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Effects of Carnitine on Ethanol-Induced Hepatic Steatosis

Tae Hyong Rhew
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To the Graduate Council:

I am submitting herewith a dissertation written by Tae Hyong Rhew entitled "Effects of Carnitine on Ethanol-Induced Hepatic Steatosis." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

Dileep S. Sachan, Major Professor

We have read this dissertation and recommend its acceptance:

Roy E. Beauchene, Hugh O. Jaynes, Sharon L. Melton

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

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Accepted for the Council:

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The Graduate School

EFFECTS OF CARNITINE ON ETHANOL-INDUCED HEPATIC STEATOSIS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Tae Hyong Rhew

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ABSTRACT

The lipotropic and hypolipidemic effects of carnitine and its precursors, namely lysine (Lys) and methionine (Met), was examined in male Sprague-Dawley rats fed ethanol as 36% of the total calories for a duration of 56 days (Experiment I). Ethanol produced significant hepatic steatosis and hypertriglyceridemia as evidenced from elevated concentrations of total lipids, triglycerides, cholesterols, and phospholipids. Supplementation of the ethanol diet with 1.0% D,L-carnitine and 0.5% L-Lys plus 0.2% L-Met significantly reduced ethanol-induced elevation of the various lipid classes with the exception of free fatty acids. The triglyceride contents in liver and plasma were inversely related to the concentrations of carnitine, acylcarnitines, and total carnitine. The effects of a combination of carnitine and its precursors were not greater than those of carnitine alone. It is suggested, therefore, that a deficiency of functional carnitine may exist in a chronic alcoholic state which can be improved by dietary carnitine.

In two following experiments, animals were fed the ethanol liquid diets supplemented with various levels (Experiment II: 0.0, 0.2, 0.6, 1.0, and 2.0%; Experiment III: 0.0, 0.1, 0.5, 0.8, 1.2, and 1.6%) of D,L-carnitine for 28 and 45 days. In the 28 day experiment, the effects of supplemental carnitine were significant only in the plasma triglycerides and reduction in hepatic lipid classes were minimal. In the 45 day experiment, various lipid

classes of the alcohol-compromised liver and plasma were significantly reduced with carnitine treatment, and the effect was dose-dependent. The optimum lipotropic and hypolipidemic effect were produced by 0.8% D,L-carnitine. Thus, it is concluded that in rats, supplemental D,L-carnitine ameliorated ethanol-induced hyperlipidemia and hepatic steatosis in a dose-dependent manner.

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CHAPTER I

INTRODUCTION

Alcoholism and alcohol abuse are major health problems. In the United States, cirrhosis of the liver is the fifth leading cause of death in the general population (1). The mechanism of the variable response to ethanol intake remains unclear, and the relationship of liver damage to alcohol metabolism is controversial. Although a great deal of evidence has been gathered to explain an etiological role of ethanol in the pathogenesis of the ethanol-induced disorders, other factors associated with alcohol abuse also play important roles.

Research interest has been centered around the relationship of ethanol intake to nutrition and lipid metabolism. Many excellent reviews are available which deal with the metabolic interrelationships of ethanol (2), nutrition (3), lipid (4), carbohydrate (5), and intermediary metabolism (2,5). Lately, with the realization that ethanol may exert its own influence by acting as a direct hepatotoxin, research interest has shifted to other areas, especially those concerned with macromolecular metabolism within the hepatocyte (6-9).

Chronic ingestion of ethanol has been reported to cause hepatic steatosis in experimental animals (10-14) as well as in humans (12-14). Primary attention has been usually given to the effects of ethanol on triglycerides, which make up the major class of hepatic lipids. However, significant effects on

cholesterol esters (10-15), phospholipids (11,15) and lipoproteins (16) have also been reported. Since ethanol is obligately metabolized in the liver (17), hepatic steatosis and associated hyperlipidemia are common features of disturbed lipid metabolism caused by ethanol intake.

Despite considerable research efforts, no satisfactory treatment for ethanol-induced fatty liver has yet been found. Many groups of investigators have studied the effectiveness of various dietary or nondietary chemical agents in preventing ethanol-induced fatty liver, but the results have not been entirely satisfactory. These studies have recently been reviewed (18). Most research for prevention of ethanol-induced disorders has been confined to fatty liver since this was the only disorder reproducible in an animal, and its mechanism was partly understood. Choline supplements protected rats, but not humans, against ethanol-induced fatty liver (19). Many drugs, including clofibrate (20), asparagine and barbiturate (21) have been credited as inhibitors against ethanol-induced fatty liver in rats, but these drugs were usually tested against a single large dose of ethanol intake. Recently in rats, a marked protective benefit of a combination of naturally occurring substances including riboflavin, dihydroxyacetone, and pyruvate has been reported (22). These agents have a potential to accept hydrogen ions and hence can correct the altered redox state of the alcoholic liver.

Among the dietary agents, the effect of dietary carnitine(3-hydroxy-4-N-trimethylaminobutyrate) has been found controversial. Some investigators suggested a state of carnitine deficiency in animals (23) and in patients hospitalized for alcoholic cirrhosis of liver (24), whereas others (25,26) demonstrated that ethanol-induced liver injury is not related to carnitine deficiency. The argument for a deficiency of carnitine in ethanol-induced fatty liver is based on the fact that carnitine is known to enhance fat metabolism by facilitating the transport of fatty acids (FA) into mitochondria where the oxidation of FA occurs. Indeed, carnitine is required for the efficient oxidation of FA (27-29). Thus, the oxidation of FA might be depressed in various organs in the absence of carnitine. Unfortunately, only three or four studies (23,24,30) have been carried out on the relationship between exogenous carnitine and ethanol-induced disturbances of lipid metabolism.

The objectives of this study were:

1. to determine effects of carnitine on ethanol-induced hyperlipemia and hepatic steatosis;
2. to establish dose dependent relationship between supplemental carnitine and lipotropy;
3. to assess the potential of alcoholic liver for biosynthesis of carnitine from its presursors.

CHAPTER II

REVIEW OF LITERATURE

1. ETHANOL METABOLISM

Ethanol is preferentially metabolized by the liver via the following reactions:

(A) $CH_3CH_2OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$

(B) $CH_3CH_2OH + NADPH + H^+ + O_2 \xrightarrow{MEOS} CH_3CHO + NADP^+ + 2H_2O$

(C) $CH_3CH_2OH + H_2O_2 \xrightarrow{Catalase} CH_3CHO + 2H_2O$

(D) $CH_3CHO + NAD^+ \xrightarrow{\text{aldehyde dehydrogenase}} CH_3COOH + NADH + H^+$

Reaction A involves the cytosolic enzyme, alcohol dehydrogenase (ADH) with nicotinamide adenine dinucleotide (NAD) as a cofactor. This reaction results in the formation of acetaldehyde and the reduction of NAD to NADH + H^+. As a result, oxidation of ethanol generates an excess of reducing equivalents in the liver primarily as NADH+H^+ (31). Ethanol is also oxidized by an alternative pathway (reaction B) that requires NADPH (reduced nicotinamide adenine dinucleotide phosphate) as a cofactor which is localized in the endoplasmic reticulum (32). Unlike ADH, the activity of microsomal ethanol oxidizing system (MEOS) increases during chronic ethanol

consumption (33). There is also an argument for a possible role of catalase (reaction C) in the oxidation of ethanol to acetaldehyde (34). However, it is generally considered that under normal conditions ethanol oxidation proceeds primarily by the alcohol dehydrogenase pathway. Acetaldehyde, the first oxidation product of ethanol, is further oxidized to acetate (reaction D) by aldehyde dehydrogenase (35). Although liver can readily metabolize acetate in vitro, most of acetate is utilized, in vivo, by extrahepatic tissues (36). The oxidative metabolic pathway of ethanol and the consequence of metabolic burden are illustrated in Figure 1 (2). Ethanol is readily absorbed from the gastrointestinal tract. Only 2-10% of the amount absorbed is eliminated through kidney and lungs. Thus, the bulk of ethanol, which has high caloric value (7.1 kcal/g), is oxidized in the liver (12). This organ specificity of ethanol metabolism is further aggravated by the absence of a storage and feedback mechanism (12). Consequently, the oxidation of ethanol produces considerable metabolic imbalances in the liver.

2. GENERAL METABOLIC EFFECTS

Many of the metabolic derangements in the liver caused by ethanol consumption seem to be attributable to the products of ethanol oxidation or its metabolites such as acetaldehyde and acetate. Most of the metabolic consequences of ethanol metabolism can be attributed to the generation of excess reducing equivalents (increased NADH + H^+/NAD ratio) or to the formation of acetaldehyde,

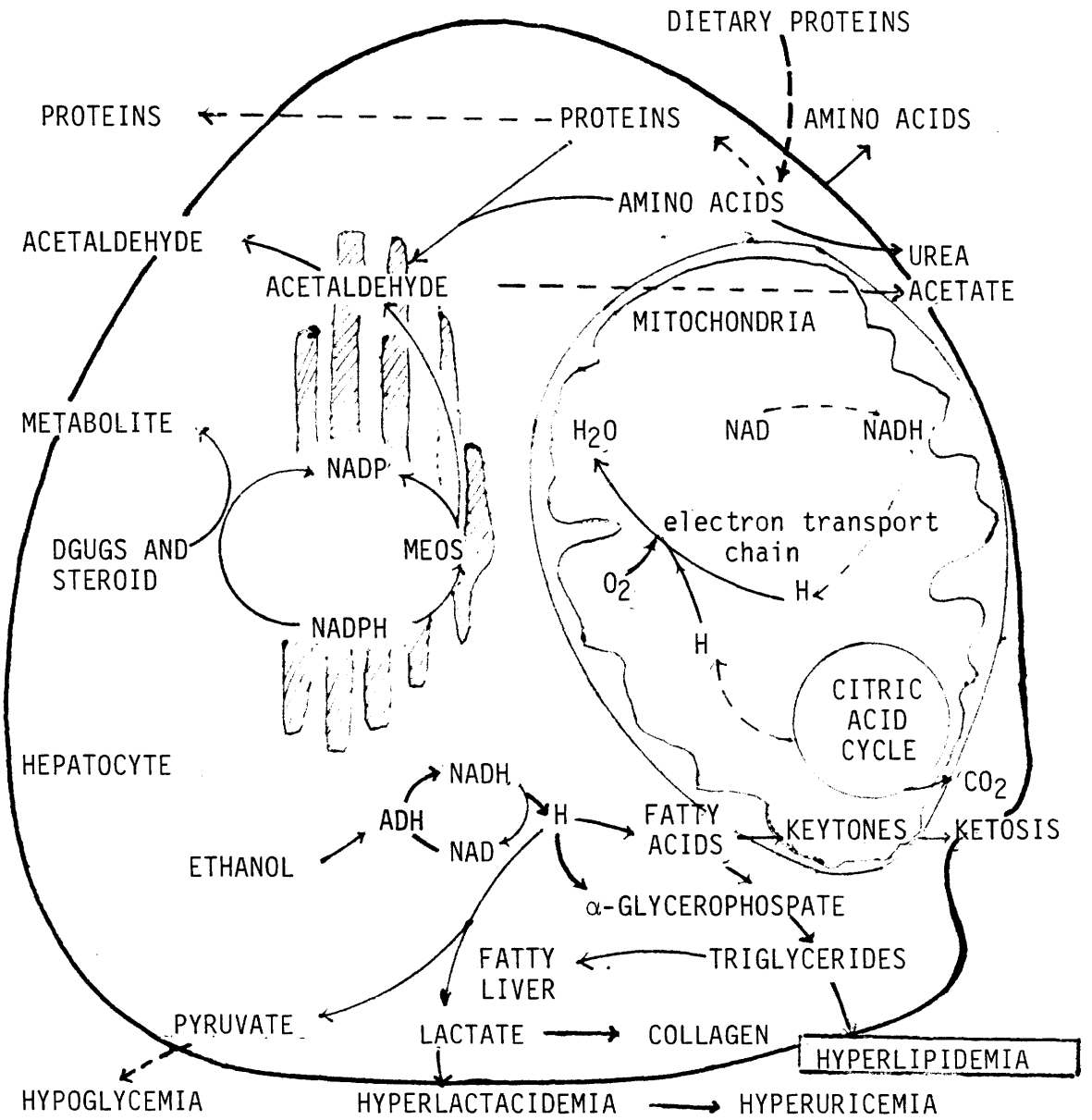

FIGURE 1. Oxidative metabolic pathway of ethanol. NAD denotes nicotinamide adenine dinucleotide; NADH, reduced NAD; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP; MEOS, the microsomal ethanol oxidizing system; and ADH, alcohol dehydrogenase (2).

an extremely reactive metabolite. Although large amounts of acetate are formed from acetaldehyde during ethanol oxidation, its effect on hepatic metabolism has remained unknown. Little was known about blood acetaldehyde concentrations in subjects consuming ethanol until Korsten et al. (37) reported a difference in blood acetaldehyde concentrations between alcoholic and nonalcoholic subjects after ethanol administration. The increased rate of ethanol oxidation that followed prolonged ethanol consumption resulted in increased acetaldehyde concentrations in alcoholics compared to nonalcoholics (42.7 ± 1.2 vs 26.5 ± 1.5 μmoles) (37). The rate of oxidation of acetaldehyde in the mitochondria does not parallel the increased rate of ethanol oxidation to acetaldehyde (38). Consequently high acetaldehyde content may exert its own toxic effect since acetaldehyde is a very reactive compound. During ethanol oxidation, the rate of oxidation of acetate in the liver is decreased because of the inhibition of the citric acid cycle (39.40). The activity of the citric acid cycle is depressed partly because of a retardation of the reactions of the cycle that requires NAD. A major site of this interaction of ethanol in the citric acid cycle is at the α-ketoglutarate oxidation step (41). Furthermore, the redox change associated with ethanol oxidation decreases the hepatic concentration of oxaloacetate (42). The concentrations of oxaloacetate is important for the efficient synthesis of citrate. The ultimate consequences of abnormal generation of acetate from ethanol in the liver remain unknown. It was also reported that

ethanol intake causes proliferation of the smooth membranes of the hepatic endoplasmic reticulum (43).

Since ethanol is exclusively metabolized in the liver, a stressful metabolic burden is imposed on the liver whenever significantly large quantities of ethanol are consumed. Generally much of the metabolic disturbance caused by ethanol intake is attributed to the generation of excess reducing equivalents in the liver and the attempts of the liver to correct this abnormal redox state.

3. SPECIFIC METABOLIC EFFECTS

Effects of Excessive Hepatic NADH Generation

The oxidation of ethanol results in the transfer of hydrogen to NAD^+. The resulting increased NADH + H^+/NAD^+ ratio produces a change in the proportion of metabolites that are dependent on the availabilities of NAD^+ and is responsible for a number of metabolic abnormalities associated with alcohol abuse.

Some of the metabolic abnormalities associated with ethanol intake are summarized below. The enhanced NADH + H^+/NAD^+ ratio induces an increased lactate/pyruvate ratio that results in hyperlactacidemia (44,45). Hyperlactacidemia causes acidosis and also reduces the capacity of kidney to excrete uric acid, leading to secondary hyperuricemia (46). On the other hand, the enhanced reducing equivalents can be transferred into the synthetic process of fatty acids (47) and α-glycerophosphate (48), which are

precursors of triglycerides. Reducing equivalents are also transferred into the mitochondria by various shuttle mechanism, e.g., "glycerophosphate shuttle" (Figure 2) or "malate shuttle" (Figure 3) (49). Presumably these are the major mechanisms for disposing of the excessive reducing equivalents in the cytosol of liver (34). Consequently, these excess reducing equivalents provide alternative mitochondrial fuel originating from ethanol rather than from fatty acid oxidation through the citric acid cycle. Fatty acids usually serve as a main source of energy in the liver; however, these are supplanted by ethanol. Decreased fatty acid oxidation by ethanol has been evidenced in rat and human liver slices (50), perfused livers (40), human liver (51) and in vivo (52). Consequences of these decreased oxidation of fatty acids are the accumulation of dietary fat in the liver, when available, or accumulation of endogenously synthesized fat in the liver when dietary fat is not available (53-56). The redox changes in liver caused by acute administration of ethanol continues after chronic ethanol feeding in rats (57). Therefore, chronic ethanol consumption produces the progression of alcoholic liver injury beyond fatty liver (58).

Effects of Acetaldehyde

Oxidation of acetaldehyde proceeds via aldehyde dehydrogenase, 80% of which is located in mitochondria (59-60). Oxidation of acetaldehyde also generates NADH + H^+, and as in the case of ethanol, some of acetaldehyde effects may be attributed to the NADH generation.

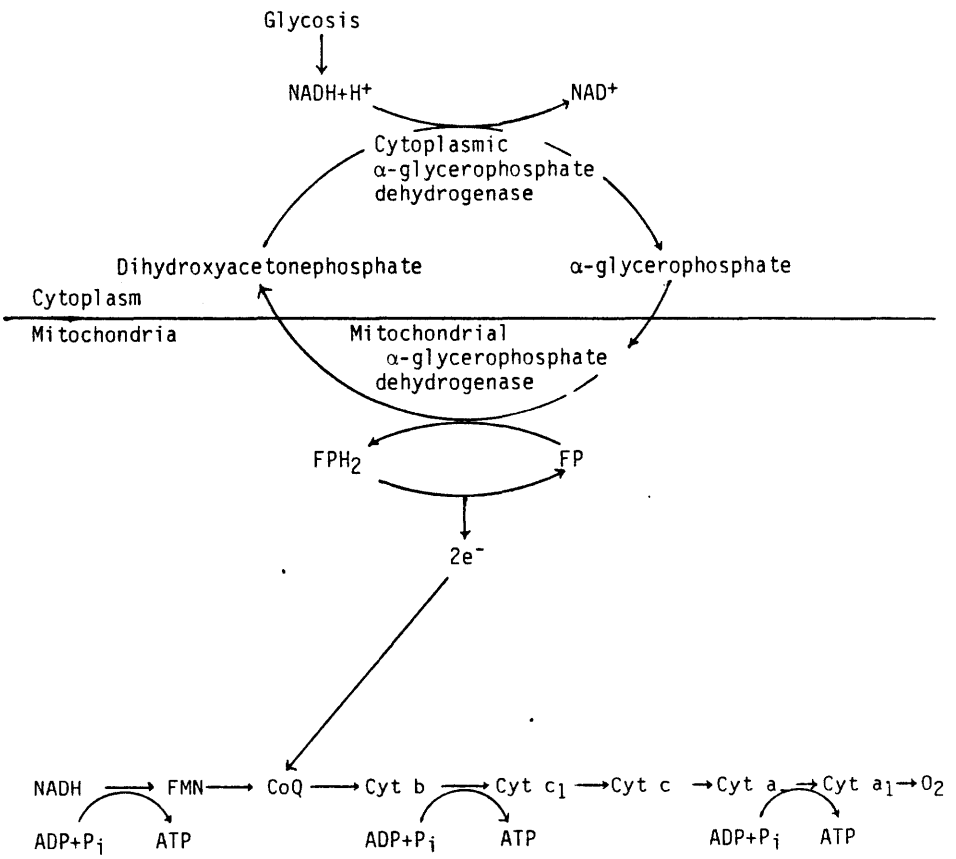

FIGURE 2. Glycerophosphate shuttle for transporting cytoplasmic reducing equivalents into the mitochondria.

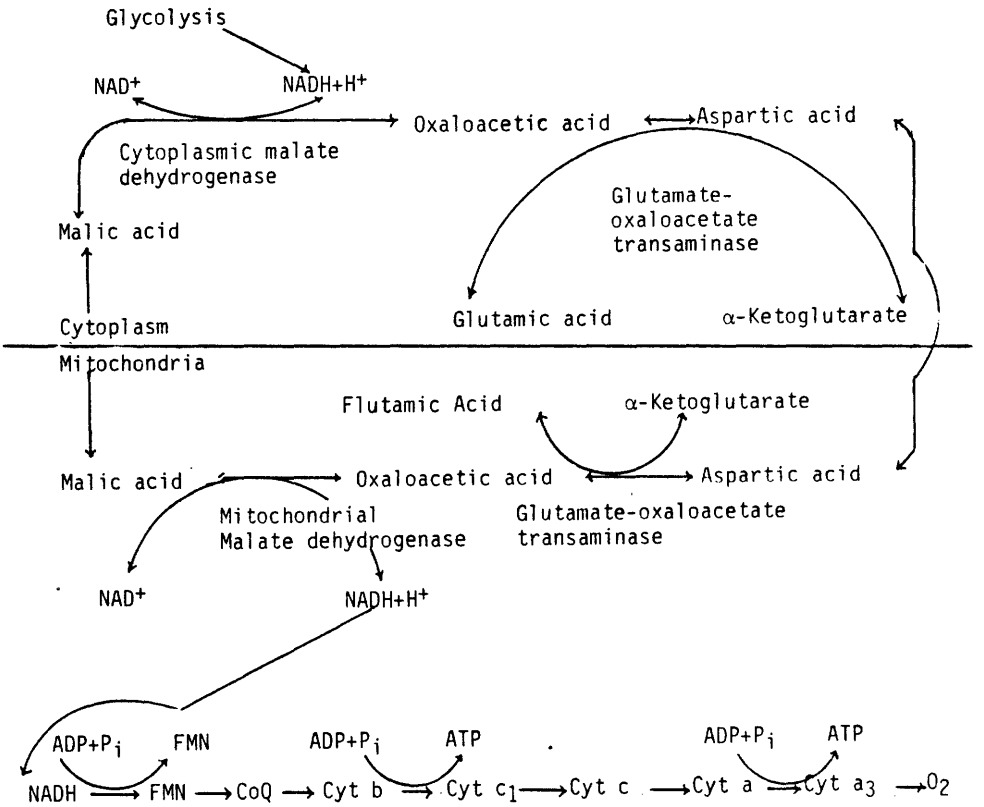

FIGURE 3. Malate shuttle for the transport of cytoplasmic reducing equivalents into the mitochondria.

However, acetaldehyde is considered to exert its own toxic effect due to its high chemical activity. The mean acetaldehyde level was shown to be significantly higher in alcoholics (42.7 $\pm$ 1.2 μmole) than in nonalcoholic subjects (26.5 $\pm$ 1.5 μmole) (37).

The reduced oxidation of acetaldehyde coupled with an enhanced production from ethanol oxidation may cause the accumulation of acetaldehyde in alcoholics. Indeed Hasumura et al. (38) found that the liver mitochondria subjected to alcohol had a significantly reduced capacity to oxidize aldehyde (rate of disappearance : 11.8 + 0.8 vs 14.6 $\pm$ 0.7 μmole/min; ethanol vs control). Acetaldehyde was found to reduce the activities of various mitochondrial shuttles involved in the disposition of reducing equivalents and to inhibit oxidative phosphorylation (61). Acetaldehyde also depresses the capacity of hepatic mitochondria to oxidize fatty acids (62) as does the ethanol (63). In some studies (64,65), addition of acetaldehyde to liver slices was shown to inhibit protein synthesis. As with ethanol, acetaldehyde generates an excess of reducing equivalents and hence may increase the lactate/pyruvate ratio.

Induction of Microsomal Enzymes by Prolonged Ethanol Intake

Various mechanisms including central nervous system adaptation and increased activity of the MEOS have been postulated as means of increased tolerance during prolonged alcohol consumption. However, from the studies

in animals (66,67) and humans (66,68), it has been observed that MEOS plays a major role in the adaptive increase in ethanol metabolism after chronic ethanol intake. Indeed, elevated cytochrome P-450 concentrations (26.0 $\pm$ 4.6 vs 8.9 $\pm$ 2.1 pmole/mg microsomal protein) and microsomal enzyme activities have been reported in the upper small intestine after chronic ethanol consumption in rats (69). The MEOS has been defined as constituting of cytochrome P-450, NADPH, cytochrome-C-reductase, and phospholipids, most of which are the component of the microsomal drug metabolizing enzyme system (70). Ethanol ingestion has been found to cause a proliferation of the smooth membranes of the hepatic endoplasmic reticulum (43), and hence, both phospholipid and total protein content of the smooth membranes were increased (71). Functional implications for these changes involve accelerated metabolism of drugs and other xenobiotics to polar metabolites. For instance, ethanol pretreatment remarkably stimulates the toxicity of carbon tetrachloride (CCl_4) in experimental animals (72). Also pretreatment of rats with phenobarbital, an inducer of the hepatic microsomal drug-metabolizing system, increased CCl_4 toxicity in the liver (73). Moreover, this induction of enzymes of the endoplasmic reticulum may promote lipid peroxidation, leading to another potential hepatic toxicity (71).

