 rats in each group. The values are group means ± SEM and those in a line bearing the same superscript letter are not statistically different ($p \geq 0.05$).

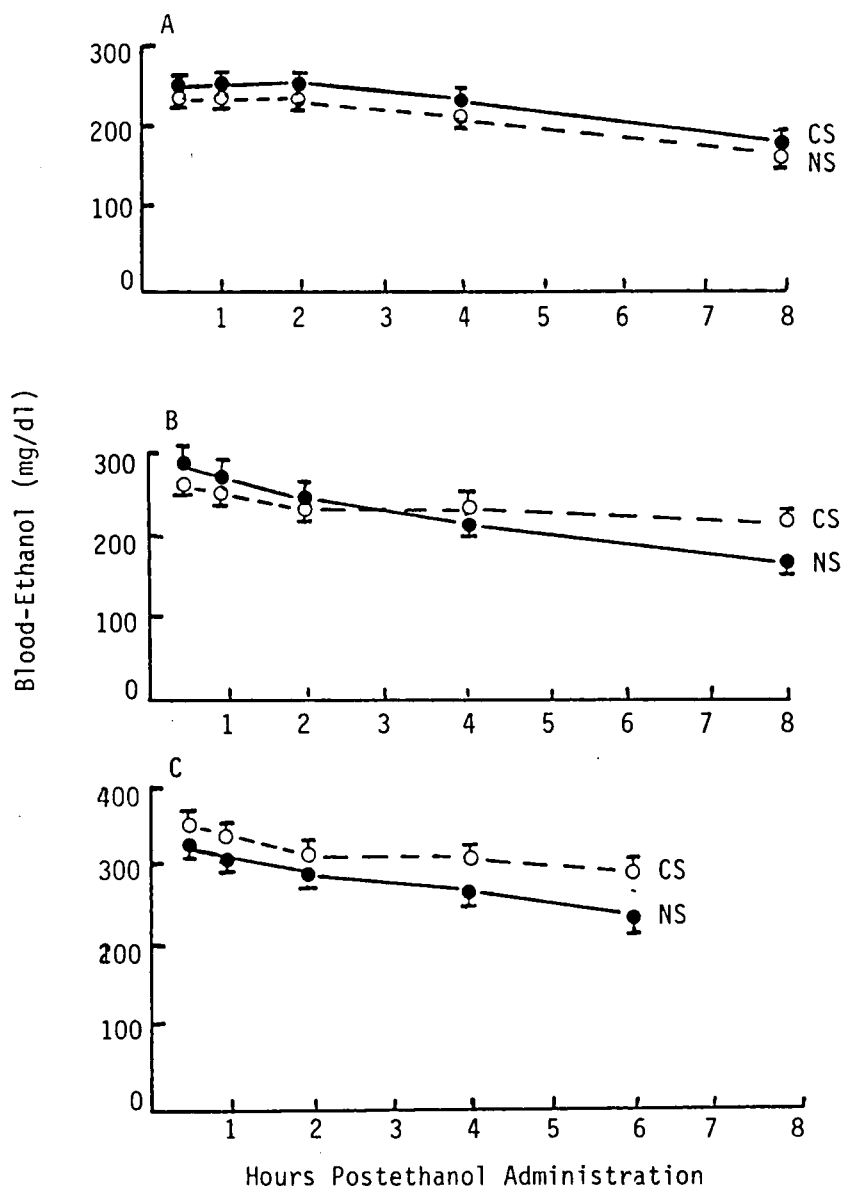


Figure 17. Blood-Ethanol Concentrations at 1 (A), 2 (B) and 3 Days (C) of Simultaneous Intravenous L-Carnitine Administration Up to 8 Hours Postethanol Dose. Each point represents the mean $\pm$ SEM for 4 animals. Slopes and correlations are as follows: A: CS; $y = (-12.4)x + 268.0$, $r = -1.00$ and NS; $y = (-10.1)x + 247.6$, $r = -1.00$. B: CS; $y = (-5.6)x + 255.7$, $r = -0.95$ and NS; $y = (-14.7)x + 279.1$, $r = -0.99$. C: CS; $y = (-10.1)x + 345.0$, $r = -0.95$ and NS; $y = (-14.9)x + 323.8$, $r = -0.98$. Slopes were determined by Linear Regression analysis after blood-ethanol peak concentrations were reached. In A, the slope differences between the groups are not significant ($p \geq 0.05$) when in B and C, the slopes for the CS groups were significantly less than those of the NS groups ($p \leq 0.05$).

Table 26
Hepatic-Ethanol Concentrations in Rats After 3 Days of Simultaneous Intravenous L-Carnitine
and Ethanol Administration

Postethanol Administration (hours)	L-Carnitine Dose (mg/kg)	Hepatic-Ethanol Concentrations (mg/g)	
		NS	CS
6	400	47.8 ± 8.1^a	24.4 ± 3.5^b

¹There were 4 rats in each group. The values are group means $\pm$ SEM and those in a line bearing the same superscript letter are not statistically different ($p \geq 0.05$).

ethanol treatment, there were significantly higher (96%) hepatic-ethanol concentrations in the NS than in the CS group; (47.8 vs. 24.4 mg/g).