Effects on Drug Metabolism

Acutely intoxicated alcoholics are markedly susceptible (increased sensitivity) to various drugs such as sedatives and

hypnotics, whereas the chronic alcoholics are extremely resistant to even large doses of similar drugs (1). In the acute alcoholics, the increased susceptibility may be partly due to combined effect of ethanol and drugs on the central nervous system (1). In addition to that, recent studies (74,75) demonstrated that ethanol inhibited the hepatic metabolism of a variety of drugs in vitro. In other studies (76,77), the simultaneous administration of ethanol and drugs decreased the rate of drug metabolism in vitro. It is thought that ethanol per se, rather than its metabolities, interferes with the binding of drug substrates of cytochrome P-450 and hence decreases their detoxification (77,78). On the other hand, in chronically intoxicated individuals, drug metabolism is increased because of proliferation of the smooth endoplasmic reticulum and increased activity of some microsomal drug-detoxifying enzymes (71,74,79). In a recent study (80), it was observed that chronic ethanol administration induced cytochrome P-450 with specific spectral and catalytic properties different from those of cytochrome P-450 of control animals and those treated with other drugs. Therefore, in addition to nervous system adaptation, the induction of microsomal drug-detoxifying enzymes may account for the resistance of chronic alcoholics to the effects of various drugs.

4. PATHOGENESIS OF ACUTE AND CHRONIC ETHANOL ADMINISTRATION

Acute Effects of Ethanol on Liver Lipids

In one study (81), it was reported that a single large dose of ethanol (4.2-6.2/kg body weight) given to rats by stomach tube or intraperitoneal injection produced a significant accumulation of triglycerides in liver within 12-16 hr. However, this fatty liver was markedly reduced when the ethanol administration was given in divided doses during certain time intervals (82). An acute dose of ethanol stimulates both hypothalamus-pituitary-adrenocortical (83) and sympatho-adrenomedullar (84) systems. The release of cathecholamines by ethanol is important because cathecholamines promote free fatty acid release from adipose tissue and accumulation of triglycerides in liver (85). This acute fatty liver can be prevented by the administration of β-adrenergic blocking agents, hypophysectomy, adrenalectomy, and spinal cord transaction (86). More importantly, ethanol per se alters lipid metabolism and is associated with a variety of secondary effects. These cause change and damage in cell population in both gastrointestinal tract (87) and peritoneum (88) resulting in systemic stress. Effects of chronic ethanol administration is much more complicated.

Chronic Effects of Ethanol on Liver Lipids

In animal experiments in which ethanol had no lipotropic effect, the alcohol was administered in drinking water at amounts equivalent to

only 10-25% of the total calories in the diet (86). However, when the intake of ethanol by rats was increased up to 36% of the total calories in liquid diets for rats, equivalent to moderate alcohol consumption in man (12), the effects were found. In this diet, isocaloric replacement of some of the carbohydrate by ethanol consistently produced 5-10 fold increases (89.4 $\pm$ 11.1 vs 11.3 $\pm$ 1.3 mg/g liver) in hepatic triglyceride concentrations in rats (12). In humans with normal livers, fatty liver also developed when ethanol was administered as a supplement to normal diets or as isocaloric substitution for carbohydrate (12,88,89). However, simple isocaloric replacements of carbohydrate by fat or administration of carbohydrate deficient diets in normal rats did not produce fatty liver as ethanol did (12). This finding indicates that in alcoholics, fatty liver is due to ethanol and not to the manipulation of the non-ethanol sources of calories (12). The lipid accumulation was apparent after a few days (19) or even after one day (90) of ethanol administration. The greatest increase is associated with the triglycerides content, although other lipid classes such as phospholipids (12) and cholesterol (10) also accumulated in the liver after chronic ethanol administration. The increase in hepatic cholesterol occurs mainly in the esterified fraction with little elevation in free cholesterol (10).

Ethanol-Induced Fatty Liver

Fatty liver is characterized by an abnormal accumulation of lipid consisting principally of triglycerides. Fatty liver occurs

when the rate of triglyceride formation exceeds its disposition from the liver. This formation of triglycerides depends on an influx of fatty acids into the liver and/or the rate of synthesis of fatty acids by the liver with subsequent esterification into triglycerides. Removal of fat from liver is carried out by its secretion as very low density lipoprotein particle (VLDL) into general circulation or by the oxidation process (to CO_2 and H_2O). Catabolism of fatty acid is mainly carried out by β-oxidation. Overall oxidation of fatty acids involves:

1. activation of fatty acid to acyl-CoA in the cytoplasm;
2. entry into mitochondria via carnitine acyltransferases;
3. oxidation (β-oxidation consists of these three steps); and
4. oxidation of acetyl CoA through the citric acid cycle to CO_2 and H_2O.

In the study by Blomstrand et al. (50), addition of ethanol to rat and human liver slices depressed the formation (3.6% vs 8.2%) of $^{14}CO_2$ from linoleic acid-1-^{14}C. In this study, as a result of ethanol addition, the esterification of triglycerides (45.6% vs 24.4%) and phospholipids (28.6% vs 19.8%) was increased, and the formation of β-ketoacid was decreased (15.9% vs 43.9%). A significant depression of the $^{14}CO_2$ excretion from ^{14}C-labelled oleic acid was observed when it was administered orally or by intravenous injection to humans as triolein with ethanol (52). These results suggest that ethanol inhibits β-oxidation of fatty acids and increases esterification of free fatty acids thus promotes accumulation of hepatic triglycerides

and phospholipids. In another study conducted by DeCarli and Lieber (10), isocaloric replacement of carbohydrate by ethanol in rat diet resulted in a significant increase of hepatic total lipids (96.8 ± 6.6 mg/g vs 46.1 ± 1.3 mg/g). In a similar study, Lieber et al. (12) also reported ethanol replacement of carbohydrate in the diet caused a morphological change of the liver in man and increased hepatic total lipids (129.4 ± 9.2 vs 43.2 ± 1.2 mg/g), triglycerides (89.4 ± 11.1 vs 11.3 ± 1.3 mg/g), and phospholipids (31.4 ± 1.1 vs 28.8 ± 1.2 mg/g) in rats fed ethanol as 36% of total calories.

Ethanol-Induced Cirrhosis

After chronic administration of ethanol, the metabolism of amino acids is depressed in the liver (91). In animals fed ethanol chronically, the concentrations of branched-chain amino acids (such as leucine) increased in both liver (273 ± 32 vs 153 ± 15 nmole/g) and plasma (235 ± 16 vs 133 ± 17 nmole/ml) (92). In liver slices of ethanol-fed animals, the production of α-amino-n-butyric acid (AANB) was increased 1-3 fold compared to control animals (93). The hepatic damage caused by ethanol includes accumulation of fat and the enlargement of hepatocytes and the diameters of hepatic cells are increased 2-3 times with an increased volume of 4-10 fold. This morphological change is generally called alcoholic hepatomegaly. The ballooning of hepatocytes coupled with necrosis causes alcoholic hepatitis, and alcoholic hepatitis further develops to alcoholic cirrhosis. However, in some populations (Europe and Japan) alcoholic cirrhosis develops without an intermediate stage of hepatitis (138).

Cellular injury is also associated with extracellular deposition of collagen. In the fatty liver stage, collagen is detectable by chemical means only (94). When collagen deposition is sufficient to become visible by microscope, it is called pericentral sclerosis. This deposition of collagen in the perivenular area (pericentral sclerosis) further develops into diffuse fibrosis and cirrhosis (95).

5. MECHANISM OF THE ETHANOL-INDUCED ACCUMULATION OF FAT IN THE LIVER

Role of Carbohydrate

Theoretically carbohydrate can contribute to the biosynthesis of fatty acids in liver by supplying the carbon skeleton. However, the role of carbohydrate in ethanol-induced accumulation of fat in liver has not been studied. On the other hand, effects of ethanol in carbohydrate metabolism is well established. In a chronic alcoholic case, increased availability of lactate may stimulate collagen production and enhance hepatic collagen proline hydroxylase activity which also play a role in collagen accumulation (96). The altered redox state may contribute to the hypoglycemia through impairment of gluconeogenesis from amino acids (97). Galactose oxidation in the liver is also inhibited by alcohol via lowering urine diphosphate glucose isomerase which is readily inhibited by NADH. However, this effect is not clinically important (97).

Role of Protein and Amino Acid

In a study (98), the high dietary protein was found to have no effect on the ethanol-induced hepatic triglyceride accumulation (55.7 $\pm$ 6.8 vs 52.3 $\pm$ 4.9 mg/g : in 12.5% of total calories as protein vs 25% of total calories as protein, respectively). However, protein contributes to the ballooning of the hepatocyte which is a conspicuous feature of alcoholic fatty liver. This hepatomegaly is mainly attributed to fat accumulation, but fat accounts for only half of the increase in liver dry weight and the other half is accounted for by an increase in protein (99). In this study, Baraona et al. have reported that the liver retained proteins such as albumin and transferrin. In chronic liver injury, there is a tendency for concentrations of the branched-chain amino acids, valine, leucine and isoleucine to increase and for concentrations of tyrosine, phenylalanine, glutamic acid, methionine, and sometimes, cystine to fall in the liver (3). Treatment with methionine, (30) has been reported to reduce ethanol-induced hepatic accumulation of triglycerides in the liver from 105.3 $\pm$ 6.1 mg/g to 69.6 $\pm$ 4.9 mg/g.

Role of Dietary Lipids

The degree of fatty liver caused by ethanol intake depends on the amount of dietary fat. Reduction of dietary fat to a level of 25% or less of total calories in the diet significantly reduced hepatic fat accumulation in rats fed ethanol (98). In this study, much more hepatic triglycerides (75 vs 17 mg/g) accumulated

with a diet of high fat content (43% of total calories as fat) than with a low-fat diet (10% of total calories as fat) when an identical amount (36% of total calories) of ethanol was administered. In addition to the amount of fat in the diet, the chain length of fatty acids is also important in the degree of fat accumulation. Replacement of dietary fat containing triglycerides of long chain fatty acids by triglycerides containing medium chain fatty acids reduced the accumulation of total lipids (48.1 $\pm$ 4.2 vs 118.8 $\pm$ 13.1 mg/g) and triglycerides (32 vs 77 mg/g) in the liver of ethanol-fed rats (30). Lieber et al. (54) found that fatty acid compositions in liver fat are similar to those of dietary fat in the case of prolonged ethanol ingestion but not with an acute dose. In another study of Lieber (53), the increase in hepatic total lipids in ethanol-fed rats was 2.6 fold with the high fat diet (4.3% fat) compared to control, but only 1.5 fold with the low fat diet (2% fat); the corresponding changes in triglycerides were 6.3 and 2.6 fold, respectively.

Dietary fat mainly consists of triglycerides containing long chain fatty acids. Triglycerides are hydrolyzed in the intestinal lumen to monoglycerides and fatty acids prior to absorption. In the intestinal mucosa, triglycerides are resynthesized and released into lymph as chylomicrons (as a packet of triglycerides, cholesterols, phospholipids, and β-apolipoprotein). Medium chain fatty acids are absorbed and carried directly to the liver via portal blood. However, chylomicron triglycerides must be hydrolyzed to fatty acids before entering the hepatocyte.

Nondietary Lipids

Unlike chronic administration of ethanol, administration of a single large dose of ethanol to rats resulted in hepatic fatty acids accumulation similar to those of adipose tissue (100). In another study (53), it was demonstrated that after a single large dose of ethanol (7.5 g/kg), concentrations of hepatic total lipids were significantly increased (81.0 $\pm$ 3.3 vs 46.8 $\pm$ 1.4 mg/g), and fatty acid compositions of hepatic triglycerides and free fatty acids were similar to those of adipose tissue. Evidently, in these cases (acute ethanol administration), increased hepatic fat is considered to be originated from increased peripheral fat mobilization. Other than this enhanced mobilization of peripheral fat, possible theoretical mechanisms of increased hepatic fat have been postulated as: (a) increased uptake of fatty acids by liver because of stimulatory effects of ethanol on hepatic blood flow (101,102); (b) increased biosynthesis of fatty acids in liver from normal carbohydrate precursors (53,54) or from the carbon skeleton of ethanol (103); (c) increased esterification of fatty acids because of increased availability of glycerides from increased dihydroxyacetone phosphate and subsequent α-glycerophosphate (41,48,104); (d) increased synthesis of cholesterol esters (105) (in ethanol-fed rats, enhanced incorporation of labeled acetate into cholesterol was reported (105); (e) decreased oxidation of fatty acids (50,52); (f) decreased hydrolysis of fatty acid esters due to depressed activity of acid lipase and esterases (106,107); (g) decreased excretion of hepatic fat into bile (major

lipids excreted into bile are phospholipids and cholesterol) (108,109); and (h) decreased release of lipoproteins (92). Therefore, as a summary, fatty liver may result from any combination of: increased influx of fatty acids into the liver; increased synthesis of fatty acids in liver; decreased oxidation of hepatic fatty acids; and impaired removal of hepatic fat. However, a recent consensus has developed that the most important mechanism responsible to ethanol-induced fatty liver is impaired oxidation of fatty acids (52,106).

6. ETHANOL-INDUCED HYPERLIPEMIA

Alcoholic hyperlipemia is classified as Type IV (110) since particulate fat behaves as VLDL on untracentrifugation and as pre-β-lipoproteins on electrophoresis. In alcoholic hyperlipemia, serum triglyceride is the main fraction which is elevated, but serum phospholipids and cholesterol are also increased (86). Very low density lipoproteins are the major triglyceride-carrying lipoproteins from liver. Ethanol enhanced the incorporation of chylomicron fatty acids into newly synthesized VLDL, as evidenced by an increased reappearance of the labelled fatty acids after injection of palmitate-^{14}C/glycerol-^{3}H which was double-labeled (8). This indicates that alcohol hyperlipemia is due partly to an increase in newly synthesized lipoproteins (8). In summary, hyperlipemia occurs when the entry of lipids into blood exceeds their removal.

Acute Effects of Ethanol on Serum Lipids

A high dose of ethanol (180g/6 hr) to chronic alcoholics produced hypertriglyceridemia (257.1 ± 31.2 vs 112 ± 22.1 mg/dl) (111). These effects of ethanol on serum triglycerides were enhanced when ethanol administration was followed or accompanied by a fat-containing diet (112,113). However, unlike in humans, acute administration of ethanol to rats did not produce consistent hyperlipemia (125 ± 26 vs 116 ± 9 mg/dl) (114).

Chronic Effects of Ethanol on Serum Lipids

Administration of 200-300g of ethanol per day (100-200 mg/dl blood) to humans for two weeks produced a 4-fold increase in serum triglycerides (480 mg % vs 140 mg %) and some increases in cholesterol (330 mg% vs 270 mg %) and phospholipids (360 mg % vs 220 mg%) (89). After two to three weeks of constant ethanol administration, these elevated levels of triglycerides returned to normal (145 mg %), despite continuation, or even an increase in ethanol intake. However, when a high fat-containing diet was accompanied by the administration of ethanol, alcoholic patients with fatty liver compared to control subjects had higher levels of serum triglycerides (108 mg/dl vs 24 mg/dl) for a longer period (6 hr vs 2 hr).

Mechanism of Ethanol-Induced Hyperlipemia

Serum triglycerides and serum fatty acids are produced in the liver and intestine, whereas cholesterol is produced in many tissues and transported to the liver for excretion as bile salts.

For the transport of these lipids from the liver, liver produces VLDL and high density lipoprotein (HDL). Very low density lipoprotein carries lipids from the liver to other tissues, and HDL serves to transport extrahepatic cholesterol to the liver for excretion (115). Serum triglycerides are hydrolyzed extensively in many organs in the body, and serum fatty acids are stored in adipose tissue or oxidized in many tissues as triglycerides. Accumulation of lipids in the blood occurs when the rate of entry into the blood exceeds the rate of removal from the liver. The theoretical mechanism of ethanol-induced hyperlipemia is illustrated in Figure 4 (86).

Interrelationship Between Serum and Liver Lipids in Ethanol-Compromised Liver

Alcoholic hyperlipemia may play a compensatory role in counteracting lipid accumulation in the liver. Indeed, full development of ethanol-induced hyperlipemia after one month of ethanol administration (36% of total calories in the diet) to rats concurred with the ceasing of the accumulation of lipids in the liver (98). Although disposal of hepatic lipids as serum lipoprotein into blood could contribute to the alleviation of hepatic steatosis, this compensatory effort is only partially effective and fatty liver is not prevented by this mechanism. When alcoholic fatty liver progresses to advanced stages of liver injury, hyperlipemia decreases. Such an inverse relationship between the degree of alcoholic hyperlipemia and the

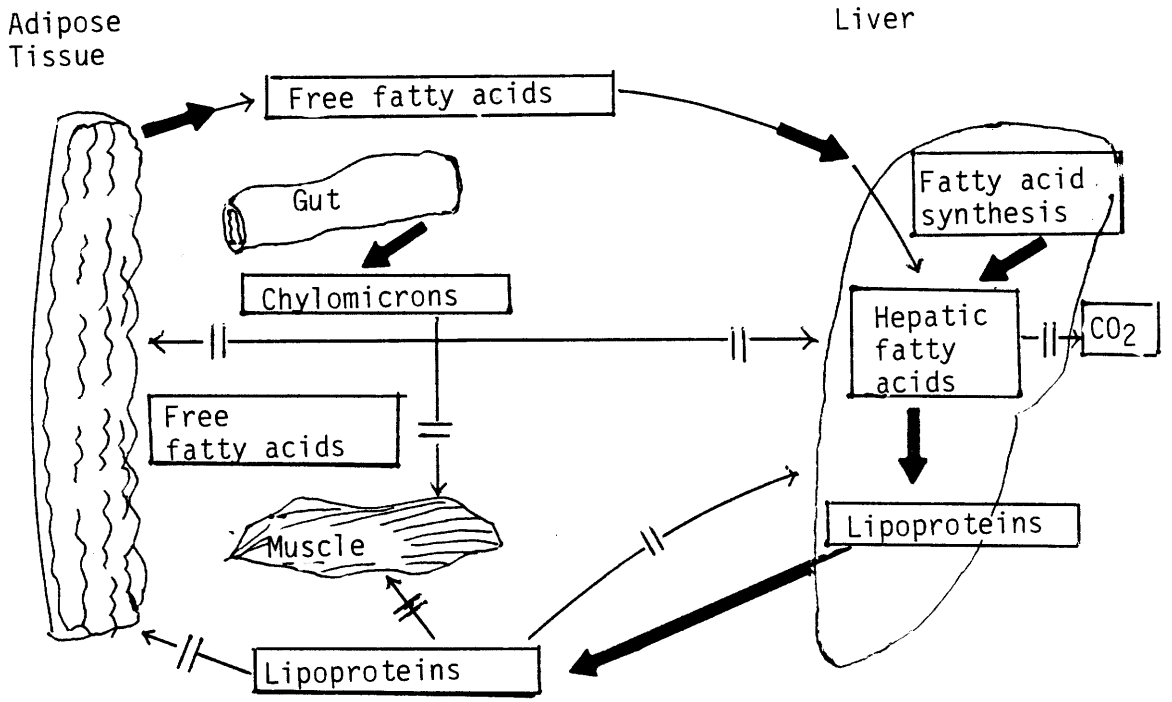

FIGURE 4. Theoretical mechanisms for ethanol-induced hyperlipidemia. Ethanol intake could result in hyperlipidemia either by enhancing the pathways illustrated with wide arrows or by blocking those illustrated with broken lines (86).

severity of liver damage have long been recognized (116). Recently, it has been observed that the decrease in VLDL, or pre-β-lipoprotein could be an early indicator of the progression of fatty liver to more severe hepatic lesions (117).

7. PREVENTION OF ETHANOL-INDUCED FATTY LIVER

The fat accumulated in the liver with ethanol consumption originates mainly from the diet and under restricted conditions, from endogenous biosynthesis and so on. In some studies, restriction of dietary fat was reported to be effective in reducing accumulation of liver fat in rats (98) and humans (54). In addition to the amount of fat in a diet, quality of the fat is also important in causing the hepatic fat accumulation. In rats, this accumulation of hepatic fat was reported to be reduced by a substitution of medium-chain triglycerides for long-chain triglycerides (40). This may be due to the preferential oxidation of medium-chain fatty acids.

A variety of dietary and nondietary chemical agents have been examined for the effectiveness in preventing fatty liver. Many drugs, including clofibrate (20); asparagine, and barbiturate (21) were found to be effective inhibiters of ethanol-induced fatty liver in rats, but these drugs were usually tested against a single large dose of ethanol. Treatment with dietary choline protected rats against ethanol-induced fatty liver but not humans (19). Recently in rats (22), a remarkable protective effect against ethanol-induced fatty liver was exhibited by naturally occurring substances,

riboflavin, dihydroxyacetone, and pyruvate. In this study, results showed that hepatic triglycerides were significantly reduced by treatment with these chemical agents (see Table 1). These agents have a potential to accept hydrogen ions and correct the altered redox state of the alcohol-compromised liver. Earlier studies (118, 119) suggested that fortification of protein in the rat diet prevented fatty liver, but more recent studies (98) have not supported this finding. Recently, Goheen et al. (120) reported a protective effect of dietary arachidonate against alcoholic fatty liver. In this study, significantly reduced concentrations of hepatic triglycerides (27.56 $\pm$ 4.34 vs 68.4 $\pm$ 6.37 mg/g) and phospholipids (19.4 $\pm$ 2.4 vs 23.1 $\pm$ 1.3 mg/g) were caused by treatment with arachidonate of ethanol-fed rats.

TABLE 1. Effects of Dietary Agents on Hepatic Fatty Acid Accumulation by Ethanol (22)

Chemical Agent	mEq/100 g liver
Ethanol	11.35 $\pm$ 0.36
Riboflavin	9.89 $\pm$ 0.48
Pyruvate + Dihydroxyacetone	9.79 $\pm$ 0.56
Pyruvate + Dihydroxyacetone + Riboflavin	9.18 $\pm$ 0.39
Pyrurate + Dihydroxyacetone + Riboflavin + Thiamin	9.56 $\pm$ 0.27

Note. Values are mean $\pm$ SEM.

Effect of Carnitine

Carnitine, which has been recognized as vitamin B_T (121), is different from most other vitamins of its nature. Carnitine can be synthesized in the mammalian system (Figure 5) and is found in large amounts in natural products such as meats (121).

In 1955, Fritz (27) observed that addition of carnitine to a reaction mixture containing palmitic acid and a rat liver homogenate caused an increase in the rate of oxidation of the fatty acids. Furthermore, it has been found that synthetic palmitic carnitine was oxidized much more rapidly by mitochondria than was palmitoyl-CoA or palmitic acid (28).

Carnitine facilitates the transport of fatty acids via carnitine acyl transferase into the mitochondria for β-oxidation (29). Fatty acids are normally oxidized in the mitochondria by β-oxidation, i.e., removal of two carbon fragment at a time from the carboxyl end of the fatty acid molecule. This process depends on carnitine acyltransferases within the mitochondrial membranes which facilitate the transfer of fatty acids into the site of β-oxidation. This process is illustrated in Figure 6 (49).