Experiment 5. Sex Differences in Carnitine-Attenuated Ethanol Metabolism

This study was designed to determine the effect of sex on ethanol metabolism. Thus, the effects of 0.5% L-carnitine supplementation on blood-ethanol concentrations was determined in both male and female rats following an acute ethanol gavage (Table 27 and Figures 18 and 19). There were no significant differences between NS and CS males at 20 and 40 minutes postethanol administration. However, at 60 minutes through 8 hours, the CS males maintained a higher blood-ethanol concentration than the NS males. The decline in blood-ethanol concentrations of the NS and CS groups paralleled each other during this period (Figure 18). While the ethanol elimination rates were not significantly different between the NS and CS males (13.2 and 13.8 mg/dl/hr, respectively), the CS group reached a significantly higher blood-ethanol peak (155.8 mg/dl) than the NS animals (123.9 mg/dl) at 2 hours postethanol administration.

The blood-ethanol concentrations in the female rats from 0-8 hours were not significantly different between the NS and CS groups at any time excluding 60 minutes postethanol dosage, despite the fact that ethanol concentrations were consistently higher in the CS than the NS female at all time points. The ethanol elimination rates were faster for the CS (27.0 mg/dl/hr) than the NS group (22.2 mg/dl/hr). The peak blood ethanol concentrations were higher in the CS (206.0 mg/dl) than

Table 27

Blood-Ethanol Concentrations at Various Times Postethanol
Administration Pre- and Post-0.5% L-Carnitine Supplementation
in Male and Female Rats¹

Postethanol Administration (minutes)	Blood-Ethanol Concentrations (mg/dl) ²	
	Male	Female
Before Feeding CS Diet:		
20	61.8 ± 3.0 ^{ac}	95.8 ± 5.2 ^{bc}
40	92.8 ± 4.8 ^{ac}	139.5 ± 11.0 ^{bc}
60	113.4 ± 4.6 ^{ac}	156.9 ± 14.1 ^{bc}
90	122.9 ± 4.2 ^{ac}	172.0 ± 20.2 ^{bc}
120	123.9 ± 3.3 ^{ac}	161.9 ± 20.2 ^{bc}
240	107.3 ± 7.5 ^{ac}	122.0 ± 25.0 ^{ac}
360	79.0 ± 7.1(8) ^{ac}	70.0 ± 19.7(8) ^{ac}
480	36.9 ± 3.2(8) ^{ac}	31.8 ± 3.9(6) ^{ac}
After Feeding CS Diet for 5 Days:		
20	65.3 ± 3.2 ^{ac}	99.7 ± 2.9 ^{bc}
40	103.1 ± 4.0 ^{ac}	150.0 ± 3.0 ^{bc}
60	144.7 ± 7.8 ^{ad}	200.9 ± 6.3 ^{bd}
90	155.8 ± 5.8 ^{ad}	206.0 ± 4.9 ^{bc}
120	147.6 ± 5.0 ^{ad}	195.3 ± 4.6 ^{bc}
240	125.9 ± 2.7 ^{ad}	146.4 ± 7.7 ^{bc}
360	99.6 ± 3.6 ^{ad}	81.1 ± 13.8(7) ^{ac}
480	60.8 ± 5.0 ^{ad}	36.7 ± 11.2(6) ^{ac}

¹There were 9 rats in each group unless otherwise indicated by number in parenthesis following a value.

²The values are group means ± SEM. Those in a line bearing the same superscript letter (a-b) are not statistically different, while those in a column bearing the same letter (c-d) signify no significant differences between an individual groups and its corresponding time prior to and after carnitine supplementation ($p \geq 0.05$).

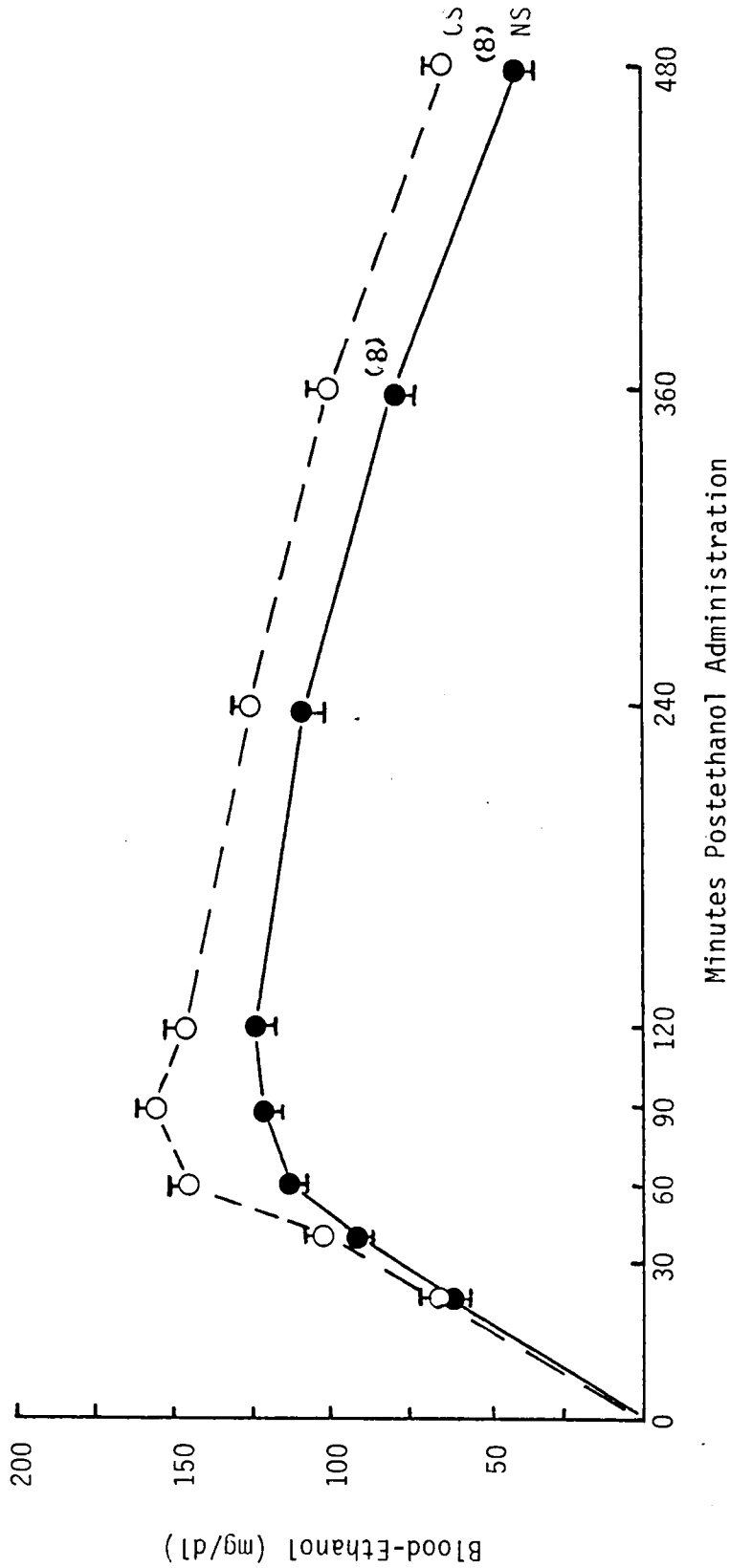


Figure 18. Time Course of Blood-Ethanol Concentration from 0-8 Hours After a Single Oral Dose of Ethanol in Male Rats Fed 0.5% Supplementary L-Carnitine for 5 Days. Each point represents the mean $\pm$ SEM for 9 animals unless otherwise indicated by the number in parenthesis above a point. Slopes and correlations are as follows: CS; $y = (-13.8)x + 178.4$, $r = -0.99$ and NS; $y = (-13.2)x + 150.3$, $r = -0.97$. Slopes were determined by Linear Regression analysis after blood-ethanol peak concentrations were reached. The slopes are not significantly different between the groups ($p \geq 0.05$).