Decreased fatty acid oxidation is incriminated as a major causative mechanism for the accumulation of lipids in the liver. Indeed, in recent studies, β-oxidation of fatty acids were reported to be decreased in rat and human liver slices (50) when alcohol was added (9.1 vs 4.2% of radioactivity recovered: control vs alcohol group with palmitic acid and 8.2 vs 3.6% of radioactivity

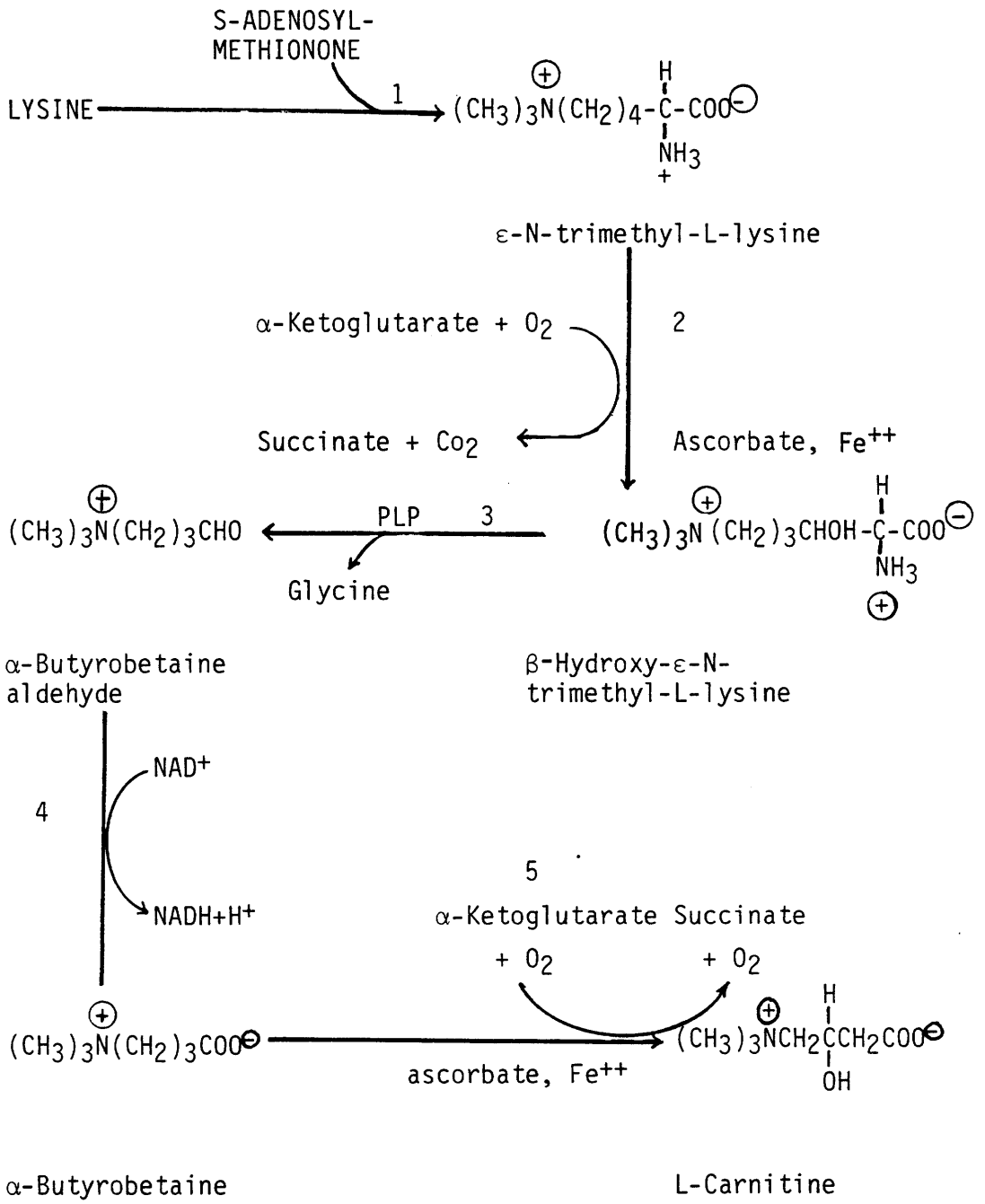

FIGURE 5. Proposed pathway of carnitine biosynthesis (122).

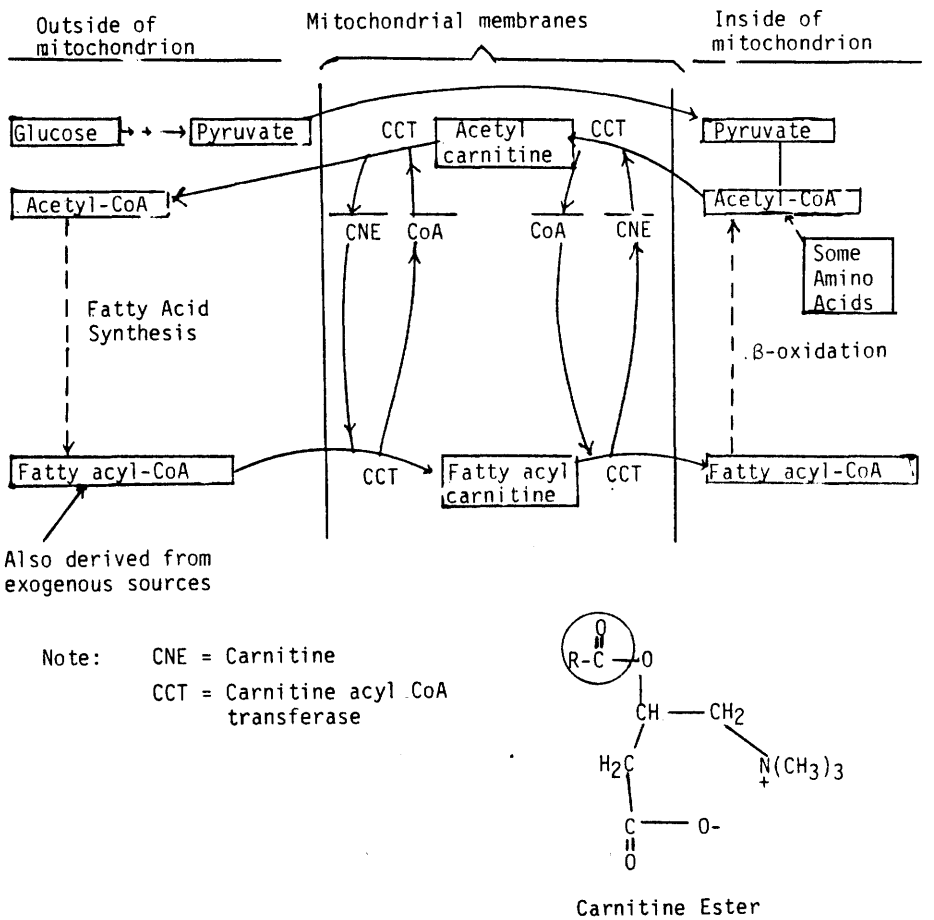

FIGURE 6. The role of carnitine in mediating acetyl and acyl transfers across mitochondrial membranes (49).

recovered with linoleic acid) and in vivo in human liver (52) given alcohol intravenously (3.5 vs 6.4% of $^{14}CO_2$/given dose in ethanol vs control in 5 hours).

Rudman et al. (23) demonstrated a carnitine deficiency in alcoholic cirrhotic patients. In this study, it was reported that the cirrhotic group had significantly lower serum total carnitine concentrations than a normal group (50% of normal concentration; 36 $\pm$ 4 vs 79 $\pm$ 3 μmole/dl) and also significantly lower serum carnitine (free) concentrations (4.9 $\pm$ 3 vs 17 $\pm$ 3 μmole/dl) compared to a normal group. The beneficial effects of dietary D,L-carnitine in ethanol-fed rats on fatty acid oxidation was reported by Hosein and Bexton (24). In this study, it was reported that D,L-carnitine treatment (0.1 mg/g body weight) significantly lowered serum triglycerides (111 $\pm$ 5.2 vs 144 $\pm$ 9.9 mg/dl) in rats which received ethanol (6 g/kg body weight) by gastric intubation. Hepatic triglycerides were also reported to be significantly reduced by carnitine administered at 0.5 mg/g body weight (75 $\pm$ 3.4 vs 92 $\pm$ 6.2 mg/g) in rats injected with ethanol (4 g/kg body weight). This argument is supported by evidence in Lieber's study (30) in which the lipotropic effect of dietary carnitine (1 mg/cal) (114 $\pm$ 17.9 vs 132.6 $\pm$ 24.3 mg/g) on ethanol-induced fatty liver was demonstrated.

CHAPTER III

PROCEDURE

1. EXPERIMENTAL DESIGN

Experiment 1

Thirty male Sprague-Dawley rats with an average body weight of 207 g (189-218 g) were purchased from Harlan Sprague-Dawley, Inc., Indianapolis, Indiana and were divided into five groups of six animals each (Table 2). Animals were individually housed in 7' x 10' x 7" wire mesh stainless steel cages and fed a nutritionally adequate liquid diet (BioMix 711 from BioServ, Inc., New Jersey) containing ethanol as 36% of the total calories (E), or a non-ethanol diet (NE) in which ethanol was substituted isocalorically by maltose-dextrin for 56 days (Table 3).

The ethanol diet was fed either as such or supplemented (W/W) with 1.0% D,L-carnitine (EC); 0.5% L-lysine plus 0.2% L-methionine (ELM); or a combination of the above levels of carnitine, lysine, and methionine (ECLM). The diets were prepared by mixing calculated amounts of solid diet and chemical agents in cold water (4°C) every other day in the morning, stored in the refrigerator, and fed at 4:00 P.M. in stoppered glass feeding tubes (Figure 7, BioServ, Inc., New Jersey). The animals were allowed to adapt to ethanol diets for three days, e.g., the ethanol was given in three increments (0%, 18%, and 36% of total calories during three days before feeding test

TABLE 2. Experimental Design for Experiment 1

Treatment	Group				
	NE	E	EC	ECL	ECLM
Number of Animals	6	6	6	6	6
Ethanol (%, V/V)	-	6.4	6.4	6.4	6.4
D, L-Carnitine (%, W/W)	-	-	1.0	-	1.0
L-lysine (%, W/W)	-	-	-	0.5	0.5
L-methionine (%, W/W)	-	-	-	0.2	0.2

Note. NE = non-ethanol, E = ethanol, EC = ethanol + carnitine, ELM = ethanol + lysine + methionine, and ECLM = ethanol + carnitine + lysine + methionine.

TABLE 3. Bio-Mix #711 Iso-Cal Liquid Rat Diets (Lieber/DeCarli Formula)

	CONTROL	ETHANOL
	----------g/l----------	
PROTEIN		
Casein (Vitamin free)	42.20	42.20
L-cystine	0.50	0.50
DL-Methionine	0.30	0.30
CARBOHYDRATE		
Maltose-Dextrins	118.64	28.90
FAT	39.49	39.59
Corn oil	28.89	28.89
Olive oil	8.59	8.59
Ethyl linoleate	2.14	2.14
Ethanol	0.00	50.0
Vitamin Mix AIN-76	2.01	2.01
Mineral Mix AIN-76	7.05	7.05

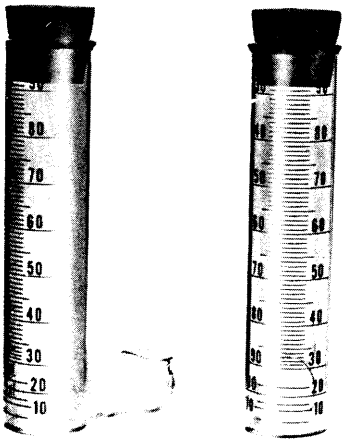

FIGURE 7. Liquid diet feeding tube.

diets). All groups of animals were pair-fed with the unsupplemented ethanol group (E). The experimental design is shown in Table 2.

Experiment 2

Twenty-five male Sprague-Dawley rats born and reared in our small animal facility with an average weight of 120 g (90-130 g), were divided into five groups of five animals each (Table 2). Animals were housed in facilities as described for experiment 1 and were fed the liquid ethanol diets described for experiment 1 (Table 3) for 28 days. The ethanol diet was given as such without supplementation (E_0) or after being supplemented (W/W) with 0.2% (E_1), 0.6% (E_2), 1.0% (E_3), and 2.0% (E_4) D,L-carnitine on a dry weight basis. Preparation and feeding of diets were carried out

in the same manner as in experiment 1. All groups of animals were pair-fed with the unsupplemented ethanol-fed group (E_0). The experimental design is shown in Table 4.

TABLE 4. Experimental Design for Experiment 2

Treatment	Group				
	E_0	E_1	E_2	E_3	E_4
Number of Animals	5	5	5	5	5
Ethanol (%, V/V)	6.4	6.4	6.4	6.4	6.4
D,L-carnitine (%, W/W)	0.0	0.2	0.6	1.0	2.0

Note. E = Ethanol diet.

Experiment 3

For this experiment, male Sprague-Dawley rats were purchased from Harlan Sprague Dawley, Indianapolis, Indiana. Thirty rats with an average body weight of 213 g (196-224 g) were divided into six groups of five animals each and were housed and fed in the same manner as experiment 1 for 45 days. The ethanol diet (Table 5) was fed either as such without any supplementation (E_0), or after being supplemented (W/W) with 0.1% (E_1), 0.4% (E_2), 0.8% (E_3), 1.2% (E_4), and 1.6% (E_5) D,L-carnitine on dry weight basis. All animals were pair-fed with the E_5 group. The experimental design is shown in Table 5.

TABLE 5. Experimental Design for Experiment 3

Treatment	Group					
	E_0	E_1	E_2	E_3	E_4	E_5
Number of Animals	5	5	5	5	5	5
Ethanol (%, V/V)	6.4	6.4	6.4	6.4	6.4	6.4
Levels of D,L-Carnitine supplemented (%,W/W)	0.0	0.1	0.4	0.8	1.2	1.6

Note. E = Ethanol diet.

2. SAMPLE COLLECTION AND ANALYSIS

After the assigned feeding period with the test diets, animals were sacrificed under sodium brevital (Eli Lilly and Co., Indianapolis, Indiana) 16 hours after the last meal on the last day of the experimental period. The blood was collected in heparinized tubes by heart puncture, and plasma was separated by centrifugation at 1500 xg and 4°C for 10 minutes in a refrigerated centrifuge. Various organs such as liver, heart, kidney, testes, skeletal muscle, and brain were excised quickly, weighed, and frozen immediately in a bottle of dry ice. Plasma and tissues were stored in a freezer at -60°C until analyzed. Plasma was used for measuring the concentrations of carnitine (123,124), proteins (125), total lipids (126), and triglycerides (127). Prior to analysis, the liver and other tissues were thawed, kept on ice and minced thoroughly. Representative

aliquots of each tissue were used for determining the concentration of DNA (128), total lipids (126), triglycerides (129), cholesterol (130), phospholipids (131), free fatty acids (132), and carnitine (123,124).

Total lipids in the liver in experiment 1 were determined gravimetrically (137) and by the phospho-vanillin method (126). Results of these two methods were compared.

3. ASSAY PROCEDURE

Carnitines Determination

A. Blood carnitine. The concentrations of plasma carnitine were determined by the radioenzymatic procedure of Cederblad and Lindstedt (123) as modified in our own laboratory (124). Total carnitine content was calculated by adding the experimental values of free carnitine, acid soluble acyl carnitine (ASAC, short chain acyl carnitine), and acid insoluble acyl carnitine (AIAC, long chain acyl carnitine) in each sample. The determination is based on the principle that carnitine is labeled from an enzymatic reaction with labeled acetyl CoA as follows:

$$\text{L-carnitine} + (1\text{-}^{14}\text{C})\ \text{acetyl CoA} \longrightarrow (1\text{-}^{14}\text{C})\ \text{acetylcarnitine} + \text{CoA-SH}$$

The reaction mixture was passed through an anion exchange resin column which trapped out the unreacted acetyl CoA molecules, while

allowing the labeled acetylcarnitine to pass through the column. Thus, the eluate was collected, and the radioactivity was detected in a liquid scintillation counter (Bekmann, LS 100C. Beckmann Instruments, Inc., Fullerton, California).

Reagents

1. Carnitine standard solution (0.5 mM): 9.88 mg of L-carnitine-HCl (Sigma Chemical Co., St. Louis, Missouri) was dissolved in cold glass distilled water (GDW), and the total volume was brought to 100 ml with additional GDW in a volumetric flask. One ml aliquots of the solution were dispensed into plastic tubes and frozen at -70°C. Each aliquot was diluted appropriately for the working standard solution.

2. L-Palmitoyl carnitine standard (22.9 mM): 1.0 ml of GDW was added to a vial containing 10 ml of L-Palmitoyl-carnitine (Sigma Chemical Co., St. Louis, Missouri). Thos solution was diluted appropriately for the working standard solution.

3. Phenol-red indicator (0.1%): 0.1 g of phenol-red was dissolved in 100 ml of absolute ethanol. Prereadied phenol-red tubes were made by adding 1 drop of phenol-red to 12 x 75 mm test tubes and allowed to dry.

4. Radioactive acetyl CoA (50 mCi/mM): 50 uCi of (1-^{14}C) acetyl CoA (Amersham Corp., Chicago, Illinois) were dissolved in 300 ml of cold GDW. Aliquots of 5.0 ml were dispensed into plastic vials and frozen at -60°C.

5. Potassium bicarbonate (2M $KHCO_3$): 20 g of $KHCO_3$ was dissolved in GDW and the total volume was brought up to 100 ml. This reagent was stored in a refrigerator.

6. Acetic anhydride (0.1 M $(CH_3CO)_2O$): 0.5 ml of $(CH_3CO)_2O$ was added to 4.95 ml of cold GDW, mixed, and used immediately for the acetyl CoA solution preparation.

7. Acetyl CoA (0.1 mM): 10 mg of coenzyme A(P-L Biochemicals, Milwaukee, Wisconsin) was dissolved in 0.5 ml of cold GDW. To this solution, 100 µl of 1 M $KHCO_3$ was added. Then, 200 µl of 0.1 M acetic anhydride was added and the volume was brought up to 80 ml with GDW. After mixing, the solution was dispensed into 5 ml volume tubes and frozen at -60°C.

8. Carnitine acetyltranferase (Pigeon Breast Muscle): carnitine-)-acetyltransferase (Sigma Chemical Co., St. Louis, Missouri) was diluted with GDW to 50 Units/ml. This enzyme is abbreviated as CAT in the procedure.

9. MOPS (1M) (3-(4-morpholino) propanesulfonic acid) (Eastman Kodak Chemical Co., Rochester, New York): 20.92 g of MOPS was dissolved in approximately 80 ml of GDW. The pH was adjusted to 7.4 with 4N KOH, and the volume was brought up to 100 ml with GDW.

10. PCA/MOPS-1: 20.9 g of MOPS was added to 50 ml of 6% perchloric acid (PCA), and the volume was made to 100 ml with GDW.

11. PCA/MOPS-2: 20.9 g of MOPS was added to 20 ml of 6% PCA, and the volume was made to 100 ml with GDW.

12. EGTA (0.1 M pH 7.0): 1.902 g of EGTA (Ethyleneglycol-bis(beta-amino-ethylether)N,N'-tetraacetic acid) (Sigma Chemical Co., St. Louis, Missouri) was dissolved in 30 ml of GDW and neutralized with 4N KOH to PH 7.0. The volume was made to 50 ml with GDW in a volumetric flask.

13. Radioactive acetyl CoA mixture (0.1 mM (1-^{14}C)-acetyl CoA solution): 2 volumes of (1-^{14}C) acetyl CoA (#3) were mixed with 1 volume of cold 0.1 mM acetyl CoA (#6) and kept on ice.

14. Reagent mixture (prepared fresh for each assay):

MOPS (1M) buffer	PH 7.4 (#8)	120 µl
EGTA-K (0.1M)	PH 7.0 (#11)	20 µl
$Na_2S_4O_6$ (0.1 M)		20 µl
Ratioactive acetyl CoA (0.1 mM) (#12)		200 µl
GDW		40 µl
	Sum	400 µl

15. Bovine serum albumine (BSA) (8% Fraction V. F. A. poor from ICN Nutritional Biochemicals, Cleveland, Ohio): 4 g of BSA was dissolved into 20 ml of GDW and stirred. The volume was made to 50 ml with GDW in a volumetric flask.

16. Scintillation fluid A: 33 g of PPO(2,5-Diphenyloxazole) and 1 g of POPOP(1,4-Bis(2-(5-phenylexazolyl))benzene) were dissolved in 4 liter of toluene ($C_6H_5CH_3$).

17. Scintillation fluid B: 2 parts of scintillation fluid A (#16) was mixed with 1 part of Triton X-100 (Fisher Scientific Co., Fair Lawn, New Jersey).

Procedure. Carnitine standards were prepared as shown in Table 6. For each sample, 50 µl of plasma was added to 200 µl of 6% PCA contained in a 12 x 75 mm test tube. Since 400 µl was desired for the final volume (to make identical volume as the standard), 150 µl of GDW was added. All test tubes were vortexed and centrifuged in a refrigerated centrifuge (Model J-6B, Beckman Instruments, Palo Alto, California) for 10 minutes at 2900 xg at 0°C. The PCA supernatant was analyzed for free carnitine and ASAC as follows:

1. Free carnitine determination: 150 µl of PCA supernatant was transfered into a 12 x 75 mm phenol red test tube. The contents turned yellowish-orange in color. Forty µl of 2M $KHCO_3$ was added to the tube (golden yellow in color), vortexed, and then set on ice uncovered for 30 minutes, after which it was centrifuged at 2900 xg for 10 minutes. The supernatant was refrigerated until assay.

2. Acid soluble acylcarnitine determination: 100 µl of PCA supernatant was transferred into a 12 x 75 mm phenol red test tube (yellowish-orange in color), 60 µl of 1N KOH was added, and vortexed (purplish-red in color). The tube was incubated for 30 minutes in a water bath for hydrolysis. The contents were neutralized with 20 µl of PCA/MOPS-2. The mixture was vortexed, precipitated by cooling on ice for 30 minutes, and then centrifuged. The supernatant was refrigerated until assay was carried out.

3. Acid-insoluble acylcarnitine determination: The remaining supernatant in the tube was drained off, and the pellet

TABLE 6. Standard Mixtures of Carnitine

0.25 mM Carnitine		0.23 mM L-Palmityl Carnitine		GDW[1]	8% BSA[2]	6% PCA[3]	Total	Total Carnitine
(µl)	(nM)	(µl)	(nM)	(µl)	(µl)	(µl)	(µl)	(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

[1]Glass distilled water.

[2]Bovine serum albumin.

[3]Perchloric acid.

was washed with additional 400 µl of 6% PCA. The supernatant was totally drained off with care until a dried pellet was obtained. After adding 1 drop of phenol red indicator on the pellet, 100 µl of 0.5N KOH was transferred into the tube. The tube was vigorously vortexed for several minutes until the pellet was completely dissolved in KOH (purplish-red in color). The tube was incubated at 65°C in a shaking incubator (100 SPM) for 60 minutes for hydrolysis. After hydrolysis, the contents were neutralized with 65 µl of PCA/MOPS-1 (light-yellow in color). The contents were vortexed and precipitated by cooling on ice for 30 minutes. The tube was centrifuged at 2900 xg for 10 minutes at 0°C and refrigerated until the assay was carried out.

4. Carnitine assay: 100 µl of supernatant from each of the three fractions: free carnitine, ASAC, and AIAC, were transferred into separate Eppendorf tubes. Four hundred µl of fresh reagent mixture was added to each tube, and 20 µl of enzyme, carnitine acyltransferase were added. The mixture was mixed by gentle tapping and then incubated at 37°C in a shaking water bath. At the end of the incubation period, 200 µl of incubation mixture was transferred on to a minicolumn (minicolumn was made by using 5-3/4" pasteur pipettes (Fisher Scientific Co., Fair Lawn, New Jersey) with a small glass wool stuffing inside the pipette above the tip, and anion exchange resin, AGI-X8, 200-400 mesh, Cl-form (Bio-Rad Laboratories, Richmond, Acl) was added up to the 9 cm mark (measured from the tip of the pipette). After the mixture was absorbed into

the resin, the column was washed with 2 portion of 500 µl of GDW into a scintillation vial (New England and Nuclear, Boston, Massachusetts). To each scintillation vial, 10 ml of scintillation fluid B was added, and the vial was capped and mixed by swirling. Radioactivity of the mixture was counted in a LS-100C Liquid Scintillation Counter (Beckman Instruments, Irvine, California). After the counts per minute (CPM) were recorded for all three fractions (free carnitine, ASAC, and AIAC), the CPM of the blank was subtracted from that of each standard fraction and from that of each sample in that fraction (corrected CPM). A standard curve was plotted by using a regression line which was calculated by a programmed least-squares method (Figure 8). The contents of carnitine for each of the three fractions was calculated as follows:

$$\text{carnitine (nmole/ml)} = \left(\frac{\text{corrected CPM} \pm b}{M}\right) \times \text{D.F.}$$

where:

b = Y-intercept in standard curve,

M = slope in standard curve,

D.F. = appropriate dilution factor.