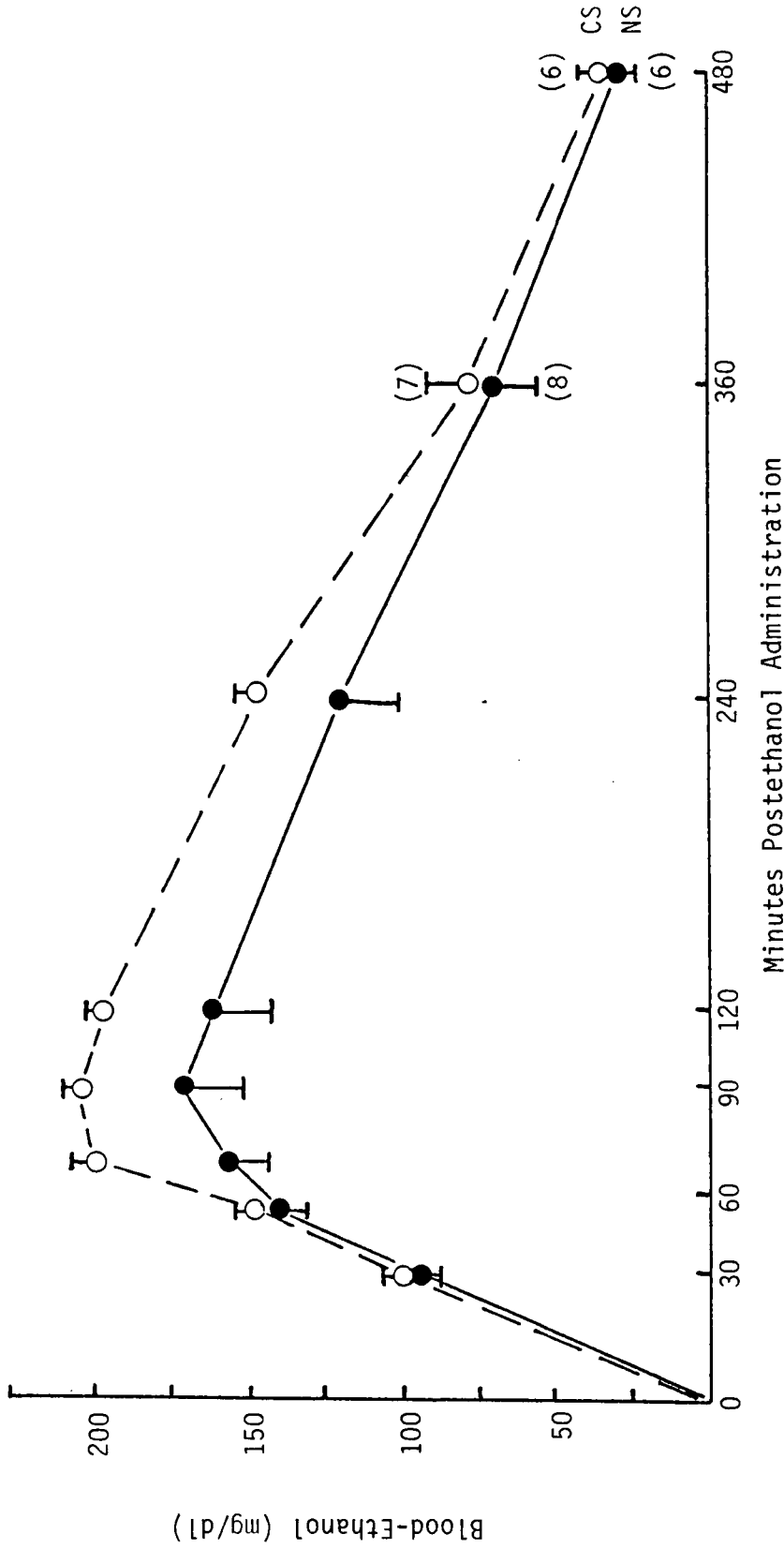


Figure 19. Time Course of Blood-Ethanol Concentration from 0-8 Hours After a Single Oral Dose of Ethanol in Female Rats Fed 0.5% Supplementary L-Carnitine for 5 Days. Each point represents the mean $\pm$ SEM for 9 animals unless otherwise indicated by the number in parenthesis above or below a point. Slopes and correlations are as follows: CS; $y = (-27.0)x + 248.0$, $r = -1.00$ and NS; $y = (-22.2)x + 205.9$, $r = -1.00$. Slopes were determined by Linear Regression analysis after blood-ethanol peak concentrations were reached. The slopes are not significantly different between the groups ($p \geq 0.05$).

in the NS females (172.0 mg/dl) at 90 minutes postethanol dosage (Table 27).

It should be noted that the female NS group reached a higher blood-ethanol peak (172.0 mg/dl) than the male NS rats (123.9 mg/dl), and the females in this group maintained significantly higher blood-ethanol concentrations at all times up to 2 hours postethanol dose compared to the males. This pattern is also observed between the female and male CS groups. The females maintained significantly elevated blood-ethanol concentrations up to 4 hours than their CS male counterparts. From Figure 18, it can be said that the ethanol elimination rates for male NS and CS groups were 13.2 and 13.8 mg/dl/hr, respectively. Conversely, the ethanol elimination rates for female NS and CS groups were 22.2 and 27.0 mg/dl/hr (Figure 19). Thus, it is concluded that the female rats reached higher blood-ethanol peaks, maintained higher blood-ethanol concentrations, and eliminated ethanol 60% and 49% faster than their NS and CS male counterparts, respectively.

NEC concentrations in the blood of all animals was determined at days 0 and 5 of supplementation, and then at days 3, 9, and 18 after supplementation was removed. Table 28 and Figure 20 illustrate that at day 0, blood-carnitine concentrations in the male rats (49.86 nm/ml) were significantly higher than that of the females (37.28 nm/ml). After 5 days of 0.5% L-carnitine supplementation, blood-carnitine concentrations rose significantly in both groups; the males exhibited a concentration of 96.10 nm/ml, while the females showed a concentration of

Table 28

Blood-Carnitine (NEC) Concentrations for Male and Female Rats Supplemented With 0.5% L-Carnitine for 5 Days and 18 Days After Supplement Removal¹

Days of Dietary Treatment	Blood-Carnitine Concentrations (nm/ml) ²	
	Males	Females
3-NS	49.86 ± 1.9 ^{ac}	37.28 ± 0.6 ^{bc}
5-CS	96.10 ± 4.2 ^{ad}	87.22 ± 4.0 ^{ad}
3-NS	55.21 ± 2.5 ^{ac}	46.22 ± 1.8 ^{bc}
9-NS	49.21 ± 1.9 ^{ac}	42.74 ± 1.1(8) ^{be}
18-NS	50.12 ± 2.2 ^{ac}	38.93 ± 1.1 ^{bc}

¹There were 9 rats in each group unless otherwise indicated by a number in parenthesis following a value. All animals were fed with 0.5% supplementary L-carnitine for 5 days after which supplement was removed.

²The values are group means ± SEM and those in a line bearing the same superscript letter (a-b) and in a column (c-e) are not statistically different ($p \geq 0.05$).

NS = nonsupplemented. CS = Carnitine supplemented.

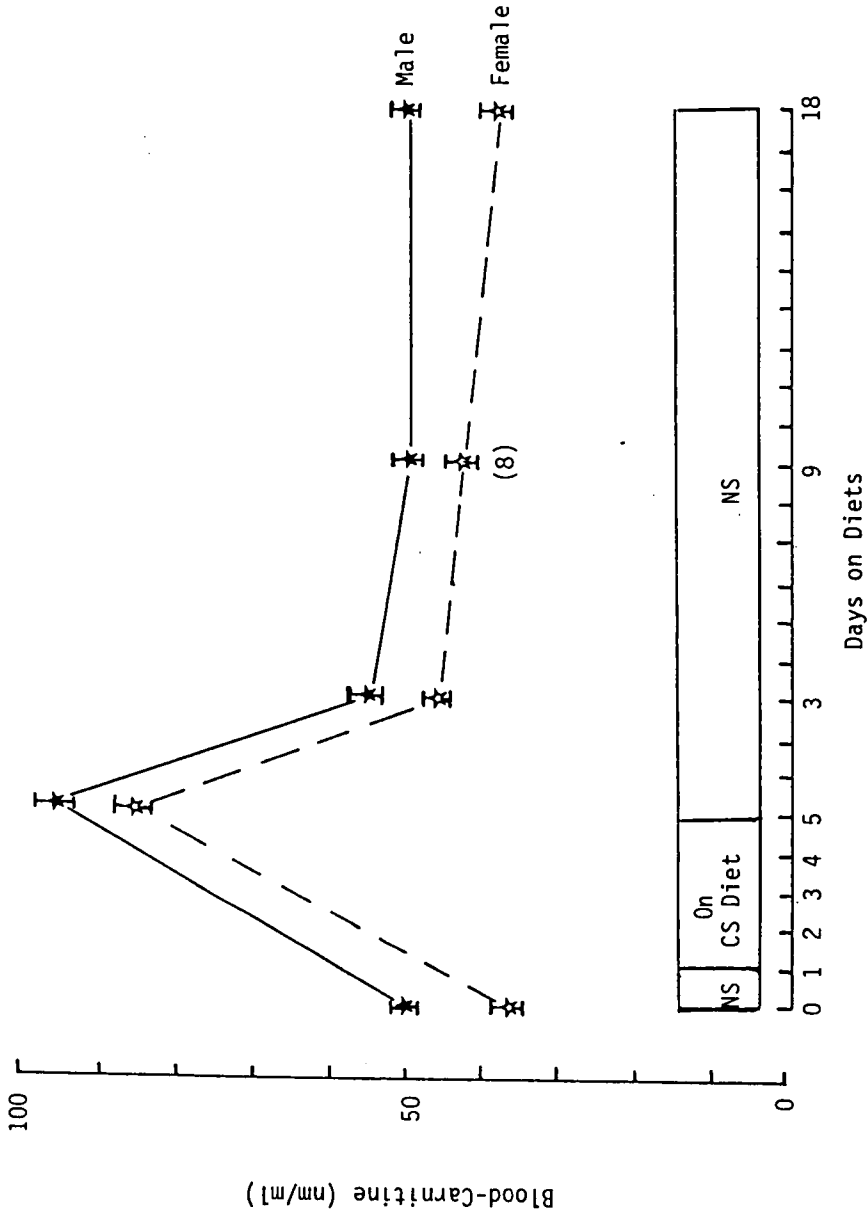


Figure 20. Blood-Carnitine (NEC) Concentrations for Male and Female Rats Supplemented With 0.5% L-Carnitine for 5 Days and Up to 18 Days After Supplement Was Removed. Each point represents the mean $\pm$ SEM for 9 rats unless otherwise indicated by the number in parenthesis below a point. NS = Nonsupplemented. CS = L-carnitine supplemented.

87.22 nm/ml and these differences between the sexes were not statistically significant. Three days after the supplement was removed, blood-carnitine concentrations showed a significant drop in both male and females (55.21 and 46.22 nm/ml, respectively). Although the blood-carnitine concentrations of the males after being off the CS diet for 3 days were not significantly different than those at day 0, the blood-carnitine concentrations of the females were still significantly higher at 3 and 9 days after being taken off the CS diet and did not return to pre-CS levels until about 18 days.

CHAPTER V

DISCUSSION

In the studies to ameliorate ethanol-induced fatty liver by D,L-carnitine, it was observed that carnitine supplemented rats were relatively more lethargic than the control rats (22). This effect of carnitine was supported by the higher blood-ethanol concentration found in the CS rats than in the NS rats under both acute and chronic ethanol treatments (25). A study involving the time course of ethanol appearance and disappearance in the blood of NS and CS rats revealed that the effect of carnitine may have been due to impaired ethanol metabolism rather than ethanol absorption from the G.I. tract (26). Since all of these studies were done using a racemic mixture of carnitine, one could not be certain if the effect was due to L-carnitine per se, or if the results were due to adverse effects of the D-carnitine moiety of the mixture. Thus it became necessary to evaluate the effects of L-carnitine on ethanol metabolism.

Optimal L-Carnitine Supplementation

Prior to investigating the effects of L-carnitine on ethanol metabolism, it was desirable to determine the levels and duration of L-carnitine supplementation needed to acquire steady state concentrations of carnitine in the blood, similar to that established for D,L-carnitine (26). Various levels of L-carnitine were supplemented in the diet for 10 days. An optimal level was found to be 0.5% L-carnitine

for 3 days (Table 12, p. 80, and Figure 8, p. 82). The 0.25% supplementary level was not quite adequate since blood-carnitine concentrations were lower than those attained with higher levels of supplementation, even though steady state concentrations were reached in 3 days. On the other hand, 0.75% and 1.0% supplementary levels did not produce significantly higher blood-carnitine concentrations than that produced by the 0.5% level of L-carnitine supplement. These results are consistent with the results of D,L-carnitine supplementary levels, where 1.0% for 5 days produced blood-carnitine steady state concentrations (26). The peak concentrations of carnitine attained in the 0.5% L-carnitine supplemented group were lower than those attained in the 1.0% D,L-carnitine supplemented group, which may be due to different times, batch of rats, and general conditions of the animals and the facilities in the earlier experiments. Blood-carnitine concentrations have been reported which were higher than these (Table 12 and Figure 8), where D,L- or L-carnitine was supplemented at a 1.0% or 0.2% level for 56 and 32 days (22, 153).