Total carnitine content for each sample was calculated by adding the values of free carnitine, true ASAC, and AIAC, in each sample. The value of true ASAC was calculated as: true ASAC = ASAC - free carnitine. This calculation was based on the principle that when the ASAC fraction was hydrolyzed by KOH, both free carnitine and

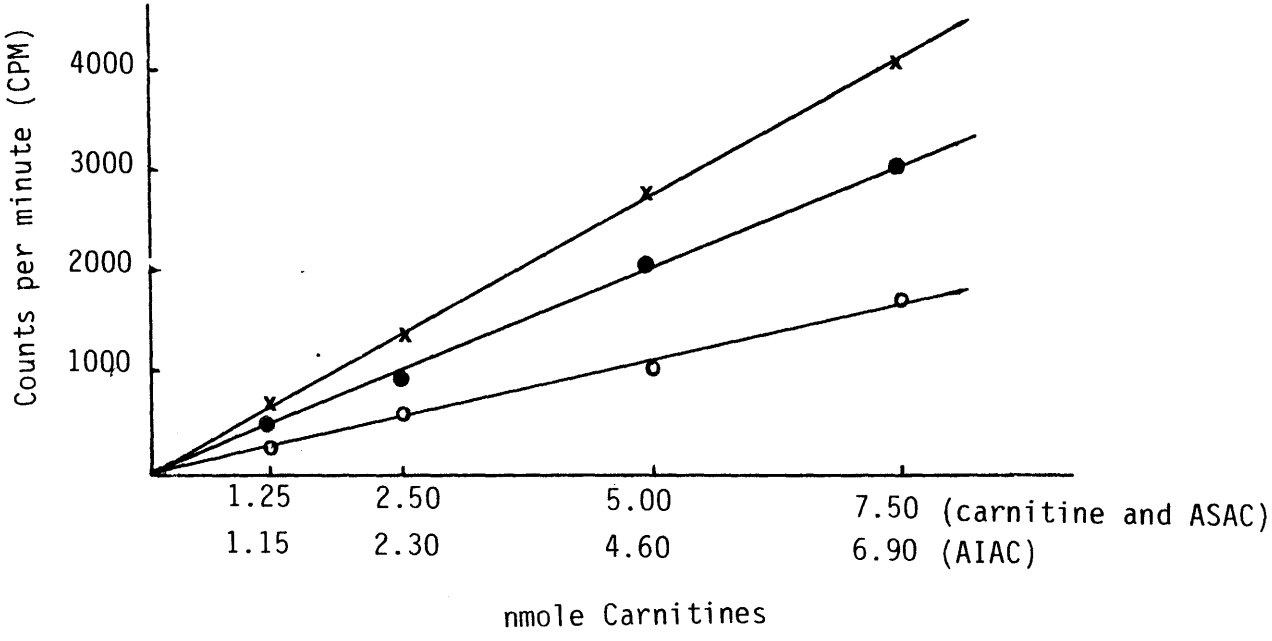

FIGURE 8. Standard curves for carnitine (CNE, -o-), acid soluble acyl carnitine (ASAC, -o-), and acid insoluble acyl carnitine (AIAC, -x-) obtained with carnitine and palmitoyl-carnitine in a LS-100C Liquid Scintillation Counter.

ASAC were liberated in that mixture. Therefore, the equation for the calculation of total carnitine is expressed as: total carnitine = free carnitine + true ASAC + AIAC.

B. Tissue carnitine determination. This procedure is different from plasma carnitine determination only in the treatment of tissue for carnitine extraction. One hundred mg of tissue was transferred into 1 ml of ice cold 3% PCA in a ground glass homogenizer and homogenized while on ice. The homogenate was transferred into a small plastic vial and homogenizing vessel was rinsed with 2 portions of 1 ml of 3% PCA into the vial (final volume = 3 ml). The contents were centrifuged at 4000 rpm for 10 minutes at 4°C. Assigned aliquots of supernatant were used for the determination of free carnitine and ASAC fraction (150 µl for free carnitine and 100 µl for ASAC), and the pellet was used for the determination of ASAC. After this step, the procedure was the same as for plasma carnitine determination.

Protein Determination

Total serum protein was determined by a conventional biuret technique described by Gornall et al. (125).

Reagents.

1. Biuret reagent: 90 grams of Rochelle salt (NaK tartarate-$4H_2O$) (Sigma Chemical Co., St. Louis, Missouri) was dissolved in about 400 ml of standard 0.2 N NaOH solution, and 10 gm of $CuSO_4 \cdot 5H_2O$ was added to it. To the solution, 10 g of KI was added, and the volume was made up to 2 liters with 0.2 N NaOH.

2. Standard serum albumin: 10 grams of crystalline bovine serum albumin (ICN Pharmacenticals, Inc., Life Sciences Group, Cleveland, Ohio) was dissolved in 100 ml of 0.9% saline. A working standard was made by diluting the stock solution to one tenth of the original concentration.

Procedure. The serum specimen (0.2 ml) was added to a test tube (1.0 x 15 cm), and the volume was made up to 5.0 ml with GDW. A reagent blank was prepared by adding GDW in place of the specimen. A standard curve was plotted by using 5 volumes (0.1, 0.2, 0.4, 0.6, and 0.8 ml) of standard serum albumin in place of the specimen. After the volumes were made up to 5.0 ml by adding GDW, all the tubes were vortexed, and 5.0 ml of biuret reagent was added to all tubes. After standing for 30 minutes at room temperature, the absorbance was measured at 540 nm against reagent blank. The length of light path of the spectrophotomeric cell used for these studies was 1 cm. A standard curve is shown in Figure 9.

Calculations. The calculations are as follows:

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

where:

Au = absorbance of unknown,

As = absorbance of standard,

Cs = concentration of standard,

D.F. = appropriate dilution factor.

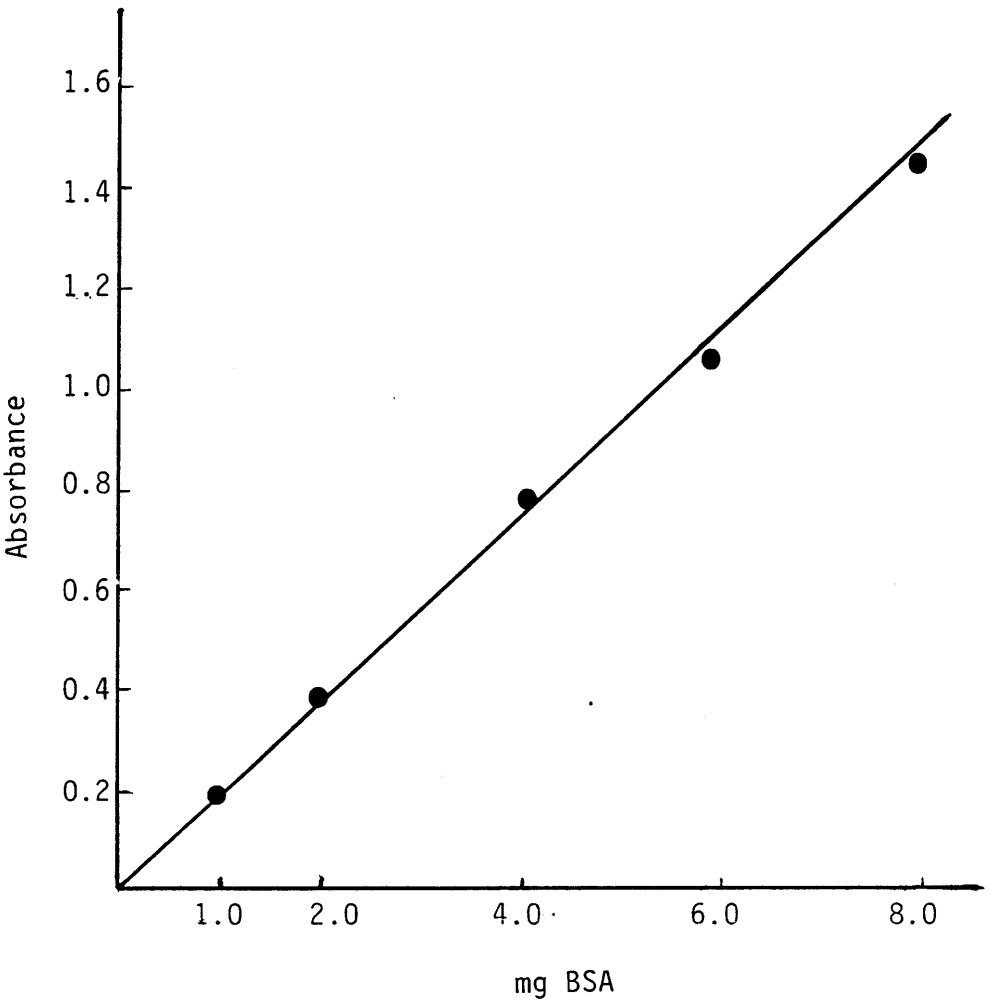

FIGURE 9. Standard curve for serum protein obtained with bovine serum albumin (BSA).

Plasma Total Lipid Determination

Total lipid contents were measured by the phosphoric acid-vanillin reaction method (126). In this method, the lipid specimen is heated with concentrated sulfuric acid; then vanillin and phosphoric acid are added to yield a pink colored product. The chemical reactions that form the basis of this method remains unknown (133). One assumption that has been stated in several published papers is that the unsaturated components of a lipid specimen first oxidize to ketones and then under the influences of acid catalysis the ketones condense with vanillin to a derivative of vanillin. Following this condensation reaction, dehydration of an aldol-type intermediate is assumed to yield a more highly unsaturated product that absorbs visible light.

Reagents. Phosphoric acid-vanillin reagent was made by dissolving 1.0 g of vanillin in 160 ml of distilled water in a 500 ml volumetric flask. Concentrated phosphoric acid was added to the mark and mixed thoroughly. A stock standard was made by mixing 1.0 g of olive oil in minimum volume of chloroform in a 100 ml volumetric flask, and the mixture was diluted to the mark with additional chloroform and mixed thoroughly. This solution was stoppered tightly and stored in a freezer (-20°C). A working standard was made by diluting the stock solution to one tenth of the original concentration. One 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 tightly and refrigerated.

Procedure. Two hundred µl of serum was transferred to each specimen tube and 200 µl of water to the blank tube; several assigned amounts (50, 100 and 200 µl) of working standards were transferred into some other tubes; the chloroform was evaporated off with a stream of nitrogen; and then 200 µl of GDW was added to the standard tubes. Five ml of concentrated H_2SO_4 was added to all the tubes, mixed immediately and thoroughly with a vortex mixer. The tubes were stoppered tightly and heated at 100°C in a heating block for 10 minutes. The tubes were allowed to cool to room temperature, and then 200 µl of the contents of each tube were transferred to small tubes (12 x 75 mm). Phosphoric acid-vanillin reagents (3.0 ml) was added to each tube and mixed thoroughly with a vortex mixer. All tubes were allowed to stand in a dark cabinet for 60 minutes. Absorbances were read at 520 nm against a reagent blank in a Beckman spectrophotometer (Model 34, Beckman Instruments, Fullerton, California). A standard curve is shown in Figure 10.

Calculations. The calculations are as follows:

$$\text{total lipids (mg/dl)} = Au/As \times Cs \times 500$$

where:

Au = absorbance for the specimens,

As = absorbance for the standards,

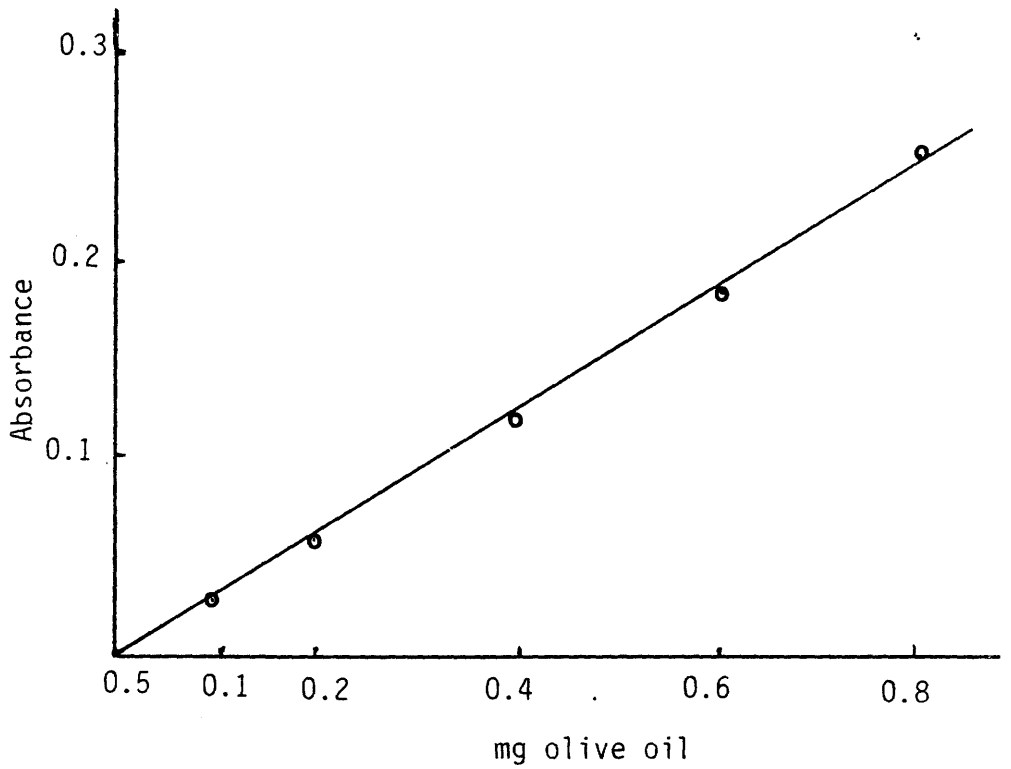

FIGURE 10. Standard curve for total lipid obtained with olive oil at 520 nm.

Cs = concentrations of standard,

500 = appropriate dilution factor (200 µl x 500 = 100 ml).

Tissue Total Lipid Determination

Tissue (0.1 g) was transferred into a ground glass homogenizer containing 1.0 ml of chloroform-methanol mixture (2:1, V/V) and hand homogenized while keeping the homogenizer in ice water. The homogenate was transferred into a small plastic vial, and the homogenizing vessel was rinsed with two portions of 1.0 ml of chloroform-methanol mixture (final volume = 3 ml). The contents were centrifuged at 2900 xg for 10 minutes at 4°C, and the supernatant refrigerated until analyzed.

Procedure. Two hundred µl of homogenate supernatant was transferred to each specimen tube and 200 µl of water to the blank tube. Several assigned amounts (50, 100, and 200 µl) of standard were transferred to some other tubes. The chloroform of both specimen and standard tube were evaporated off with a steam of nitrogen, and 200 µl of water was added. The next step was carried out in the same manner as for the determination of plasma total lipids.

Plasma Triglycerides Determination

The contents of triglyceride in plasma were measured by the method of Giegel et al. (127). The procedure is based on the principle that lipids are partitioned between water/isopropanol

and noname phases with the triglycerides extracted into the nonane; glycerol is liberated from the triglycerides by sodium hydroxide and oxidized to formaldehyde by periodate; and the formaldehyde reacts with 2,4-pentanedione to form 3,5-diacetyl-1,3-dihydrolutidine, the colored compound.

Reagents.

1. Extraction reagent: n-Nonane/isopropanol (2.0/3.5 by volume).

2. Triolein standard: triolein* 100 mg/dl in extraction reagent (1.13 mM/liter) *(Sigma Chemical Co., St. Louis, Missouri).

3. Dilute sulfric acid, 40 mM/liter.

4. Transesterifying reagent: NaOH in isopropanol (100mM/liter).

5. Oxidizing reagent: sodium periodate, 18 mM/liter in 2.0 M/liter acetic acid.

6. Color buffer: ammonium acetate (6.0 M/liter, PH 6.0 at 25°).

7. Working color reagent: 4.0 ml of acetylacetone was added to 100 ml of the color buffer; shaked vigorously; allowed to stand for at least 15 minutes before use. Fresh solution was made for every batch of analysis.

Procedure. The specimen (0.2 ml) was extracted with a mixture of 5.0 ml of extraction reagent in a screw-capped test tube (16 x 100 mm, #986), and 1.0 ml of dilute sulfuric acid was added to the tube. A reagent blank was prepared by adding water in place of the specimen. A standard curve was plotted by using three

volumes (0.1, 0.2, and 0.4 ml) of standard in place of the volume of extraction reagent. All tubes were centrifuged at 100 rpm for 10 minutes to obtain a clear upper phase. An aliquot (0.5 ml) of each extraction upper phase was mixed with 0.5 ml of transesterifying reagent and allowed to stand for five minutes at room temperature. Oxidizing reagent (0.5 ml) was added to each tube, and the mixture was allowed to stand for at least two minutes at room temperature. A two-phase system was developed at this point. Six ml of working color reagent was added to each tube and mixed. 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.

Calculations. The calculations are as follows:

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

where:

Au = absorbance of unknown,

As = absorbance of standard,

Cs = concentration of standard,

D.F. = appropriate dilution factor.

Tissue Triglycerides Determination

Tissue triglycerides were measured by a spectrophotometric method described by Sardesai and Manning (129). This procedure is based on the principle that involves extraction and saponification

of triglycerides, the oxidation of glycerolmoiety to formaldehyde, and the conversion of aldehyde to a color compound, 3,5-diacetyl-1-4-dihydrolutidine.

Reagents.

1. Chloroform-methanol mixture: two parts of chloroform in one part of methanol (V/V).

2. Silicic acid mixture: one part of silicic acid (Sigma Chemical Co., St. Louis, Missouri) mixed with one part of Hyflo Super-Cel (Sigma Chemical Co., St. Louis, Missouri). The mixture was activated by heating in an oven at 100°C overnight.

3. Alcoholic potassium hydroxide: 2 g of KOH was dissolved in minimum amount of water and diluted to 100 ml with 95% ethanol.

4. Sulfuric acid, 0.2 N.

5. Sodium arsenite, 0.05 N.

6. Acetyl acetone reagent, PH 6.0 at 25°C: ammonium acetate (150 g), 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were added in GDW and diluted to 1 liter (kept in a refrigerator).

7. Triglycerides stock standard: one gram olive oil was dissolved and diluted to 100 ml with chloroform and kept in a refrigerator.

8. Sodium metaperiodate, 0.05 N.

Procedure. Procedure one was the calibration curve. Working standards ranging from 10 to 250 µg/ml (10, 50, 100, 150, 200, and 250) were made from the stock standard by dilution with chloroform.

One ml of each of the standards was transferred into a 16 x 100 mm test tube. One ml of chloroform was pipetted into another test tube (blank). Chloroform was evaporated from the tubes in a boiling water bath for 10 minutes; subsequently, 0.5 ml of alcoholic potassium hydroxide was added, and all tubes were incubated 15 minutes in a 60°C water bath. After incubation, 0.5 ml of 0.2 N sulfuric acid was added to each tube followed by 0.1 ml of sodium metaperiodate (to oxidize the glycerol released on saponification). After exactly 10 minutes, the oxidation was stopped by adding 0.1 ml of sodium arsenite. After 5 minutes (to allow free iodine to escape), 0.8 ml of GDW was added to each tube, followed by 2 ml of acetyl acetone reagent, and mixed thoroughly. The mixture was incubated in a water bath at 58°C for 10 minutes, cooled to room temperature and the optical densities were determined at 412 nm against reagent blank. Standard curve is shown in Figure 11.

Procedure two was with the tissue. Preminced tissue (500 mg) was accurately weighed and homogenized with 2 ml of saline in a ground glass homogenizer. The homogenizing vessel was rinsed with an additional two portions of saline (2 and 1 ml), and final volume was made up to 5 ml. Aliquote of homogenate (0.5 ml) was transferred into a 16 x 100 mm test tube, and 9.5 ml of extraction solvent (chloroform-methanol mixture) was added. The mixture was filtered through nonlipid-containing filter paper (ether treated) into a 60 ml separatory funnel containing 10 ml of GDW and shaken vigorously. After two layers separated, the lower layer was passed

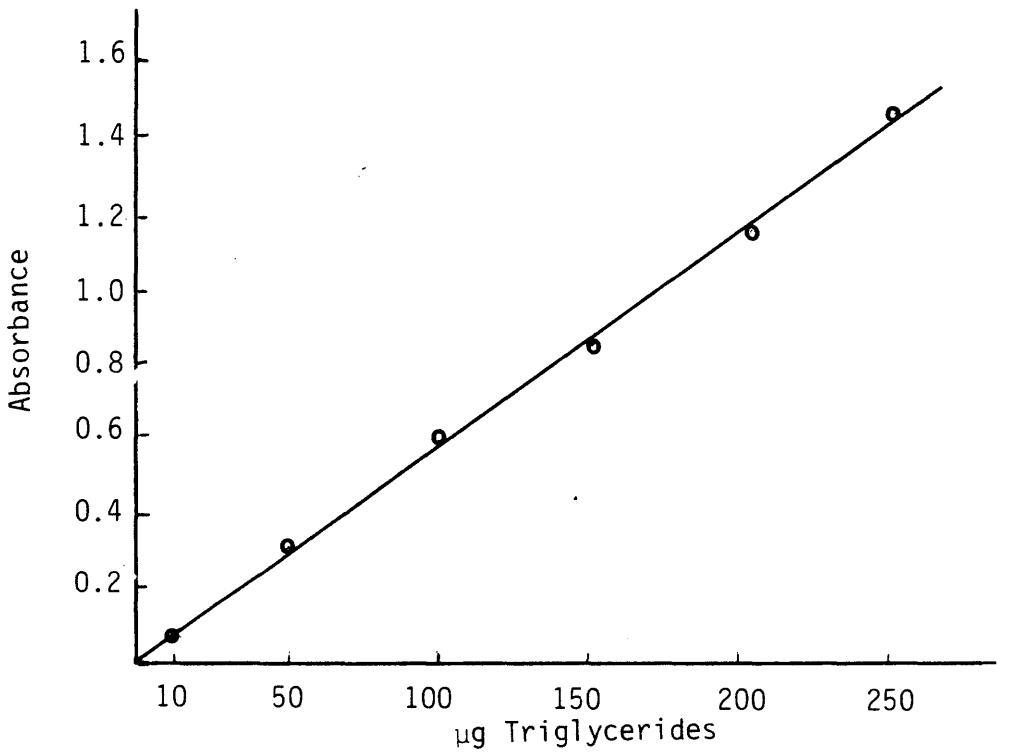

FIGURE 11. Standard curve for triglyceride obtained with trioleine at 412 nm.

through a column (column was made by using 1 cm I.D. x 30 cm glass column, 0.5 g of activated silicic acid mixture was packed into the column, and wash with 5 ml of chloroform). The eluate was collected into a 10 ml volumetric flask and the column washed with additional chloroform until eluate was up to the 10 ml mark. An aliquot (3 ml) of eluate was transferred into a test tube (made 3 test tubes: 2 of them were duplicated for saponified samples and the other one was for unsaponified sample). All test tubes were incubated in a heating block at 100°C for 10 minutes. Subsequently, 0.5 ml of alcoholic potassium hydroxide was added to the first two test tubes (saponified), and 0.5 ml of 95% ethanol was added to the other test tube (unsaponified). After this step, the procedure was carried out as described for the preparation of the calibration curve. The absorbance of the saponified sample minus that of the unsaponified represented the absorbance of the triglycerides in the amount of the sample analyzed.

DNA Determination

Deoxyribonucleic acid contents in each sample were measured by a modified method of Ceriotti (128).

Reagents.

1. PCA, 0.6 N (Fisher Scientific Co., Fair Lawn, New Jersey): 25.7 ml of 70% PCA was diluted to 500 ml of GDW.

2. PCA, 0.2 N: 17.2 ml of 70% PCA was diluted to 1 liter with GDW.

3. PCA, 1.2 N: 51.5 ml of 70% PCA was diluted to 500 ml with GDW.

4. KOH, 0.3 N (Fisher Scientific Co., Fair Lawn, New Jersey): 8.42 g of KOH was added to minimum amount of GDW and diluted to 500 ml.

5. KOH, 0.1 N: 2.81 g of KOH was dissolved in minimum amount of GDW and diluted to 500 ml.

6. Indole, 0.04% with 60 μmole $CuSO_4$ (Fisher Scientific, Pittsburgh, Pennsylvania): 40 mg of indole was added to a 100 ml volumetric flask and 25 ml of GDW was followed. The flask was incubated in a boiling water bath for one hour. At the end of incubation, 0.06 ml of 0.1 M $CuSO_4$ was added, and the volume was made up to 100 ml with GDW. The solution was stored in a refrigerator.

Procedure. A portion of liver (0.3 g) was homogenized with 2 ml of cold GDW in a ground glass homogenizer and rinsed with an additional 2 ml of cold GDW two times (total volume was 6 ml). An aliquot (5 ml) of homogenate was transferred into round bottom tubes in duplicate. To each tube, 2.5 ml of cold PCA was added and mixed with vortex mixer (caution was taken to avoid precipitation on the walls of tubes). The tubes were allowed to stand on ice for 10 minutes. The mixture was centrifuged at 1500 xg for 15 minutes, and the supernatant was discarded. The precipitate was washed with 5 ml of cold 0.2 N PCA, mixed, and centrifuged for 12 minutes at 1500 xg for 12 minutes, and the supernatant was carefully

discarded. The washing was done twice. The precipitate was dried by inverting the tubes over filter paper. To the tubes, 5 ml of 0.3 N KOH was added, and the tubes were capped and incubated at 50°C in a water bath (vortexed every 15 minutes). The mixture was transferred into a 50 ml volumetric flask. To the flask, 12 ml of 0.3 N KOH was added, and the volume was made to 50 ml with GDW. A 2.0 ml aliquot of the extract was added to a test tube (1.0 x 15 cm), and 1.0 ml of indole solution was subsequently added and mixed. To the mixture, 1.0 ml of concentrated HCl was added, and it was mixed again. The tube was capped and incubated in a boiling water bath for 10 minutes. The mixture was cooled on ice for 10 minutes. To the tube, 4.0 ml of chloroform was added, and the tube was capped and shaken vigorously for 15 seconds. The mixture was centrifuged at 1500 xg for 5 minutes. The pink layer at the bottom was aspirated from the tube to another test tube (without losing yellow colored DNA). After repeating the steps from addition of chloroform to aspiration, the tube was allowed to stand at room temperature for 30 minutes. The yellow colored water phase was separated, and the absorbance was measured at 490 nm against GDW as a blank. A standard curve was plotted by using 5 volumes (0.1, 0.2, 0.4, 0.6, and 0.8 ml) of standard in place of sample. A standard curve is shown in Figure 12.