The urinary-carnitine profile resembled the blood-carnitine profile for the levels and duration of L-carnitine supplementation. While blood-carnitine steady state was reached after 3 days of 0.5% L-carnitine supplementation, urinary-carnitine steady state was not reached until 5 days (Table 13, p. 84, and Figure 9, p. 86). It appears that once blood-carnitine peaks were attained, the excess carnitine was spilled over into the urine. From this data it can be concluded that urinary-carnitine steady state concentrations can be reasonable

predictors of blood-carnitine steady state concentrations. The urinary-TC excretion in the NS groups (6.47 ± 0.30 μ moles/24 hours) were in agreement with those found in the NS rats of Tao et al. (171) and Gudjonsson et al. (172) which excreted 2.4-5.7 μ moles carnitine/24 hours for a 300 g rat. Conversely, urinary-TC excretion by NS rats in this study was about 2-3 fold higher than those reported by Brass and Hoppel (173) and Maccari et al. (174) which may be due to the higher dietary carnitine content (64.2 nmoles/g dry weight) of our diet as opposed to those used in the studies quoted (173, 174).

In a subsequent experiment (Table 28, p. 121, and Figure 20, p. 122) the course of blood carnitine concentration was determined following cessation of feeding an 0.5% CS diet. These determinations were the follow-up of the study examining the effect of sex difference on the carnitine-induced alterations of ethanol metabolism in male and female rats (Experiment 5). The peak concentrations of L-carnitine were nearly identical to those found in Experiment 1A after 5 days of feeding the CS diet. When the CS diet was replaced by the NS diet on the 6th day through the 18th day, the blood-carnitine concentrations steadily decreased reaching pre-CS concentrations by the 3rd day after being switched to the NS diet in the males (Table 28 and Figure 20). Although peak blood-carnitine concentrations in the female rats were also reached after 5 days of feeding the CS diet, they did not return to pre-CS diet concentrations for at least 9 days (9-18 days) after being switched to the NS diet (Table 28 and Figure 20).

A striking feature of urinary-carnitine data of this study is the concentration of AIAC in the urine (Table 14, p. 87, and Figure 10, p. 88). The approximately 20 fold higher excretion of AIAC observed in the CS animals has not been before observed, although claims have been made about carnitine mobilizing depot fat (175). It is unusual for AIAC fractions to be higher than ASAC fractions which is seen in this study (Figure 10). Part of the reason for this observation may be because the pellet which contains the AIAC fraction was not washed (see carnitine assay procedure). In our experience, washing the PCA pellet decreases the AIAC almost 50%. Even if these values for AIAC were cut in half, they would still remain higher than those of the ASAC. In absence of an adequate explanation for these high values, we must accept these data until a suitable rebuttal is found in the course of future studies. Should these results be reproduced, they will certainly help to explain the lipotropic effect of carnitine reported in the literature (22, 174).

The knowledge of urinary-carnitine steady state excretion can be very useful in planning and executing basic and clinical trials in humans (7-12, 16-20) and animals (13-15). An easy assessment of blood-carnitine steady state concentrations without having access to the blood, may promote better cooperation from human participants as well as a speedy approval by the committee on research involving human subjects. Should these profiles be reproduced in human volunteers, it will provide a basis for establishing carnitine requirements under various conditions in people by simply analyzing their urinary-carnitine profiles. However, additional data on urinary-carnitine concentration increases with

each increment of dietary carnitine level over and above the steady state levels, must be obtained for urinary-carnitine values to serve as a tool in determining nutritional status with regard to carnitine in human populations.

None of the supplementary levels of carnitine feeding for 10 days altered skeletal muscle-carnitine concentrations (Table 15, p. 90, and Figure 11, p. 91) in these studies. Perhaps a longer duration of feeding would have concurred with the results of Rebouche (170), who showed increases of 46 and 60% in the skeletal muscle-carnitine concentrations of rats after feeding diets supplemented with 0.1 and 1.0% L-carnitine, respectively, for 32 days. Sachan et al. (unpublished) also showed that 1.0% D,L-carnitine supplementation for 56 days resulted in significantly higher carnitine concentrations in skeletal muscles of CS than NS chronic alcoholic rats. Thus, although 10-day feeding of 0.5% L-carnitine supplemented diet was sufficient to reach steady state carnitine concentrations in the blood and urine, it was not administered long enough to alter carnitine concentrations in skeletal muscle. Because carnitine concentrations were not determined in other organs of the rats, as done by other investigators (21, 22, 163, 170, 174), it can not be certain whether carnitine concentrations in the other organs (i.e., heart, liver, kidneys, testes) of these rats were altered.

Supplementary L-Carnitine Affects Ethanol Metabolism

Pharmacokinetics of ethanol in the CS animals showed that the rates of ethanol appearance in the first 60 minutes postethanol administration

were not significantly different between NS and CS rats (Figure 3, p. 35) (26). However, the peak concentrations were not reached at the same time in the two groups which left room for some concern over the conclusion that carnitine affected only the metabolism and not the absorption of ethanol at all. Therefore, ethanol concentrations were monitored in the portal and systemic blood of NS and CS rats simultaneously (Table 16, p. 92). Initial rates of ethanol appearance in the portal blood were not significantly different between NS and CS groups (120 vs. 124 mg/dl/min). However, the appearance of ethanol in the systemic blood of the CS animals was more than doubled compared to that found in the NS rats (52 vs. 20 mg/dl/min) during the same period of time. The rates of ethanol disappearance from the portal blood of the various CS groups (0.25-1.0%) ranged from 8.9-12.8 mg/dl/min compared to 17.5 mg/dl/min found in the NS group. Because these disappearance rates were significantly slower in all CS groups than to the NS group, the data presented here strengthen the conclusion that carnitine attenuates ethanol metabolism. Further support for this conclusion is drawn from the studies where ethanol was administered intraperitoneally and intravenously to NS and CS rats, and there again, CS rats maintained a higher systemic blood-ethanol concentration than did the NS rats (Experiments 3A, 3B, 4B, 4C).