Calculations. The calculations are as follows:

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

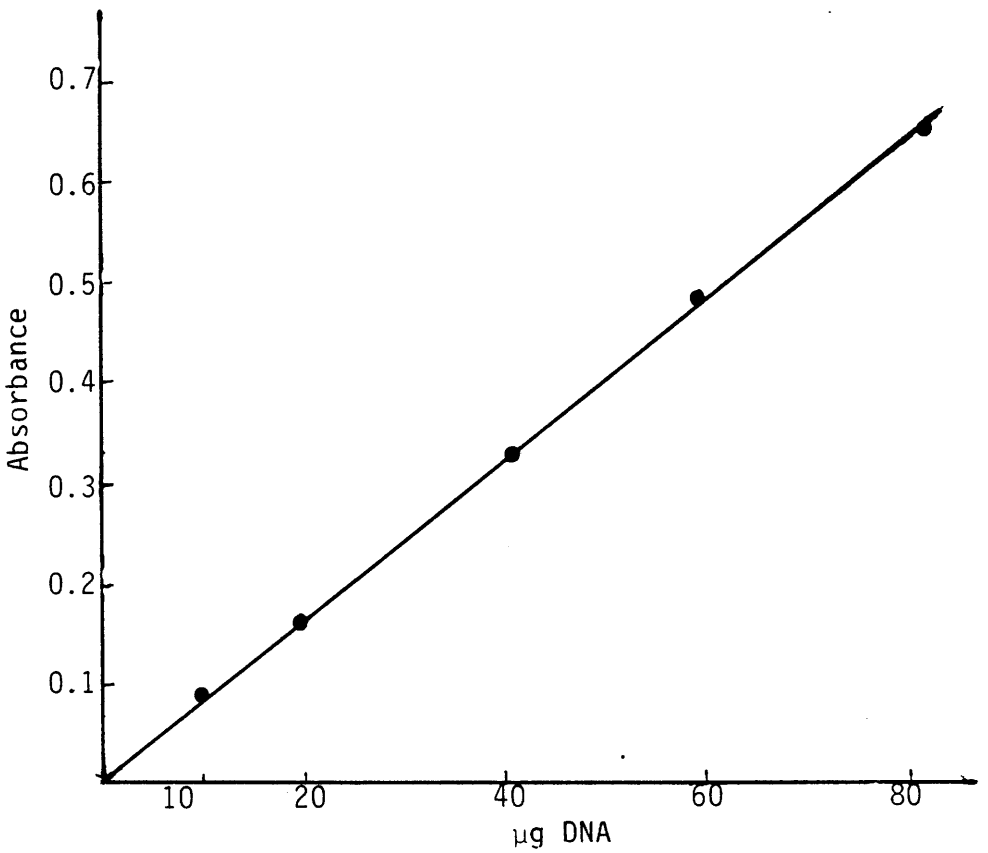

FIGURE 12. Standard curve for DNA obtained at 490 nm.

where:

Au = absorbance for the unknown,

As = absorbance for the standards,

Cs = concentrations of standards

D.F. = appropriate dilution factor.

Cholesterols Determination

All classes of cholesterol of serum and tissue were measured by the method of Searcy and Berquist (130). This procedure is based on the principle that an orange color is formed when cholesterol and a solution of $FeSo_4$ in glacial acetic acid is treated with H_2SO_4; the colored product can be quantitated at a wave length of 490 nm.

Reagents.

1. $FeSO_4$-acetic acid reagent: a saturated solution was prepared by adding several grams of $FeSO_4 \cdot 7\ H_2O$ to 1 liter of reagent glacial acetic acid. After a few minutes of mixing, a clear solution was obtained by filtration.

2. Digitonin solution (5%): in a 200 ml volumetric flask, 1.0 g of digitonin was dissolved in 100 ml of absolute ethanol at 60°C. After addition of 2.0 ml of 10% acetic acid, the mixture was diluted to volume with distilled water.

3. Acetone/absolute ethanol: equal parts (V/V) were mixed.

4. Cholesterol standard: a 60 mg/100 ml standard was prepared by dissolving 120 mg of purified cholesterol in 200 ml

of acetone ethanol. The solution was kept tightly stoppered in a freezer and renewed frequently.

Procedure. Total cholesterol, a 1 to 10 dilution was made by adding 0.1 ml of specimen to 0.9 ml of acetone/ethanol. The mixture was stoppered, mixed well, and centrifuged at 1600 xg for five minutes. A 0.4 ml of aliquot of the clear supernatant was placed in a test tube (10 x 150 mm) and diluted with 6.0 ml of $FeSO_4$ reagent. A 2.0 ml portion of H_2SO_4 was added to the mixture and mixed immediately using a vortex mixer to obtain uniformity. Ten minutes were allowed to elapse before colorimetric measurement. The absorbance were measured by a Beckman spectrophotometer (Model 34, Beckman instruments, Fullerton, California) at a wavelength of 490 nm against a reagent blank prepared with 0.4 ml of acetone/ethanol. A standard curve is shown in Figure 13.

Calculations. The calculations are as follows:

$$\text{Total cholesterol (mg/dl)} = \frac{Au}{As} \times C_S \times \text{D.F.}$$

where:

Au = absorbance of unknown,

As = absorbance of standard,

Cs = concentration of standard,

D.F. = appropriate dilution factor.

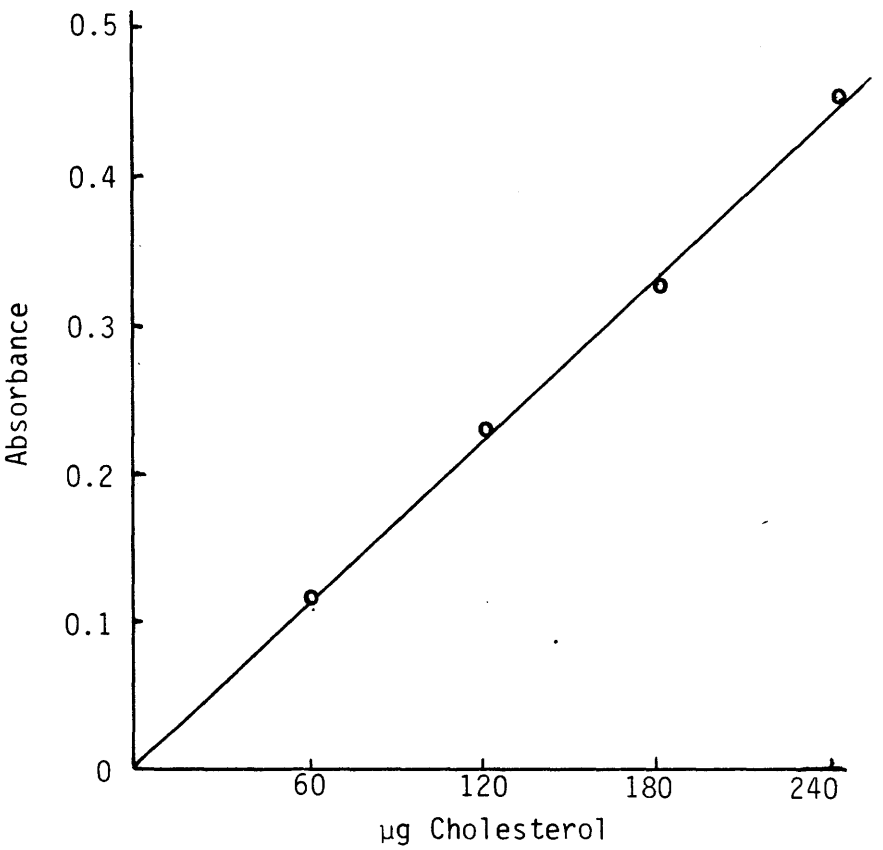

FIGURE 13. Standard curve for cholesterol obtained with cholesterol at 490 nm.

Free cholesterol. A 1.0 ml aliquot of specimen was placed in a conical centrifuge tube and mixed well with 1.0 ml of digitonin solution. After a 1 hour precipitation period, the mixture was centrifuged at 1500 xg for 10 minutes. The supernatant was discarded and the precipitate was suspended in acetone-ether. The mixture was centrifuged again at 1600 xg for 10 minutes, and the supernatant was carefully discarded. The cholesterol precipitate was then dissolved in 6.0 ml of the $FeSO_4$ reagent; the mixture was incubated at 37°C for 5 minutes in a water bath to dissolve the digitonide. The solution was cooled to room temperature before mixing thoroughly with 2.0 ml of H_2SO_4. The reaction mixture was transferred to a spectrophotometric cell and the absorbance measured in a Beckman spectrophotometer at 490 nm against a reagent blank.

Calculations. The calculations are as follows:

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

where:

Au absorbance of unknown,

As absorbance of standard,

Cs concentration of standard,

D.F. appropriate dilution factor.

Esterified cholesterol contents were calculated by subtracting the values of free cholesterol from those of total cholesterol in each sample. The equation is expressed as: esterified cholesterol = total cholesterol - free cholesterol.

Phospholipid Determination. The phospholipid content of each sample was measured by a colorimetric method described by Connerty et al. (131). A trichloroacetric acid (TCA) filtrate of plasma or tissue was treated with ammonium molybdate which reacts with phosphate to form ammonium molybdophosphate. This is thought to have the formula $(NH_4)_3 [PO_4(MoO_3)_{12}]$. The addition of a suitable reducing agent such as Elon (P-methyl aminophenol) produces a blue color of heteropolymolyldenum blue.

Reagents.

1. Digestion mixture: a 50 ml of GDW was mixed with 25 ml of concentrated H_2SO_4 and 25 ml of 70% perchloric acid.

2. A 2.5% aqueous ammonium molybdate solution.

3. Elon solution: one gram of Elon (P-methyl aminophenol sulfate) was dissolved in 100 ml of a 3% solution of sodium bisulfite.

4. Trichloroacetic acid (TCA), 5% W/V concentrations.

5. Sodium acetate, 50% W/V solution.

6. Phosphorus standard (1 mg/ml of P): a 4.394 g of dried potassium dihydrogen phosphate (KH_2PO_4) was dissolved in sufficient water to make 1 liter and acidified with 2 ml of concentrated H_2SO_4 as a preservative before diluting to the mark.

7. Working standard: One ml of the phosphorus standard was diluted to 250 ml with GDW (1 ml = 4 µg of P).

Procedure. A specimen (0.2 ml) was transferred to a 16 x 150 mm glass-stoppered 10 ml graduated tube. Five ml of 5% TCA was added drop by drop, mixing by lateral shaking, and then centrifuged at 1100 xg for 10 minutes until the precipitate was tightly packed. The supernatant fluid was decanted and discarded and the tube was inverted over filter paper until all the droplets of fluid adhering to the wall of the tube was drained away. One ml of digestion mixture was added along with a small glass bead to the protein-phospholipid precipitate and heated at 200°C for 15 minutes until a colorless (or faint yellow) digest was obtained (one-half hour was sufficient). The tubes were cooled for 2 minutes, 1 ml of GDW was added cautiously, and heated to boiling for 15 seconds to convert any pyrophosphoric acid into orthophosphoric acid. One ml of 50% solution of sodium acetate was added and GDW up to the 10 ml mark. One ml of 2.5% solution of ammonium molybdate was added and mixed with vortex mixer. One ml of Elon reagent was added and thoroughly mixed again with a vortex mixer. The tubes were allowed to stand for 15 minutes and the optical densities were measured at 700 nm against a reagent blank. The reagent blank was made by mixing 0.2 ml of concentrated H_2SO_4, 1 ml of 50% solution of sodium acetate, 1 ml of 2.5% solution of ammonium molybdate, 1 ml of Elon reagent, and 8.75 ml of GDW. A standard was prepared by mixing 20 µg of phosphorus (0.5 ml of dilute standard) to the same mixture as the blank. Standard curve is shown in Figure 14.

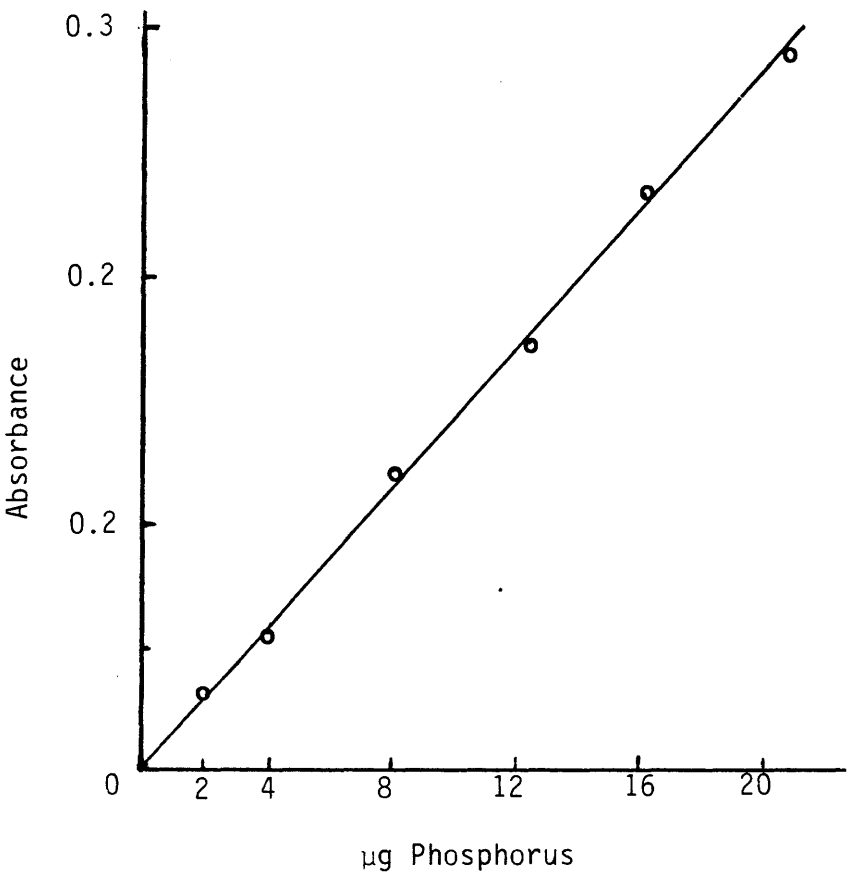

FIGURE 14. Standard curve for phospholipid obtained with phosphorus at 690 nm.

Calculation. The standard contains 20 μg of phosphorus and is compared with the phospholipid-phosphorus present in 0.2 ml of serum.

$$\text{Phospholipids (mg/dl)} = Au/As \times Cs \times 25 \times D.F.$$

where:

Au = absorbance of unknown,

As = absorbance of standard,

Cs = concentration of standard,

25 = conversion factor for the fact that phosphorus constitutes about 4% of average weight of phospholipids.

D.F. = appropriate dilution factor.

Free Fatty Acid Determination

The content of free fatty acid (FFA) of each sample was measured by a colorimetric ultramicro method described by Novak (132). The method is based on the color reactions of copper soaps. Cobalt forms a soap with FFA that gives a color reaction with alpha-nitroso-bata-naphthol (134). The absorbance was read at 500 nm in the Beckman spectrophotometer against the reagent blank.

Reagents.

1. Solution A (Cobalt nitrate-acetic-potassium sulfate): 6.0 g of $Co(NO_3)_2 \cdot 6H_2O$ and 0.8 ml of glacial acetic acid were added to a solution of K_2SO_4 (saturated while boiling, stored in contact

with excess crystals and filtered before use) go give a total volume of 100 ml at 37°C.

2. Solution B (saturated Na_2SO_4 solution): sodium sulfate was added to boiling water until saturated, and kept at 37°C overnight.

3. Cobalt reagent (triethanolamine, 1.35 volume). this was made up to 10 volumes with solution A. Solution B (7 times the volume of triethanolamine) was added to this and the mixture was shaken. The reagent is not stable so that fresh solution was prepared for every batch of analysis. Solutions A and B were kept at 37°C.

4. Indicator (stock solution, 0.4% alpha-nitro-beta-naphththol in 96% ethanol): this was diluted with ethanol by a factor of 12.5 before use.

5. Extraction mixture: was prepared according to Dole (135) in the procedure (isopropyl alcohol: heptane: 1N H_2SO_4 = 40:10:1, V/V).

6. Chloroform-heptane, 5:1 (V/V).

Procedure. A 50 µl portion of sample was added to 250 µl of Dole's extraction mixture in an Eppendorf tube. The contents were mixed with vortex mixer (care being taken not to allow them to reach the stopper). The tubes were cooled for 10 minutes in a bath of melting ice. A 300 µl volume of heptane was added; 500 µl of GDW followed; the contents were thoroughly mixed with a

vortex mixer. After two phases had separated, 300 µl was drawn from the upper heptane phase and transferred to another Eppendorf tube. A 400 µl portion of chloroform-heptane was added, followed by 500 µl of cobalt reagent, and the contents were thoroughly mixed by vortexing for 3 minutes. The mixture was centrifuged for 15 minutes at 1160 x g, and 600 µl of the upper chloroform/heptane phase was transferred to another Eppendorf tube containing 750 µl of indicator solution. For the blank and standard, 50 µl of GDW and standard respectively, instead of serum, were carried through the operation. Two standards (palmitic acid in Dole's extraction mixture, 0.2 and 0.4 meq/liter) were prepared. After 30 minutes standing, absorbance was read at 500 nm in a Beckman spectrophotometer. A standard curve is shown in Figure 15.

Calculations. The calculations are as follows:

$$FFA\ (mg/dl) = Au/As \times Cs \times D.F.$$

where:

Au = absorbance of unknown,

As = absorbance of standard,

Cs = concentration of standard,

D.F. = appropriate dilution factor.

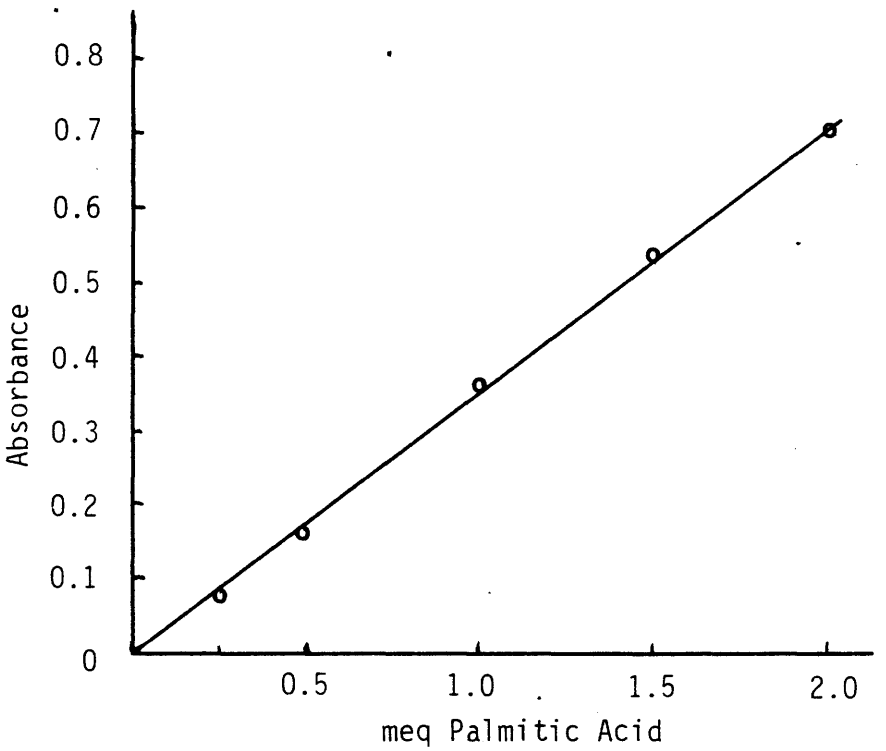

FIGURE 15. Standard curve for free fatty acid obtained with palmitic acid at 500 nm.

4. STATISTICS

All data were expressed as mean $\pm$ SEM.* The data were analyzed by using Analysis of Variance and Duncan's Multiple Range Test for statistical significance at $P<0.05$ (136).

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*SEM = standard error of mean.

CHAPTER IV

RESULTS

1. EXPERIMENT I

The effects of carnitine and its precursors, namely lysine and methionine, on ethanol-induced hepatic steatosis were examined in three groups of healthy rats for an experimental period of 56 days and were compared with either an unsupplemented ethanol group of rats or an unsupplemented nonethanol group of animals.

Unsupplemented ethanol-fed animals (E) consumed significantly more food than non-ethanol (NE) animals or supplemented ethanol-fed animals (Table 7). Supplementation with carnitine and its precursors significantly decreased daily food consumption, which was attributed partly to poor intake during the first week of the feeding period. The relative gain in body weight was NE>E>EC>ELM>ECLM (Table 6). The weight gain for ELM and ECLM were significantly lower than for the NE and E groups. However, gains in body weight (g) per unit volume (ml) of diet consumed were fairly consistent for all ethanol-fed animals (E, EC, ELM, and ECLM: 2.1, 2,2 2,2, and 2.0, respectively) but were lower than nonethanol-fed animals (NE, 2.5). The weight of livers of the E group animals were significantly heavier than those of the NE group of animals, as well as the supplemented ethanol-fed groups (EC, ELM, and ECLM) (Table 6). However, the weights of hearts, brains, kidneys, and testes showed no significant differences among the group (Table 7).

TABLE 7. Effects of Carnitine and its Precursors on Body Weight, Food Consumption, and Weight of Organs in Ethanol-Fed Rats (Experiment I)

	Group				
	NE	E	EC	ELM	ECLM
Food consumption (ml/day)	61.79±0.38[a]	64.64± 1.31[b]	54.56±0.84[c]	53.67±0.94[c]	54.73±0.90[c]
Weight gain (g)	157.52±6.25[a]	136.50±12.05[b]	118.90±8.16[bc]	112.20±6.98[c]	107.70±5.14[c]
Liver (g)	11.22±0.14[a]	13.11± 0.72[b]	10.95±0.31[a]	11.18±0.40[a]	10.69±0.48[a]
Heart (g)	1.14±0.03[a]	1.12± 0.05[a]	1.10±0.05[a]	1.10±0.03[a]	1.06±0.04[a]
Brain (g)	1.91±0.05[a]	1.87± 0.05[a]	1.86±0.04[a]	1.82±0.04[a]	1.83±0.04[a]
Kidney (g)	2.44±0.06[a]	2.37± 0.08[a]	2.31±0.09[a]	2.32±0.11[a]	2.30±0.07[a]
Testes (g)	3.67±0.10[a]	3.68± 0.02[a]	3.73±0.13[a]	3.37±0.13[a]	3.78±0.10[a]

Note. The values are mean ± SEM for six animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). NE = non-ethanol, E = ethanol, EC = ethanol + carnitine, ELM = ethanol + lysine + methionine, and ECLM = ethanol + carnitine + lysine + methionine.

Plasma concentrations (mg/g) of total carnitine contents were significantly lower in the E and ELM group compared with the NE group (Table 8 and Figure 16). However, all supplemented ethanol-fed animals (EC, ELM, and ECLM) showed significantly elevated levels of total carnitine when compared with the E group. A markedly elevated concentration of plasma total carnitine was found in the EC group which is higher than even that of the ECLM group (Table 8 and Figure 16). The highest plasma concentrations (mg/dl) of triglyceride were found in the E group, followed by similar levels in the ELM group. The plasma concentrations of triglyceride were lowest in the animals of the EC group but were not statistically significant from those of the NE and ECLM groups (Table 8 and Figure 16). The concentrations (mg/g) of plasma protein were not different among the groups (Table 8).

Hepatic DNA contents (mg/g) were not altered by treatment, whereas all hepatic lipid classes were significantly affected by supplemented agents (Table 9). The concentrations of total lipid, triglyceride, esterified cholesterol, free cholesterol, total cholesterol, and phospholipid were significantly increased in the livers of the E group compared with the NE group and were significantly reduced (except for phospholipid in the ELM and ECLM groups) from those in the E group by treatment with supplemented agents (EC, ELM, and ECLM groups (Table 9). The values of both total lipid and triglyceride were lowest in the livers of the EC group among the supplemented groups (Table 9 and Figure 17). On the other hand,

TABLE 8. Effects of Carnitine and its Precursors on Plasma Concentrations of Carnitine, Triglyceride, and Protein (Experiment I)

Plasma Component	Group				
	NE	E	EC	ELM	ECLM
Total carnitine (nmoles/ml)	249.98±11.63^{c}	120.33±8.73^{a}	334.90±25.50^{d}	169.23±7.57^{b}	238.91±10.00^{c}
Triglycerides (mg/dl)	29.83± 3.45^{ab}	49.64±9.92^{c}	22.90± 0.55^{a}	43.72±4.92^{bc}	30.20± 3.05^{ab}
Protein (mg/ml)	67.83± 3.20^{a}	72.07±2.17^{a}	65.73± 2.18^{a}	78.75±7.22^{a}	72.80± 4.58^{a}

Note. The values are mean ± SEM for six animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). NE = non-ethanol, E = ethanol, EC = ethanol + carnitine, ELM = ethanol + lysine + methionine, and ECLM = ethanol + carnitine + lysine + methionine.