Thus, it is apparent that the elevation of blood-ethanol concentration was not due to enhanced gastrointestinal emptying, such as caused by the cholinergic drugs, Nicotine or clofibrate (101), nor did carnitine increase ethanol absorption, as caused by the cholinergics or H₂-blocking agents like Cimetidine® (104, 105).

Specific Effect of L-Carnitine on Ethanol Metabolism

During the course of these studies questions have been raised about the specificity of the carnitine effect on ethanol metabolism. It was questioned as to whether ethanol metabolism could be attenuated by carnitine precursors (i.e., lysine, methionine, trimethyllysine and γ -butyrobetaine), carnitine derivatives (i.e., acetyl and acylcarnitines) and/or carnitine-like lipotropic agents (i.e., choline and lecithin). While the answer to all these questions is not known yet, it has been established that the effect of carnitine is specific and can not be reproduced by choline (Table 17, p. 94, and Figure 12, p. 95). Choline was chosen because it is structurally similar to carnitine, readily available, and has been shown to be lipotropic (176-178).

When choline was supplemented at the same equimolar concentration as 0.5% L-carnitine for 7 days, blood and urinary-carnitines were not significantly different from the control rats (Table 18, p. 96). This showed that supplementary choline did not increase blood- or urinary-carnitine concentrations. Although Tsai et al. (179) showed that liver and muscle carnitine concentrations were increased by feeding dietary supplements of choline (0.2 and 0.4%) to rats for 26 days, these carnitine pools were not addressed in this study. Thus it is concluded that choline, although possessing the lipotropic properties of carnitine, does not alter ethanol metabolism, and that the effect of carnitine is specific. Additional studies should be carried out to answer concerns about the other compounds previously mentioned.

Carnitine Effect in Relation to Dosage of Ethanol

In order to further define the carnitine-mediated attenuation of ethanol metabolism, it became necessary to examine the doses of ethanol at which carnitine may be effective. Since only one dose of ethanol (3 g/kg b.w.) has been used in all experiments up to this point, it was not known if 0.5% supplementary carnitine will also retard the metabolism of ethanol at high and low dosages. A series of five different single doses of ethanol were administered intraperitoneally to NS and CS rats (90 days on 0.5% CS diet) and their blood-ethanol concentrations were followed for 8 hours postethanol administration (Table 20, p. 101, and Figure 14, p. 103). The CS rats had consistently higher blood-ethanol concentrations at all dosages of ethanol, however, statistically significant differences were only found at and beyond 0.2 g/kg b.w. dosages. The differences at certain time points at 0.4 and 0.8 g/kg b.w. dosages were on the borderline of significance, perhaps due to the large standard deviations which were present. Part of the reason for the smaller differences in blood-ethanol concentrations of NS and CS rats following various dosages of i.p. ethanol may be due to the small differences which were found in their blood-carnitine concentrations (98.4 vs. 128.5 nmoles/ml) (Table 21, p. 105). In previous experiments differences between NS and CS blood-carnitine concentrations were much greater (31.0 vs. 71.0 nmoles/ml) while so were the blood-ethanol (168.0 vs. 257.0 mg/dl) concentrations. Although there were no significant differences in blood-ethanol concentrations between the NS and CS groups

at the lower i.p. ethanol doses (0.05 and 0.1 g/kg b.w.), ethanol clearance rates were still slower by 167% for both doses in the CS rats than the NS rats. This finding may be due to either: (1) the doses of ethanol administered were too low to observe a true carnitine-mediated effect, or (2) ethanol may not have been absorbed or distributed evenly at these very low doses. At the i.p. ethanol doses of 0.2, 0.4, and 0.8 g/kg b.w. the carnitine effect was seen, with the optimal dose at 0.2 g/kg b.w. (Table 20, p. 102, and Figure 14, p. 103). It is evident all doses (0.05-0.8 g/kg b.w.) of ethanol resulted in clearance rates of 167, 167, 73, 77, and 23%, respectively in CS rats which were slower than those in the NS rats.

While serum triglyceride concentrations of NS and CS rats were not significantly different (Table 21, p. 105), hepatic triglyceride and total lipids were significantly lower in the CS than the NS rats, which is consistent with the lipotropic effect but not with the hypolipidemic effect of carnitine (22, 146).

Sequence and Duration of Carnitine and Ethanol Administration Via Different Routes

The simultaneous administration of carnitine with ethanol via different routes (oral, i.p., i.v.) was ineffective in retarding ethanol metabolism in rats (Tables 22, 24, 25, pp. 107, 110, 103, and Figures 15-17, pp. 108, 111, 114). However, a significant difference in blood-ethanol concentration in CS rats was seen 32 hours (8 hours post-ethanol dosage) following the oral carnitine dose (Table 22 and Figure 15A). When a second ethanol gavage was administered 52 hours

following the carnitine dose (Table 22, p. 107, and Figure 15B, p. 108) no significant difference was observed between NS and CS rats up to 3 hours postethanol gavage. Although the carnitine effect was not significant by blood-ethanol concentrations at 4 hours postethanol administration (60 hours after carnitine dosage) (Tables 23, p. 109), the hepatic concentrations of ethanol in NS rats (57.7 mg/g) were nearly twice those of CS rats (30.0 mg/g). A similar effect was observed with i.p. and i.v. doses of carnitine and ethanol. Although the carnitine effect was not observed by blood-ethanol differences between NS and CS groups, except at 4 and 8 hours postethanol dosage on day 3 (Tables 24, 25, pp. 110, 113, and Figures 16, 17, pp. 111, 114), hepatic-ethanol concentrations of the NS rats (47.8 mg/g) were nearly twice those of the CS animals (24.4 mg/g) in the i.v. study (Table 26, p. 115). Hepatic-ethanol concentrations were not analyzed in the i.p. study (Experiment 4B).