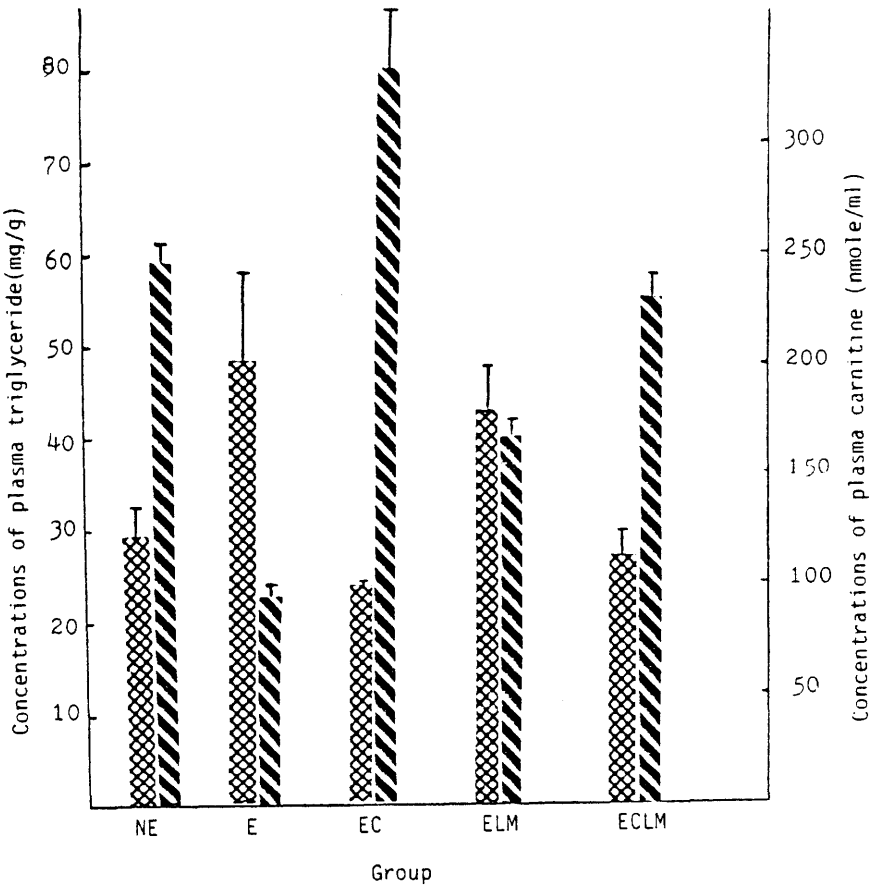

FIGURE 16. Effects of carnitine and its precursors on plasma concentrations (mean + SEM.) of triglyceride (▨) and total carnitine (▨) in ethanol-fed rats.

TABLE 9. Effect of Carnitine and its Precursors on Hepatic DNA and Lipids in Ethanol-Fed Rats (Experiment I)

Parameters	Groups				
	NE	E	EC	ELM	ECLM
	(mg/g wet weight of liver)				
DNA	3.30±0.16[a]	2.97± 0.10[a]	3.10±0.07[a]	3.07±0.07[a]	3.01±0.14[a]
Total lipids	48.59±3.80[a]	78.05± 9.50[b]	55.95±4.30[a]	59.52±3.80[a]	56.82±3.00[a]
Triglycerides	19.39±6.05[a]	61.81±11.46[b]	23.42±6.02[a]	33.62±3.59[a]	33.02±8.68[a]
Esterified cholesterol	5.72±0.71[a]	8.56± 0.58[b]	6.13±0.51[a]	6.52±0.50[a]	5.93±0.49[a]
Free cholesterol	1.11±0.05[a]	1.35± 0.07[b]	1.16±0.39[a]	1.20±0.02[a]	1.16±0.04[a]
Total cholesterol	6.84±1.78[a]	9.92± 1.57[b]	7.30±1.23[a]	7.72±1.23[a]	7.10±1.24[a]
Phospholipids	5.78±0.50[a]	8.02± 0.59[b]	6.42±0.40[a]	6.94±0.38[ab]	7.85±0.36[b]
Free fatty acids	9.91±0.84[a]	12.01± 1.79[ab]	13.12±1.24[bc]	15.86±1.81[c]	14.91±1.06[bc]

Note. The values are mean ± SEM for six animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). NE = non-ethanol, E = ethanol, EC = ethanol + carnitine, ELM = ethanol + lysine + methionine, ECLM = ethanol + carnitine + lysine + methionine.

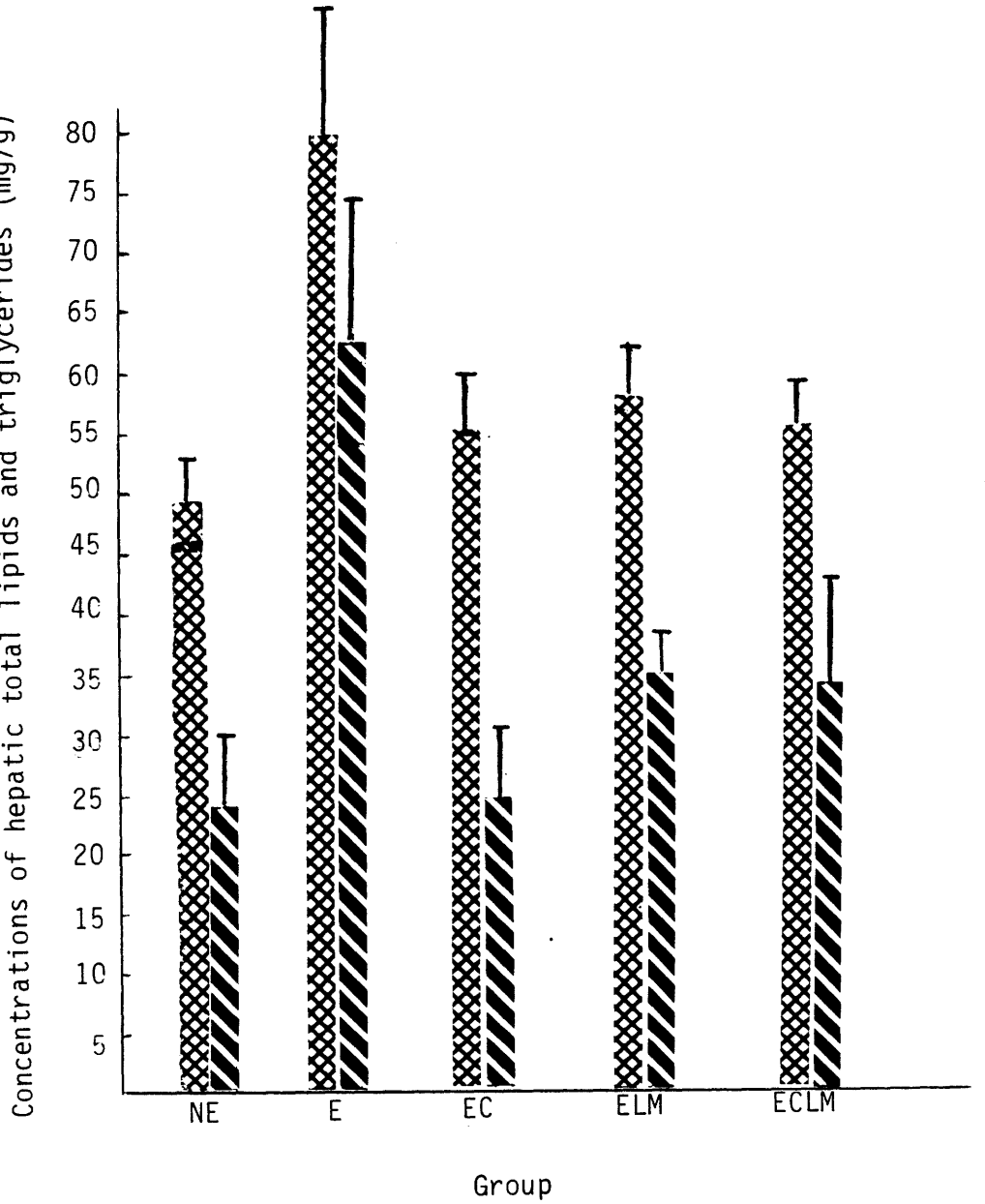

FIGURE 17. Effects of carnitine and its precursors on plasma concentrations (mean + SEM) of total lipid (▨) and triglyceride (▨) in ethanol-fed rats.

the concentrations of free fatty acid were significantly increased in all ethanol-fed animals compared with the NE group (Table 9). Furthermore, of the supplemented ethanol-fed groups, significantly higher concentrations of free fatty acid were found in the ELM group compared with the NE and E groups (Table 9).

Although the gravimetric method (137) that was initially used in the present study produced values more consistent with that reported by Lieber and DeCarli (30) who also used gravimetric procedure for total lipid determination in their studies, the spectrophotometric (phosphoric acid-vanillin) method (126) yielded results in close agreement with those by summing of the total lipid components of a sample (Table 10 and Figure 18). Therefore, the values of total lipid content presented in this study are those determined by the spectrophotometric phosphoric acid-vanillin method.

The concentrations (nmole/g) of hepatic carnitine (free carnitine) were significantly elevated in the supplemented ethanol-fed groups (EC, ELM, and ECLM) compared with unsupplemented non-alcoholic animals (NE) (Table 11 and Figure 19). Among the supplemented groups, only the EC group had significantly higher concentrations of carnitine than those of the E group (Table 11). The concentrations of carnitine in the E group were also elevated compared to the NE group, but the difference was statistically not significant (Table 11). However, the concentrations of ASAC did not differ among groups (Table 11). The concentrations of AIAC were significantly elevated in the supplemented groups: EC, ELM,

TABLE 10. Comparison of Analysis Methods for Total Hepatic Lipids (Experiment I)

Sources	Group				
	NE	E	EC	ELM	ECLM
	(mg/g)				
Gravimetric method	33.7± 3.3	139.2± 4.2	82.9± 7.1	91.4±3.6	89.6±13.0
Spectrophotometric method (phosphovanillin)	48.6± 3.8	78.1± 9.5	56.0± 4.3	59.5±3.8	56.8± 3.0
Summation[a]	41.9±14.5	50.3± 8.2	50.3± 8.2	64.1±5.7	62.9±10.6
Literature[b]	58.0± 2.5	132.6±24.3	114.5±17.9	-	-

[a]Summation = triglyceride + phospholipid + total cholesterol + free fatty acids.

[b]Literature values are cited from values reported by Lieber, C. S. and DeCarli, L. M. (30) where gravimetric method was used for total lipids determination. Values are mean ± SEM.

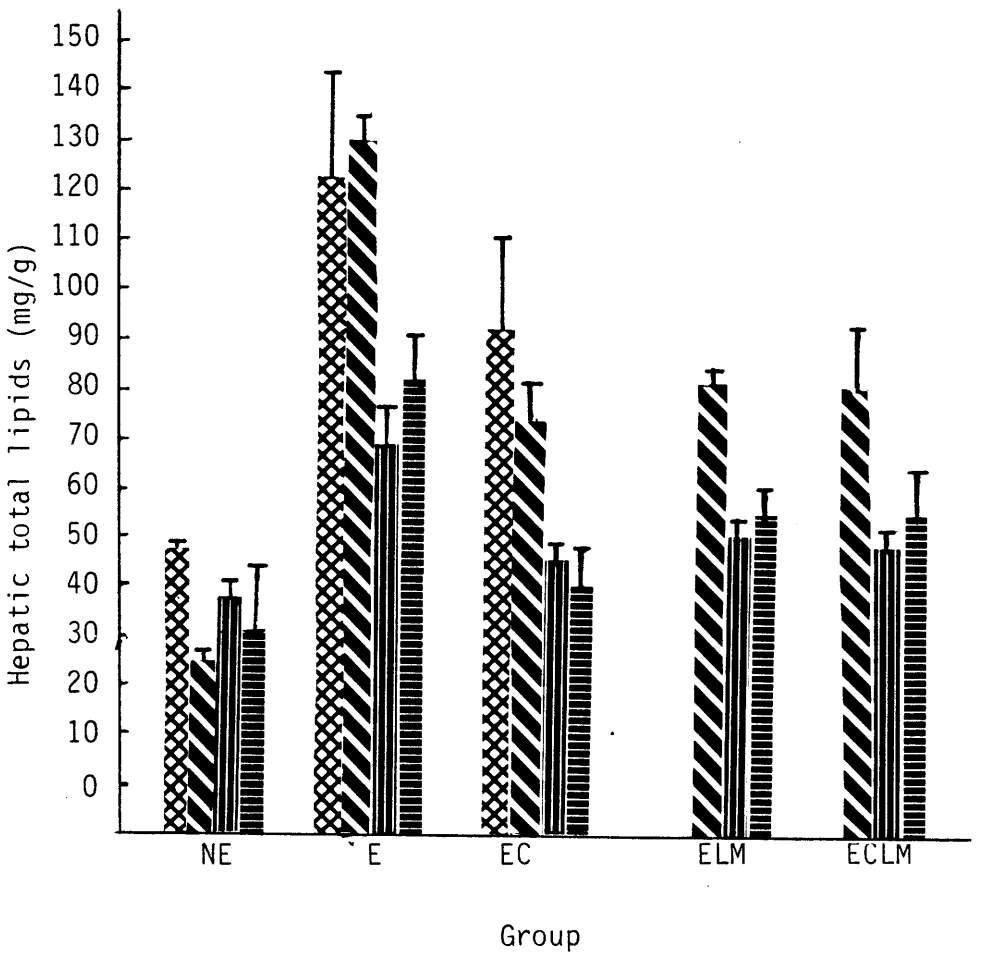

FIGURE 18. Comparison of hepatic total lipid values found in literature (▩) with values (mean ± SEM) obtained by gravimetric (▨), spectrophotometric (▥), and summation (▤) procedures.

TABLE 11. Concentrations of Hepatic Carnitines in Rats Fed With and Without Carnitine and its Precursors (Experiment I)

Parameters	Groups				
	NE	E	EC	ELM	ECLM
	(nmoles/g wet weight of liver)				
Carnitine	365.2±23.4^{a}	486.4±26.6^{ab}	705.2±63.8^{c}	533.8±26.0^{b}	639.7±41.0^{bc}
ASAC	121.2±25.4^{a}	209.0±57.1^{a}	218.2±27.4^{a}	143.2±28.2^{a}	213.0±23.4^{a}
AIAC	5.7± 0.3^{a}	8.5± 0.9^{ab}	11.5± 0.8^{b}	10.8± 0.5^{b}	11.4± 1.0^{b}
Total carnitine	492.1±26.2^{a}	703.9±58.3^{ab}	934.8±85.5^{c}	687.8±15.9^{ab}	864.0±51.2^{bc}
$\frac{\text{ASAC + AIAC}}{\text{carnitine}}$	0.35	0.45	0.33	0.29	0.35

Note. The values are mean ± SEM for six animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). ASAC = acid soluble acyl carnitine; AIAC = acid insoluble acyl carnitine; NE = non-ethanol, E = ethanol, EC = ethanol + carnitine, ELM = ethanol + lysine + methionine, ECLM = ethanol + carnitine + lysine + methionine.

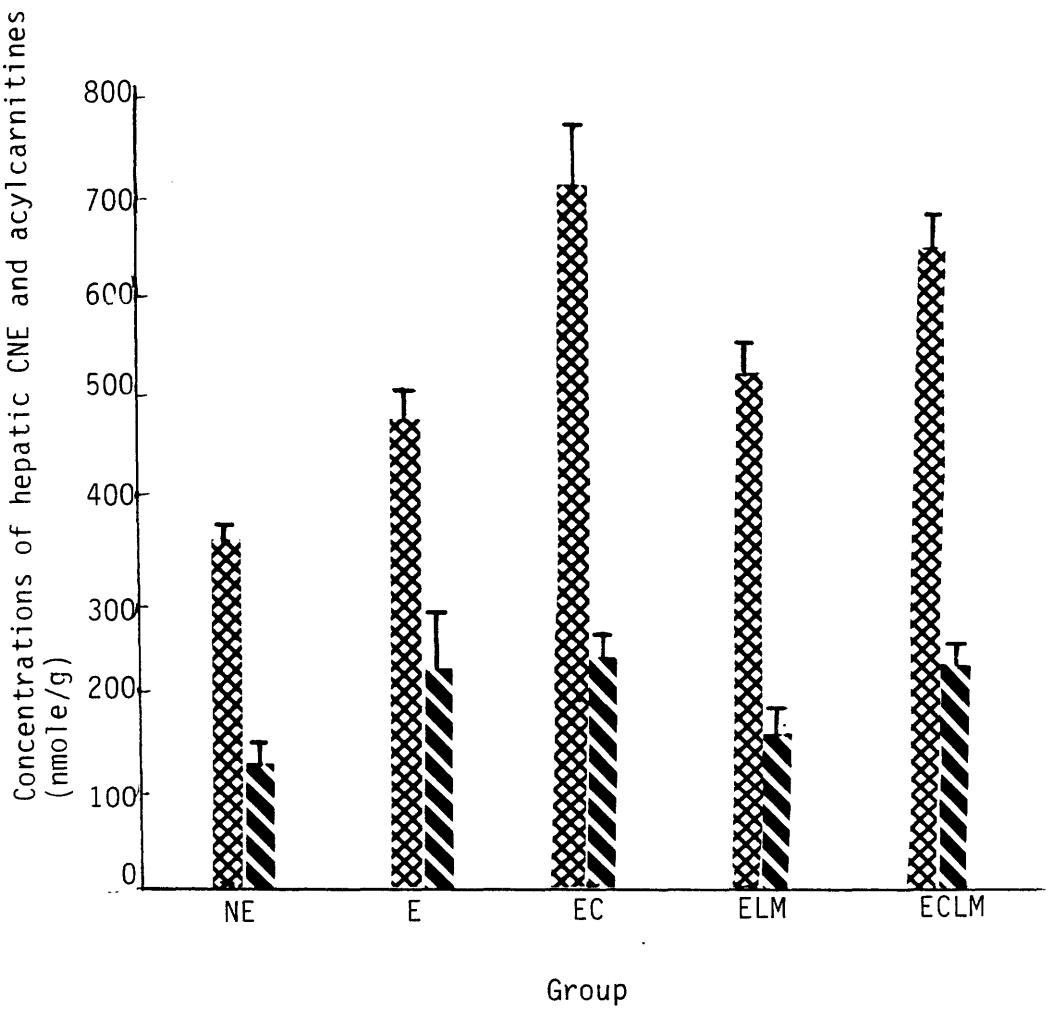

FIGURE 19. Effects of carnitine and its precursors on the concentrations (mean + SEM) of hepatic carnitine (▩) and acylcarnitines (▨) in ethanol-fed rats.

and ECLM, compared to the NE group and also elevated in the E group compared with the NE group but the latter difference was not significant (Table 11). The concentrations of total carnitine were significantly elevated in the EC and ECLM groups when compared to NE group (Table 11). When compared with the NE group, the unsupplemented alcoholic group (E) showed elevated concentration of all species of carnitine but the differences were not significant (Table 11). The ratios of acylcarnitine (ASAC + AIAC) to carnitine in the supplemented groups (EC, ELM, and ECLM) were much closer to those in the NE group than those in the E group (Table 11).

As shown in Table 12, the concentrations of total lipid in organs such as skeletal muscle, brain, testes, and heart were not statistically different among groups. However, the concentrations of lipids in kidney were significantly higher in both unsupplemented alcoholic group (E) and supplemented alcoholic groups (EC, ELM, and ECLM) when compared to NE group (Table 12). There appeared to be a trend that the elevated concentrations of total lipid in the kidney of ethanol-fed animals were decreased in the supplemented groups (EC, ELM, and ECLM). The concentrations of triglycerides in the organs showed similar pattern of changes as those of total lipid (Table 13). All the organs except kidney had no significant difference in the concentrations of triglycerides among groups (Table 13). The kidney had significantly elevated concentrations of triglycerides in the E group compared to the NE group and reduced concentrations in supplemented groups

TABLE 12. Effects of Carnitine and its Precursors on the Concentrations of Total Lipid in Organs Other Than Liver (Experiment I)

Organs	Group				
	NE	E	EC	ELM	ECLM
	(mg/g of wet weight)				
Kidney	14.20±0.72[a]	20.14±2.53[b]	18.22±1.91[b]	18.70±0.72[b]	18.14±1.91[b]
Skeletal muscle	16.31±1.00[a]	18.52±4.20[a]	15.43±5.61[a]	17.32±5.20[a]	15.35±3.15[a]
Brain	38.01±7.26[a]	33.58±4.56[a]	35.03±3.20[a]	34.95±4.63[a]	34.14±4.11[a]
Testes	9.69±0.81[a]	11.16±1.52[a]	10.72±1.52[a]	11.34±0.75[a]	11.13±0.62[a]
Heart	17.81±1.71[a]	20.93±3.68[a]	16.49±3.18[a]	17.18±3.89[a]	19.52±1.23[a]

Note. The values are mean ± SEM for six animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). NE = non-ethanol; E = ethanol; EC = ethanol + carnitine; ELM = ethanol + lysine + methionine, and ECLM = ethanol + carnitine + lysine + methionine.

TABLE 13. Effects of Carnitine and its Precursors on the Concentrations of Triglyceride in Organs Other Than Liver (Experiment I)

Organs	Group				
	NE	E	EC	ELM	ECLM
	(mg/g of wet weight)				
Kidney	6.98±1.52[a]	10.85±2.13[b]	7.62±1.26[ab]	6.60±0.82[a]	7.00±1.88[ab]
Skeletal	10.19±1.45[a]	11.48±1.45[a]	8.92±1.02[a]	6.60±1.08[a]	8.18±1.07[a]
Brain	14.35±2.04[a]	11.78±2.98[a]	11.49±2.89[a]	11.14±3.17[a]	11.40±2.63[a]
Testes	3.59±0.15[a]	4.07±0.47[a]	3.88±0.77[a]	4.09±0.53[a]	4.04±0.67[a]
Heart	10.39±0.66[b]	11.49±0.82[b]	7.05±1.25[a]	10.10±1.78[b]	9.95±1.33[b]

Note. The values are mean ± SEM for six animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). NE = non-ethanol; E = ethanol; EC = ethanol + carnitine; ELM = ethanol + lysine + methionine, and ECLM = ethanol + carnitine + lysine + methionine.

(EC, ELM, and ECLM) compared with the elevated concentrations in the E group with the lowest concentrations being in the ELM group (Table 13).

2. EXPERIMENT II

This study was designed to determine whether the lipid-lowering effect of dietary carnitine on ethanol-induced fatty liver was dose-dependent or not. To determine the most effective lipotropic dosage of dietary carnitine, various levels of D,L-carnitine were supplemented in the nutritionally adequate liquid ethanol diet used in Experiment I (E_0: 0.0%, E_1: 0.2%, E_2: 0.6%, E_3: 1.0%, and E_4: 2.0% carnitine) (Table 4, page 36). There appeared to be no significant difference among all groups in the daily diet consumption (Table 14). Similarly, liver weights were not significantly different among groups (Table 14). The relative gain in body weight (g) was as follows: $E_1 > E_0 > E_2 > E_4 > E_3$ (Table 14). However, the gain in body weight (g) per unit volume (ml) of diet consumed showed a trend that the higher supplementation of carnitine was associated with less gain in body weight (E_0: 1.16, E_1: 1.33, E_2: 0.87, E_3: 0.45, and E_4: 0.48, respectively) (Table 14). The concentrations of plasma total carnitine (nmole/ml) were significantly elevated in all the carnitine supplemented groups (E_1, E_2, E_3, E_4) when compared to unsupplemented ethanol-fed animals (E_0), but did not differ statistically among supplemented groups (Table 15 and Figure 20). Plasma levels of triglyceride were not affected

TABLE 14. Effects of Various Levels of Carnitine on Food Consumption, Liver Weight, and Body Weight Gain (Experiment II)

Parameters	Groups				
	E_0	E_1	E_2	E_3	E_4
Food intake (mg/day, rat)	32.2 ±1.61[a]	29.5 ±2.32[a]	29.06±1.88[a]	26.9 ±2.06[a]	27.5 ±2.15[a]
Liver (g)	5.40±0.35[a]	5.38±0.28[a]	5.46±0.29[a]	4.73±0.33[a]	4.75±0.02[a]
Body weight gain (g)	36.9±3.56[bc]	38.6 ±1.39[c]	24.8 ±3.19[b]	12.2 ±3.17[a]	12.8 ±2.68[a]

Note. The values are mean ± SEM for five animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). E = ethanol.