It is concluded that carnitine is ineffective in attenuating ethanol metabolism when administered simultaneously with ethanol. However, after 3 days of carnitine administration via any route prior to ethanol dosage, carnitine was quite effective. The effect of carnitine on blood-ethanol clearance was different from that of chloral hydrate (107), chlorpromazine® (108), or any of the pyrazoles (31, 109, 110) which act by directly inhibiting ADH. It was also different from that of disulfiram (106) which directly inhibits aldehyde dehydrogenase (ALDH). However, the action of carnitine on ethanol metabolism is

similar to that carnitine has on ammonia metabolism and the metabolism of valproic acid (20), by reducing the effects of hyperammonemia.

Sex Effect on Carnitine-Ethanol Interactions

Carnitine-mediated attenuation on ethanol metabolism observed in male rats (Table 27, p. 117, and Figure 18, p. 118) could not be produced in the female rats (Table 27 and Figure 19, p. 119). Although CS males maintained significantly higher blood-ethanol concentrations than NS males after 40 minutes postethanol dosage, there were no significant differences in blood-ethanol concentrations in CS and NS female rats, although the blood-ethanol concentrations of CS females were about 20% higher than those of the NS females (Table 27 and Figure 19). Two observations must be noted in this study (Experiment 5): (1) both NS and CS female rats reached higher blood-ethanol peaks (172.0 and 206.0 mg/dl) than their corresponding male counterparts (123.9 and 155.8 mg/dl), and (2) both NS and CS females had significantly faster blood-ethanol elimination rates (27.0 and 22.2 mg/dl/hr) than their male counterparts (13.8 and 13.2 mg/dl/hr). These results are supported by Eriksson (180) who showed that when blood-ethanol peaks were attained, blood-ethanol elimination rates were 20-30% faster in the females than in the male rats of the same strains. Other investigators (92, 181) have reported that female rats reach higher blood-ethanol peaks than their male counterparts, which may be due to faster absorption rates or lower ADH activities in females than in males. It was also shown (180) that female rats had a significantly lower mitochondrial NADH/NAD ratio

than did males under the same alcoholic conditions, and may be explained by the greater oxygen consumption attained by the females. The increased oxygen consumption would cause an increased mitochondrial reoxidation of NADH, which in turn, would cause an increased ethanol oxidation. This observation was supported by the fact that the livers of the female rats had greater acetaldehyde outputs than those of the male rats after ethanol administration (180). In any case, the increased ethanol oxidation rate observed in female rats seems to override the carnitine effect. One possible explanation for the greater oxygen consumption which causes increased ethanol oxidation rates in female rats, may be due to the increased oxygen demand in female rats needed for the metabolic breakdown of hormones, such as the estrogens. Thus, the increased rate of ethanol metabolism observed in female rats may be due to a greater activity of the microsomal enzyme system.

CHAPTER VI

SUMMARY

It was established that 0.5% (w/w) L-carnitine supplementation for 3 days was optimum to attain blood-carnitine steady state concentrations in Sprague-Dawley rats. The urinary-carnitine profile was similar to that of blood-carnitine, and the peak concentration of carnitine in the urine was attained after 5 days of supplementation. It was suggested that urinary-carnitines may be reasonable predictors of blood-carnitine steady state concentrations. The knowledge of urinary-carnitine steady state excretion may be a useful parameter in assessing blood-carnitine steady states in clinical situations where access to blood may not be practical. Unfortunately, blood- and urinary-carnitine steady state concentrations could not be used in predicting muscle-carnitine concentrations under the conditions of these experiments.

L-carnitine, like D,L-carnitine, attenuated ethanol metabolism because (1) higher blood-ethanol concentrations were maintained by the animals given carnitine orally, i.p., or i.v. than their respective controls, and (2) while portal blood-ethanol concentrations did not differ significantly, the systemic blood-ethanol concentrations were significantly elevated in the CS rats during the same period of time postethanol administration. Thus it is concluded that carnitine altered ethanol metabolism and not the absorption from the G.I. tract. While carnitine was effective at all routes and doses of ethanol administration, the magnitude of the effect was variable.

Since the simultaneous administration of carnitine with ethanol by any route did not attenuate ethanol metabolism, it is proposed that carnitine may modulate the hepatic cell structure or enzyme systems, perhaps in some type of inductive manner. The carnitine-mediated attenuation of ethanol metabolism was specific to carnitine since it could not be mimicked by an analogous compound, choline.

Further, the effect of carnitine on ethanol metabolism was influenced by the sex of the animal, since only the males were significantly affected in a consistent manner, while the female rats, although affected, were not so to the same extent. This difference may be attributed to differences in hormonal activity, metabolic rates, volume of distribution, and adiposity of males and females.

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VITA

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