TABLE 15. Effects of Various Levels of Carnitine on the Plasma Concentrations of Total Carnitine and Triglyceride (Experiment II)

	Groups				
	E_0	E_1	E_2	E_3	E_4
Total carnitine (nmole/ml)	30.54 ± 2.36^a	91.03 ± 4.79^b	105.10 ± 1.83^b	98.22 ± 2.25^b	106.93 ± 2.62^b
Triglycerides (mg/dl)	30.16 ± 1.37^b	29.19 ± 1.64^b	27.90 ± 1.58^b	26.29 ± 0.94^{ab}	22.58 ± 1.59^a

Note. The values are mean ± SEM for five animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). E = ethanol.

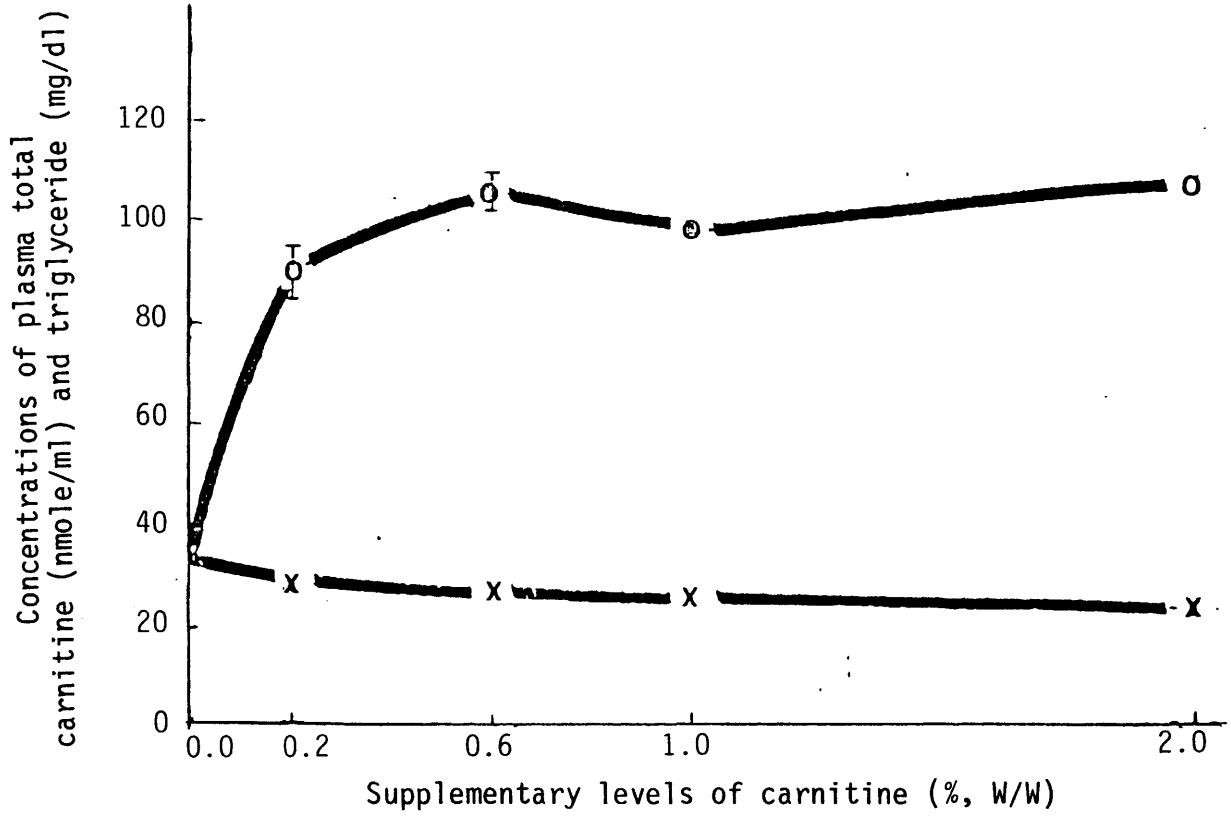

FIGURE 20. Effects of various levels of carnitine on plasma concentrations (mean ± SEM) of total carnitine (-O-) and triglyceride (-x-) in ethanol-fed rats.

significantly by treatment with carnitine supplementation up to the 0.6% level (E_1 and E_2), but were slightly depressed by 1.0% carnitine supplementation (E_3) compared to the E_0 group (Table 15 and Figure 20).

With 2.0% carnitine supplementation (E_4), the levels of plasma triglyceride were significantly lower compared to the unsupplemented group (E_0) (Table 15). These changes can be expressed as a 3.2%, 7.5%, 12.8%, and 25.1% decrease in the E_1, E_2, E_3, and E_4 groups, respectively, compared to the E_0 group. None of the hepatic lipid parameters showed any significant changes in the carnitine-supplemented groups (Table 16). However, the concentrations of hepatic total lipid tended to be depressed by carnitine supplementation (Table 16 and Figure 21) compared to the unsupplemented group (E_0), the levels of total lipid were decreased as much as 2.7%, 7.6%, and 9.2% in the E_1, E_2, and E_3 groups, respectively, but increased 0.74% in the E_4 group. The levels of hepatic triglyceride showed a similar pattern as total lipids (Table 16 and Figure 21). The percent change in hepatic triglecerides were 1.7%, 6.1%, 11.5%, and 6.2% decrease in the E_1, E_2, E_3, and E_4 groups respectively, when compared to the unsupplemented group (E_0). Free cholesterol decreased by 5.4%, 14.1%, 0.6%, and 29.5% in the E_1, E_2, E_3, and E_4 groups, respectively. On the other hand, esterified cholesterol increased by 7.3%, 26.2%, and 15.0% in the E_1, E_2, and E_3 groups, respectively, but there was a 12.9% decrease in the E_4 group compared to the E_0 group. Thus, total cholesterol increased by

TABLE 16. Effects of Various Levels of Carnitine on Hepatic Concentrations of Lipids (Experiment II)

Parameters	Group				
	E_0	E_1	E_2	E_3	E_4
	(mg/g wet weight of liver)				
Total lipids	48.80±5.85[a]	47.46±2.41[a]	45.07±3.27[a]	44.32±2.58[a]	49.16±6.98[a]
Triglycerides	16.74±1.99[a]	16.09±0.84[a]	15.72±1.06[a]	14.82±0.64[a]	15.71±1.59[a]
Free cholesterol	1.49±0.09[a]	1.41±0.09[a]	1.28±0.10[a]	1.48±0.21[a]	1.05±0.06[a]
Esterified cholesterol	2.33±0.32[a]	2.50±0.58[a]	2.94±0.16[a]	2.68±0.15[a]	2.03±0.32[a]
Total cholesterol	3.29±0.51[a]	3.55±0.57[a]	4.22±0.22[a]	3.94±0.23[a]	3.10±0.25[a]
Phospholipids	4.55±0.27[a]	4.88±0.26[a]	4.45±0.43[a]	4.85±0.17[a]	4.77±0.28[a]
Free fatty acids	4.22±0.18[a]	4.28±0.25[a]	5.02±0.12[a]	5.52±0.15[a]	5.32±0.19[a]

Note. The values are mean ± SEM for five animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). E = ethanol.

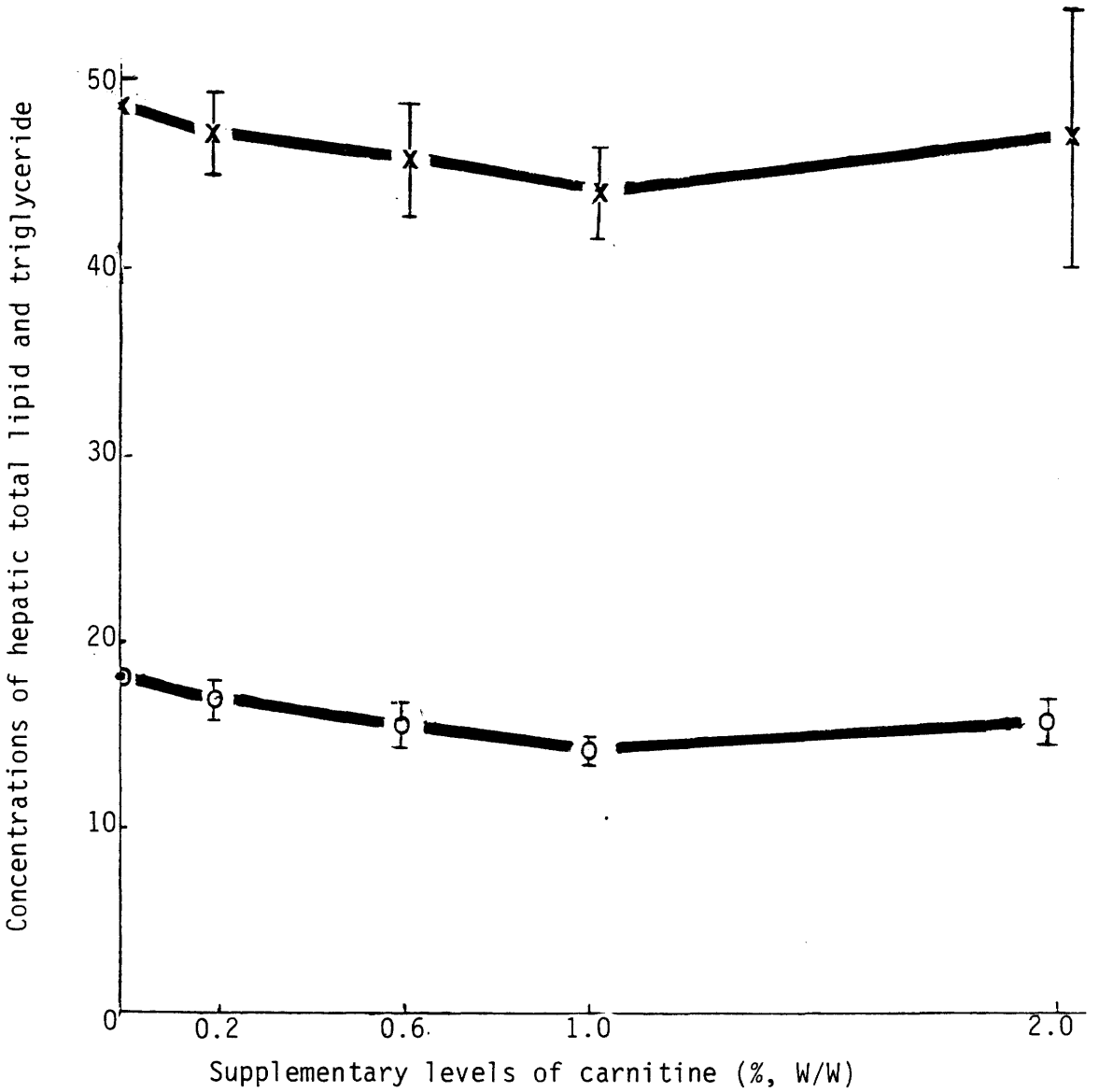

FIGURE 21. Effects of various levels of carnitine on hepatic concentrations (mean ± SEM) of total lipid (-x-) and triglyceride (-o-) in ethanol-fed rats.

7.9%, 28.3%, and 19.8% in the E_1, E_2, and E_3 groups, respectively, but there was a 5.8% decrease in the E_4 group. Changes in phospholipid concentrations did not show any consistent pattern. There was a 7.2%, 6.6%, and 4.8% increase in the E_1, E_3, and E_4 groups respectively, but a 2.2% decrease in E_1 compared to the E_0 group. However, when compared to the E_0 group, there appeared to be consistent increases in hepatic free fatty acid concentrations in the supplemented groups (1.4%, 19%, 30.8%, and 26.1% increase in the E_1, E_2, E_3, and E_4 group, respectively) (Table 16).

3. EXPERIMENT III

This study was carried out to further substantiate the dose-response effect of carnitine-supplementation, as demonstrated in Experiment II. This was especially important in Experiment II in which the animals were too young, and received the ethanol test diet for only 28 days. The levels of carnitine supplementation tested were changed (see Experimental design).

In this experiment, the study was expanded in population of animals (from 25 to 30) and in experimental period (from 28 to 45 days). As shown in Table 17, only the E_5 group showed a significantly higher diet consumption than the other groups. However, there was no significant difference among groups in either body weight gain or weights of organs e.g., liver, kidney, heart, and small intestine (Table 17).

TABLE 17. Effects of Various Levels of Carnitine on Food Consumption, Body Weight Gain, and Weights of Organs in Ethanol-Fed Rats (Experiment III)

Parameters	Group					
	E_0	E_1	E_2	E_3	E_4	E_5
Food intake (ml/day, rat)	56.91±0.85[a]	56.67±1.48[a]	57.17±1.32[a]	55.31± 2.52[a]	54.89±1.32[a]	64.78±2.96[b]
Body weight gain (g)	71.6 ±1.09[a]	68.0 ±5.37[a]	66.6 ±5.37[a]	56.1 ±10.03[a]	59.4 ±5.63[a]	62.4 ±9.84[a]
Liver (g)	10.97±0.53[a]	10.27±0.36[a]	9.45±0.38[a]	8.83± 0.79[a]	8.61±0.20[a]	10.45±0.97[a]
Kidney (g)	2.14±0.19[a]	1.91±0.06[a]	2.08±0.24[a]	2.44± 0.37[a]	2.27±0.27[a]	2.18±0.20[a]
Heart (g)	1.22±0.21[a]	1.18±0.22[a]	1.15±0.22[a]	1.55± 0.29[a]	1.37±0.21[a]	1.24±0.21[a]
Small intestine (g)	5.53±0.27[a]	6.12±0.13[a]	6.67±0.59[a]	5.69± 0.37[a]	6.17±0.38[a]	6.07±0.58[a]

Note. The values are mean ± SEM for five animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). E = ethanol.

Plasma concentrations (mg/g) of total lipids were significantly reduced by carnitine treatment with the lowest contents being found in the E_3 group (Table 18 and Figure 22). Higher levels of carnitine supplementation than 0.8% (E_3) did not further decrease total lipid concentrations (Figure 22). Plasma concentrations (mg/g) of triglyceride showed a similar pattern as total lipids (Table 18 and Figure 22). The concentrations of triglycerides were significantly lowered by carnitine supplementation with the lowest concentrations found in the E_3 group. Again, higher levels of carnitine than 0.8% did not further decrease triglyceride concentrations either (Figure 22).

Plasma concentrations (nmole/ml) of carnitine were elevated in all the supplemented groups, and compared to the E_0 group, the elevation was statistically significant in the groups supplemented with 0.5% carnitine or more (E_2, E_3, E_4, and E_5) (Table 19 and Figure 23). The highest concentrations of plasma carnitine were found in the E_4 and E_5 group (Table 19 and Figure 23). However, the plasma concentrations of ASAC and AIAC were not significantly altered by carnitine supplementation (Table 19 and Figure 23). Among the supplemented groups, the highest concentrations of these acylcarnitines were found in the E_3 group (Table 19 and Figure 23). Changes in total carnitine content showed a fairly similar pattern as those of the carnitine content and were elevated in all carnitine-supplemented groups, and the elevations were statistically significant in all the supplemented groups compared to the E_0 group except for E_1 (Table 19 and Figure 23).

TABLE 18. Effects of Various Levels of Carnitine on Plasma Concentrations of Total Lipid and Triglyceride (Experiment III)

Parameters	Group					
	E_0	E_1	E_2	E_3	E_4	E_5
	(mg/dl)					
Total lipids	81.43 ± 1.75^{c}	61.43 ± 5.74^{ab}	51.79 ± 4.09^{ab}	47.50 ± 4.53^{a}	58.57 ± 6.00^{ab}	67.14 ± 5.97^{bc}
TG	23.33 ± 1.94^{c}	18.61 ± 1.04^{ab}	16.67 ± 0.98^{ab}	14.23 ± 1.63^{a}	17.01 ± 1.71^{ab}	19.10 ± 1.71^{b}

Note. The values are mean ± SEM for five animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). E = ethanol.

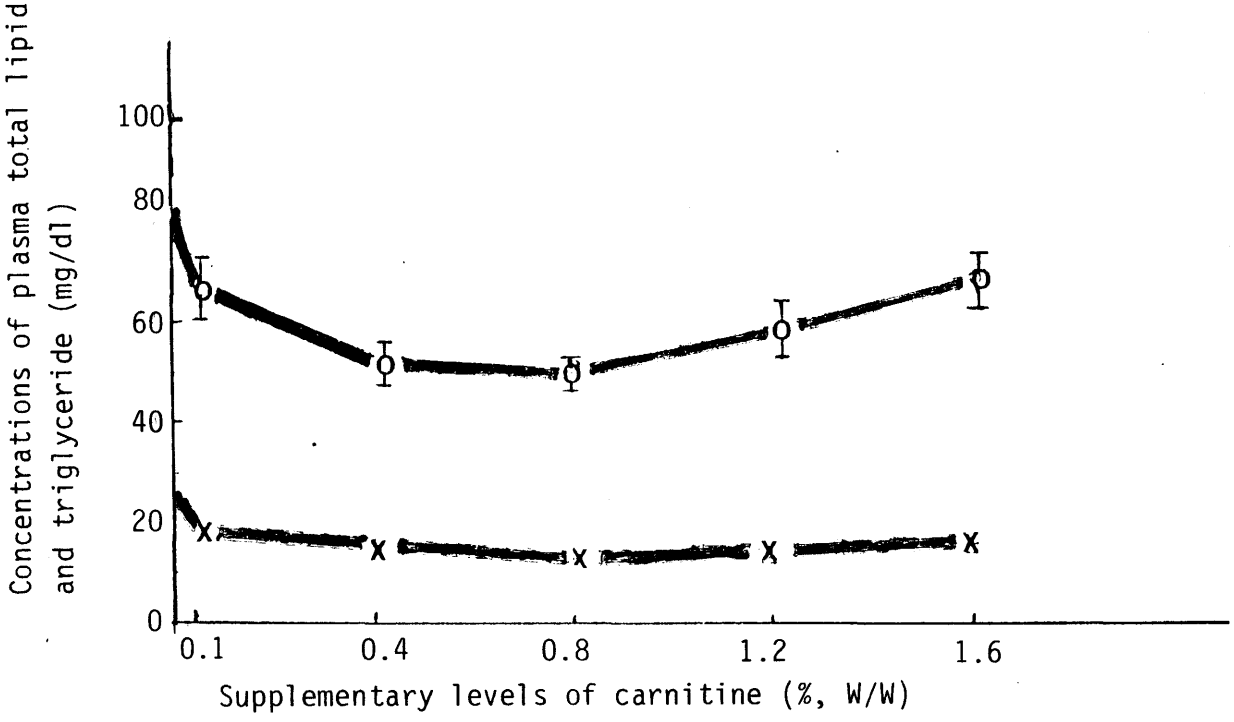

FIGURE 22. Effects of various levels of supplementary carnitine on the plasma concentrations (mean ± SEM) of total lipid (-o-) and triglyceride (-x-) in ethanol-fed rats.

TABLE 19. Effects of Various Levels of Carnitine on Plasma Concentrations of Carnitine (Experiment III)

Parameters	Group					
	E_0	E_1	E_2	E_3	E_4	E_5
	(nmole/ml)					
CNE	34.57±6.68^a	48.86±7.35^{ab}	56.38± 5.44^b	69.43±5.41^{bc}	80.48±3.58^c	77.92±5.98^c
ASAC	10.31±1.98^a	11.66±3.26^a	14.82± 3.95^a	19.02±2.22^a	16.49±4.15^a	16.30±3.11^a
AIAC	5.30±0.87^a	6.15±1.25^a	8.70± 2.37^a	9.08±1.46^a	6.36±1.01^a	8.60±0.87^a
Total CNE	50.89±8.15^a	66.67±8.00^{ab}	79.91±11.03^{bc}	97.53±7.52^c	103.33±4.53^c	102.83±7.71^c

Note. The values are mean ± SEM for five animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). CNE = carnitine, ASAC = acid soluble acyl carnitine, AIAC = acid insoluble acyl carnitine. E = ethanol.

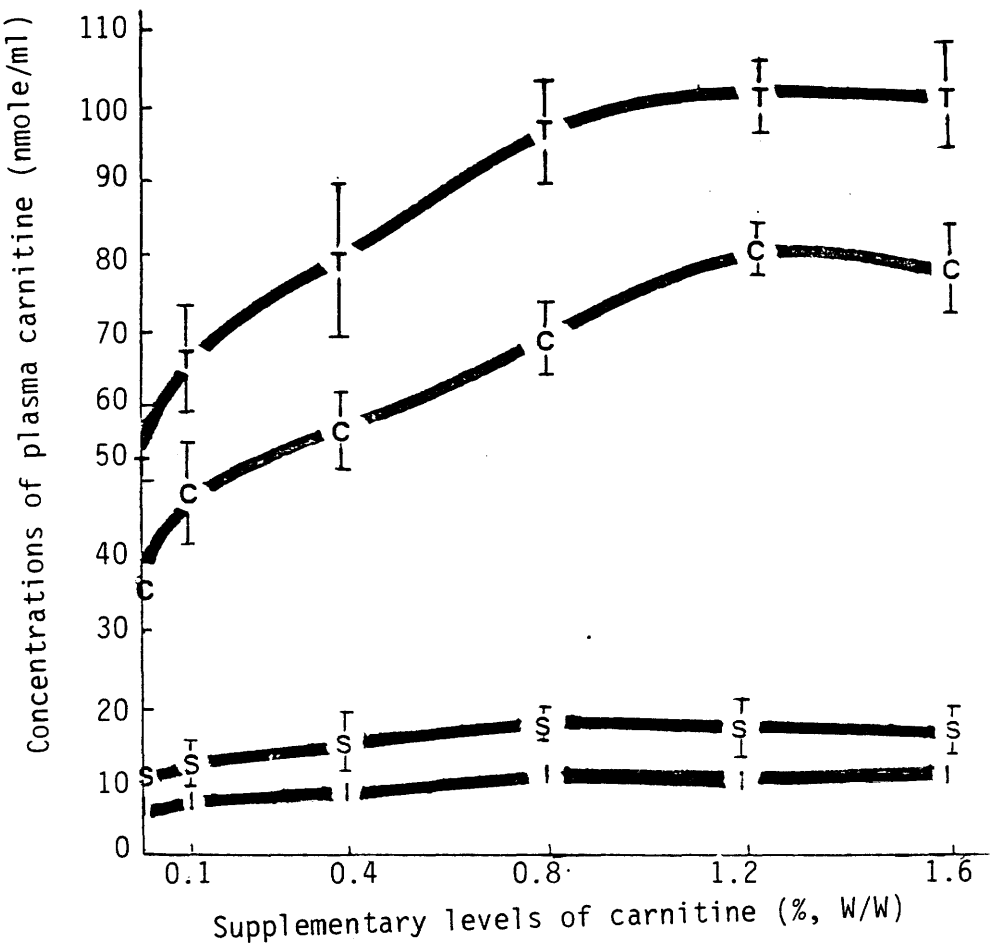

FIGURE 23. Effects of various levels of carnitine on concentrations (mean ± SEM) of plasma total carnitine (-T-), carnitine (-C-), ASAC (-S-), and AIAC (-I-) in ethanol-fed rats.

The difference in the concentration (mg/g) of hepatic total lipids was not statistically significant between the E_0 group and the E_1 group, but the concentration was significantly lower in the other supplemented groups (E_2, E_3, E_4, and E_5) compared to the E_0 group (Table 20 and Figure 24). The changes in hepatic triglycerides showed a similar pattern as those of hepatic total lipids, and the levels were significantly reduced in the supplemented groups (except for the E_1 group) compared to the E_0 group (Table 20 and Figure 24). The lowest concentrations in both total lipids and triglycerides were found in the E_3 group (Table 20 and Figure 24). Hepatic concentrations (mg/g) of both free and esterified cholesterol did not significantly differ among groups, but those of total cholesterol were significantly depressed in the E_3 group compared with other groups, E_0, E_1, and E_5 (Table 20). The concentrations (mg/g) of both phospholipids and free fatty acids in the liver were not significantly altered by carnitine supplementation, but there was a consistently increasing trend in free fatty acid content with increasing carnitine supplementation (percent increase: 4.58, 15.81, 18.96, 19.14, and 25.16 in E_1, E_2, E_3, E_4, and E_5, respectively) (Table 20).

Hepatic concentrations (nmole/g) of carnitine were significantly elevated in all the carnitine supplemented groups compared with those in the E_0 group (Table 21 and Figure 25). The changes in the concentrations of hepatic ASAC showed a similar pattern as carnitine and were elevated in all the supplemented groups compared

TABLE 20. Effects of Various Levels of Carnitine on Concentrations of Hepatic Lipid (Experiment III)

Parameters	Groups					
	E_0	E_1	E_2	E_3	E_4	E_5
	(mg/g wet weight of liver)					
Total lipids	69.35 ± 4.11^{c}	61.41 ± 4.69^{c}	46.41 ± 4.00^{b}	31.10 ± 4.26^{a}	36.51 ± 1.95^{ab}	43.25 ± 7.49^{ab}
Triglycerides	31.61 ± 2.67^{c}	30.41 ± 2.85^{c}	21.86 ± 2.73^{b}	11.45 ± 2.52^{a}	12.23 ± 2.07^{a}	15.15 ± 3.84^{ab}
Free cholesterol	3.31 ± 0.34^{a}	3.27 ± 0.15^{a}	2.98 ± 0.12^{a}	2.85 ± 0.17^{a}	3.07 ± 0.07^{a}	3.23 ± 0.12^{a}
Esterified cholesterol	7.62 ± 0.35^{a}	7.40 ± 0.54^{a}	7.30 ± 0.48^{a}	6.47 ± 0.35^{a}	7.21 ± 0.24^{a}	8.33 ± 0.35^{a}
Total cholesterol	10.93 ± 0.46^{bc}	10.67 ± 0.57^{bc}	10.29 ± 0.44^{ab}	9.32 ± 0.39^{a}	10.28 ± 0.26^{ab}	11.57 ± 0.33^{c}
Phospholipids	6.12 ± 0.30^{a}	6.40 ± 0.59^{a}	5.61 ± 0.41^{a}	5.12 ± 0.51^{a}	5.85 ± 0.64^{a}	5.13 ± 0.16^{a}
Free fatty acids	11.13 ± 0.81^{a}	11.16 ± 0.65^{a}	12.89 ± 0.40^{a}	13.24 ± 0.76^{a}	13.26 ± 0.60^{a}	13.93 ± 0.37^{a}

Note. The values are mean ± SEM for five animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). E = ethanol.

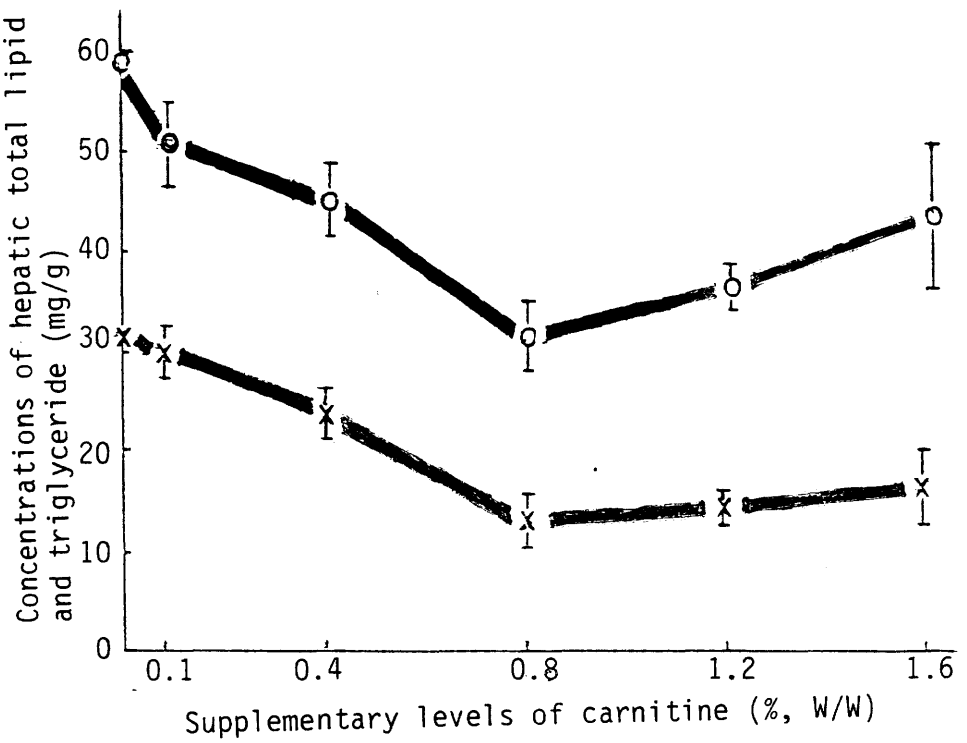

FIGURE 24. Effects of various levels of carnitine on hepatic concentrations (mean ± SEM) of total lipid (-o-) and triglyceride (-x-) in ethanol-fed rats.

TABLE 21. Effects of Various Levels of Carnitine on Concentrations of Hepatic Carnitines (Experiment III)

Component	Groups					
	E_0	E_1	E_2	E_3	E_4	E_5
	(nmole/g wet weight of liver)					
Carnitine	691.11^{a} ± 79.20	1138.69^{b} ± 112.09	1186.26^{b} ± 111.15	1350.68^{b} ± 53.06	1366.53^{b} ± 128.59	1069.40^{b} ± 28.44
ASAC	215.97^{a} ± 56.66	584.98^{b} ± 52.46	687.40^{b} ± 37.16	633.91^{b} ± 100.57	569.90^{b} ± 46.92	588.14^{b} ± 59.22
AIAC	4.50^{a} ±0.56	6.05^{bc} ± 0.23	5.54^{abc} ± 1.42	6.93^{c} ± 0.95	4.89^{abc} ± 0.98	3.36^{a} ± 0.13
Total carnitine	862.26^{a} ±101.93	1663.26^{b} ± 78.11	1941.73^{bc} ± 84.22	1991.52^{c} ± 46.32	1941.32^{bc} ± 136.28	1660.89^{b} ± 71.74

Note. The values are mean ± SEM for five animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). ASAC = acid soluble acyl carnitine; AIAC = acid insoluble acyl carnitine.

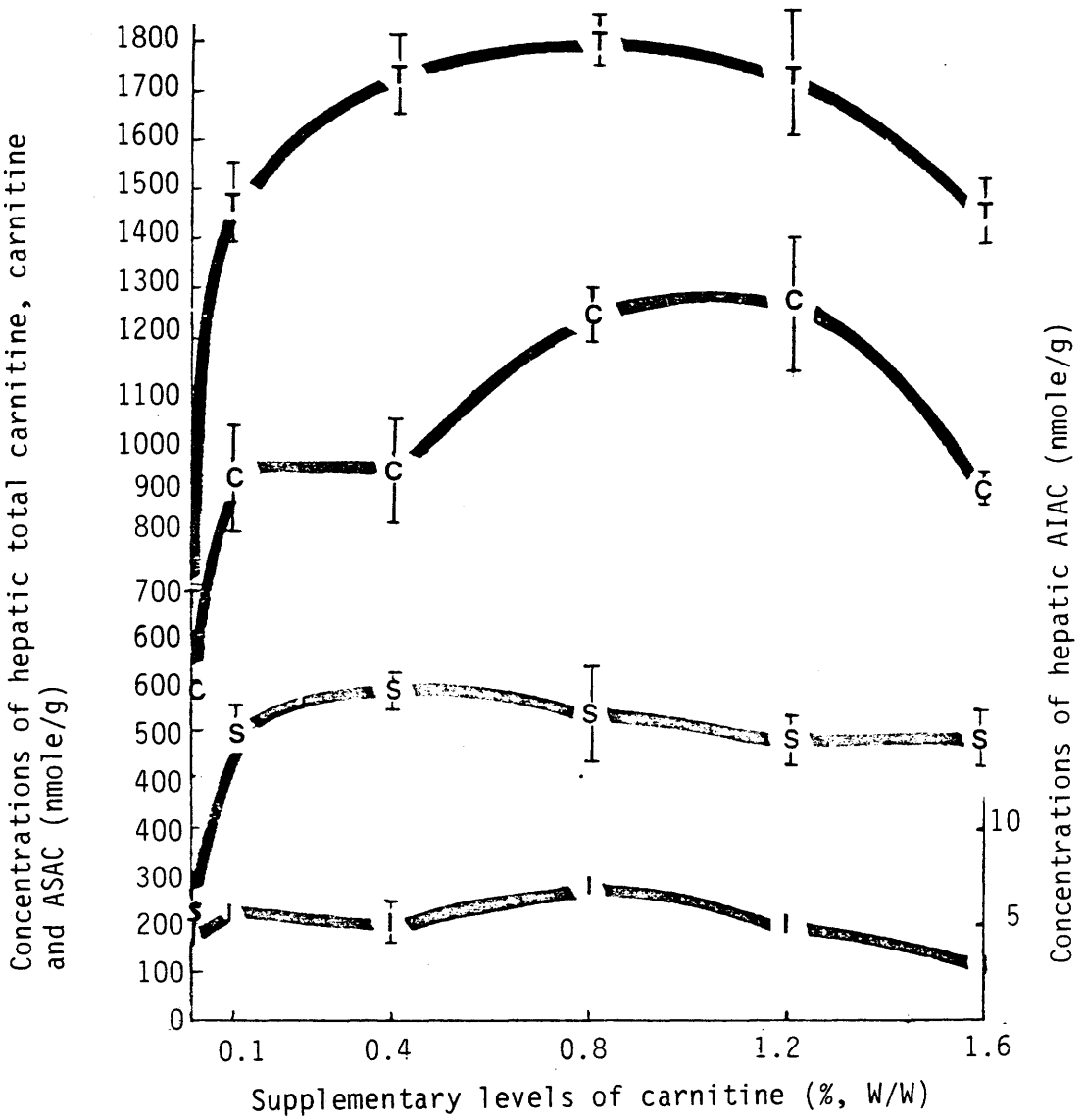

FIGURE 25. Effects of various levels of carnitine on hepatic concentrations (mean ± SEM) of total carnitine (-T-), carnitine (-C-), ASAC (-S-), and AIAC (-I-) in ethanol-fed rats.

to the unsupplemented group (E_0). However, changes in hepatic AIAC were not consistent with concentrations being significantly increased over the E_0 group only in the E_1 and E_3 groups among the supplemented groups (Table 21 and Figure 25). The concentrations of hepatic total carnitine content showed a consistent and significant increase in all the supplemented groups, and the highest concentrations were found in the E_3 group (Table 21 and Figure 25).

The concentrations of total lipids in extrahepatic organs such as kidney, skeletal muscle, and heart were not significantly different (Table 22). However, the concentrations of triglycerides in some of the organs changed significantly with carnitine supplementation (Table 23). Compared with the E_0 group, the following changes were found: kidney showed significantly reduced concentrations of triglycerides in E_2, E_4, and E_5 groups, and those in skeletal muscle were significantly lowered in the E_3, E_4, and E_5 groups (Table 23). No significant changes in the levels of triglyceride content in the heart were found among groups (Table 23).

TABLE 22. Effects of Various Levels of Carnitine on the Concentrations of Total Lipid in Organs Other Than Liver (Experiment III)

Parameters	Group					
	E_0	E_1	E_2	E_3	E_4	E_5
	(mg/g of wet weight)					
Kidney	14.12±1.91[a]	13.94±0.42[a]	13.62±1.14[a]	13.67±2.17[a]	13.90±2.05[a]	11.95±3.28[a]
Skeletal muscle	8.09±1.84[a]	9.35±2.64[a]	10.23±1.56[a]	8.95±1.48[a]	9.12±1.61[a]	7.16±1.39[a]
Heart	14.45±2.34[a]	13.70±2.76[a]	13.91±3.37[a]	11.59±1.60[a]	13.30±0.56[a]	12.53±1.38[a]

Note. The values are mean ± SEM for five animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). E = ethanol.

TABLE 23. Effects of Various Levels of Carnitine on the Concentrations of Triglyceride in Organs Other Than Liver (Experiment III)

Parameters	Group					
	E_0	E_1	E_2	E_3	E_4	E_5
	(mg/g of wet weight)					
Kidney	11.03 ± 0.36^{b}	9.89 ± 0.87^{ab}	9.00 ± 0.68^{a}	9.67 ± 0.65^{ab}	8.76 ± 0.54^{a}	8.45 ± 0.32^{a}
Skeletal muscle	5.56 ± 0.09^{c}	5.60 ± 0.13^{c}	5.53 ± 0.28^{bc}	4.34 ± 0.28^{ab}	3.89 ± 0.38^{a}	4.07 ± 0.34^{a}
Heart	6.54 ± 0.27^{a}	6.23 ± 0.39^{a}	6.19 ± 0.37^{a}	5.45 ± 0.51^{a}	6.67 ± 0.40^{a}	6.62 ± 0.36^{a}

Note. The values are mean ± SEM for five animals. Values without a common superscript letter are significantly different from each other ($P<0.05$). E = ethanol diet.

CHAPTER V

DISCUSSION

1. EFFECTS OF CARNITINE AND ITS PRECURSORS ON TISSUE CONTENTS OF LIPIDS AND CARNITINES

Ethanol feeding to rats at a level of 36% of the total calories in the diet produced fatty liver and accompanying hypertriglyceridemia (Tables 8 and 9, pages 78 and 80, and Figures 16 and 17, pages 79 and 81). These results are consistent with the observations reported in the vast amount of literature available on ethanol-induced fatty liver (8,10,11,13-16,30,38,99). The concentration of total lipid in chronically ethanol-compromised liver has been found to increase approximately 115% (84.3 - 132.6 mg/g) over the normal liver (41.0 - 58.0 mg/g) (8,10,30) which is in agreement with our results where an increase of 60% (78.1 $\pm$ 9.5 mole/g) above the NE group (48.6 $\pm$ 3.8 mg/g) was found. The ethanol-induced increases in hepatic triglycerides of about 21.8% (61.81 $\pm$ 11.46 vs 19.39 $\pm$ 6.05 mg/g) and in phospholipids, 38% (8.02 $\pm$ 0.59 vs 5.78 $\pm$ 0.50 mg/g) observed in the current studies (Table 9, page 80) are also comparable with literature values of a 691% increase (89.4 $\pm$ 11.1 vs 11.3 $\pm$ 1.3 mg/g), and 9% increase (31.4 $\pm$ 1.1 vs 28.8 $\pm$ 1.2 mg/g) for triglyceride and phospholipid, respectively (37). Similarly, the 22% increase in hepatic free cholesterol (1.35 $\pm$ 0.07 vs 1.11 $\pm$ 0.05 mg/g) and 50% increase in esterified cholesterol (8.56 $\pm$ 0.58 vs

5.72 ± 0.71 mg/g) found in the present studies are supported by ethanol-induced increases reported in literature values such as 10% elevation (2.2 ± 0.13 vs 2.0 ± 0.15 mg/g) in free cholesterol and 5-fold increase (2.92 ± 0.14 vs 0.59 ± 0.05 mg/g) in esterified cholesterol (10). The ethanol-induced increase in plasma triglyceride of 66% (49.64 ± 9.92 vs 29.83 ± 3.45 mg/dl) was consistent with reported literature values of a 97% increase (144 ± 9.9 vs 73 ± 2.1 mg/dl) (24).

Supplementation of ethanol diet with 1.0% D,L-carnitine significantly reduced the concentration of lipids in the livers Table 9, page 80, and Figure 17, page 81) of alcoholic rats. The lipotropic effects of dietary carnitine were definitely evident in the changes in total lipid, triglyceride, free cholesterol, and phospholipid contents of the liver (Table 9). The results are consistent with the observations of Hosein and Bexton (24) who reported a significant lipid lowering effect of tube-fed carnitine (0.1 mg/g body weight) on rats; total lipids were reduced 19% (from 77 ± 2.4 to 62 ± 4.4 mg/g) in livers of ethanol-fed rats (6 g/kg body weight) by gastric intubation. Lieber and DeCarli (30) also demonstrated a lipotropic effect of dietary carnitine (1.0 mg/cal) in rats on total lipids (114.5 ± 17.9 vs 132.6 ± 24.3 mg/g) and triglycerides (34 ± 4.5 vs 39.5 ± 4.5 mg/g). In the present studies, the hyperlipidemia commonly associated with chronic ethanol consumption was also minimized by carnitine (Table 8, page 78); these results are comparable with the decrease in plasma

triglyceride (from 144 ± 9.9 to 111.0 ± 5.2 mg/dl) observed by Hosein and Bexton (24) following carnitine treatment of ethanol-fed rats by gastric intubation. If the reduced hepatic and plasma lipid contents in the carnitine supplemented group (Tables 3 and 9, pages 78 and 80) were due to depressed consumption of diet in this group (Table 7, page 76), one would have expected the same effects in the ELM and ECLM groups where food consumption (53.7 and 54.7 ml/day, respectively) were akin to the EC group (54.6 ml/day). The decrease in hepatic total lipid or triglyceride contents were more closely related to the hepatic carnitine contents than to the diet consumption (Figures 17 and 19, pages 81 and 86, and Tables 9 and 11, pages 80 and 85). In other studies also, animals given carnitine or other supplements consumed identical quantities of diet but maintained different concentrations of hepatic lipids (138).

The concentrations of carnitine and its species were monitered to assess whether the lipotropic effect of supplemental carnitine was due to increased tissue pool of carnitine and acylcarnitine. Hepatic carnitine and total carnitine concentrations were elevated in both supplemented ethanol-fed groups (EC, ELM, and ECLM; 46.2 - 93.1%) and unsupplemented (E; 33.2%) group compared to those in the NE group. However, the concentrations of acylcarnitines, especially the AIAC, were significantly increased only in the supplemented ethanol-fed groups (E, EC, and ECLM) (Table 11, page 85). Though the hepatic carnitine content of the supplemented ethanol-fed group was not significantly greater than that of the

NE group, it was akin to that reported by Kondrup and Grunnet (25). They reported increased hepatic concentrations of carnitine of 52% (213 ± 54 from 140 ± 15 nmole/g); acetylcarnitine, 47% (203 ± 31 from 138 ± 42 nmole/g); and total carnitine, 47% (425 ± 76 from 290 ± 51 nmole/mg) in comparison of a carnitine-supplemented group to a control group.

Thus, the question arises as to why the increased concentration of carnitine in livers of the E group was unable to prevent hepatic steatosis like was found in the supplemented groups. Part of the answer may lie in the formation of AIAC which was significantly elevated only in the supplemented groups. Perhaps the endogenously pooled carnitine by liver was below the critical concentration needed for exerting protective action and/or that this carnitine was being concentrated for purposes yet to be discovered.

Prevention of fat accumulation in the livers of carnitine supplemented ethanol-fed groups (Table 9, page 80) found in the present study is supported by the observations of Hosein and Bexton (24). They demonstrated a 18% decrease in total hepatic lipids of carnitine treated rats compared to noncarnitine treated rats. Also, observations in the current study lend support to the carnitine deficiency hypothesis of Rudman et al. (23) in patients with chronic alcoholic cirrhosis (in cirrhotic and noncirrhotic patients, respectively, 36 ± 4 and 78 ± 9 μmole total carnitine and 17 ± 3 and 49 ± 3 μmoles free carnitine/dl of serum). Unfortunately, it can not be a simple classical deficiency of

carnitine as may have been construed. It is, however, possible that there is a deficiency of functional carnitine i.e., a lack of carnitine destined for acylation of fatty acids.

Precursors of carnitine, namely lysine and methionine also reduced hepatic accumulation of lipid though these were not as effective as the carnitine (Table 9, page 80). These data are comparable to observations of Lieber and DeCarli (30) in ethanol-fed rats (36% calories as ethanol) where a significant lipid lowering effect (69.6 $\pm$ 4.9 from 105.3 $\pm$ 14.6 mg total lipid/g liver) of D,L-methionine (2.5 mg/cal) was observed. Whether the effect of lysine plus methionine was being mediated through endogenous biosynthesis of carnitine from these precursors or by enhancing lipoprotein secretion cannot be clearly determined from the current study. A part of the lipotropic effect in the ELM group may be caused by methionine which is a known lipotropic agent (30) or by lysine which is known to promote lipoprotein secretion in ethanol-treated livers (8). It seems unlikely that lysine and methionine exerted their lipotropic effect through additional biosynthesis of carnitine, because the carnitine concentrations in the plasma (Table 8, page 78) and livers (Table 11, page 85) of the ELM group were not higher than those found in plasma and livers of the E group. Additionally, a combination of carnitine, lysine, and methionine failed to produce an additive effect in reducing hepatic lipid accumulation in alcoholic rats (Table 9, page 80). The presence of lysine and methionine with carnitine (ECLM) may even

be interfering with the effect of carnitine (Table 9, page 80, EC vs ECLM), since the lipotropic effect of the combination of all three agents was lower than that of carnitine alone. In the study by Rudman et al. (23), administration of lysine-rich, methionine-rich, and carnitine-free diet to the normal humans maintained normal serum carnitine concentrations (55-103 μmole), but the identical diet to cirrhotic subjects produced serum carnitine concentrations only 25% of normal. In another study (138), supplementation of the basal diet with 0.42% lysine maintained hepatic concentrations of carnitine close to those in the livers of rats fed 0.2% D,L-carnitine diets. These observations indicate that biosynthesis of carnitine from the precursor, lysine was possible when the liver was functioning normally. The data reported in Table 11, page 85 of this study are only an indirect evidence that the biosynthesis of carnitine from its precursors is impaired in the ethanol-compromised liver since the concentrations of carnitine neither in plasma (Table 8, page 78) nor in liver (Table 11, page 85) were significantly increased in the ELM group over that of the E group. It is very possible that supplementary lysine and methionine did not reach the liver in sufficient concentrations because the intestinal absorption of amino acids is usually impaired by ethanol (139). Since carnitine biosynthesis require cofactors like ascorbic acid, pyridoxal, and niacin, their inadequate supply which is not uncommon in alcoholic conditions may further cripple the biosynthetic processes to produce carnitine (122). There is also a possibility

that the enzyme, 4-N-trimethyl-aminobutyraldehyde dehydrogenase may compete for NAD^+ against the alcohol dehydrogenase which may be increased in alcoholic cases (124). Definite evidence for possible impaired carnitine biosynthesis in ethanol-compromised liver must be provided by further research.

Ethanol-induced hypertriglyceridemia (Table 8, page 78, and Figure 16, page 79) is closely related with hypocarnitinemia. Supplementation of carnitine was effective in reducing the plasma triglyceride concentrations close to those found in the plasma of non-ethanol-fed animals (NE). These data are supported by the observations of Hosein and Bexton (24) who reported a reduction in plasma triglycerides (111 $\pm$ 5.2 from 114 $\pm$ 9.9 mg/dl of plasma) by carnitine in rats receiving ethanol (6g/kg body weight) by gastric intubation as a 40% solution (W/V) in saline containing carnitine to provide a dose of 0.1 mg/g body weight. Also in another study by Askew et al. (140) it was reported that 1.0% dietary D,L-carnitine reduced serum triglyceride contents by 21% (77.1 $\pm$ 12.0 to 61.0 $\pm$ 5.1 mg/dl) in non-ethanol-fed rats. On the other hand, the precursors, lysine and methionine failed to reduce plasma triglyceride concentrations significantly, even though they were able to enhance plasma concentrations of total carnitine significantly over that of the E group (Table 8, page 78, and Figure 16, page 79). The hypolipidemic effect of a combination of all three agents (carnitine, lysine, and methionine) was inferior to that of carnitine. Like in the case of liver, it seems that the presence of lysine and

methionine with carnitine interferes with the hypolipidemic effect of carnitine (Table 8, page 78, EC vs ECLM). Reduced concentrations of plasma carnitine in the E group (Table 8, page 78) may be ascribed to confiscation of carnitine by the livers of these rats (Table 11, page 85). In other words, elevated concentrations of carnitine in the alcoholic liver (E) (Table 11, page 85) may be at the cost of plasma carnitine and perhaps the other extrahepatic tissues.

It can be argued that the lipotropic effect of carnitine is due to transformation of carnitine to acylcarnitine, thus, consuming the fatty acid pool which is known to increase in chronic ethanol consumption. However, such a simple explanation can be only a part of the total answer because the increases in acylcarnitines in the liver were too small to affect the large magnitude of decrease in hepatic lipid contents in the supplemented ethanol-fed groups (Tables 9 and 11, pages 80 and 85). In addition, the consistently high concentrations of free fatty acids in the livers of these groups does not concur with this idea. Therefore, while a partial contribution made by acylcarnitine formation may not be entirely disregarded, it would be reasonable to say that carnitine mediates a lipotropic effect via some other mechanisms which need to be investigated.

The lipotropic effect of carnitine in alcoholic fatty liver must be evaluated in comparison with that of some of other dietary agents reported by other investigators. Stanko et al. (22) reported that chronic ethanol consumption (33% of calories as ethanol) caused

a significant increase in the concentrations of triglyceride (67 ± 7.5 vs 22.7 ± 2.5 mg/g) and supplementing the ethanol diet of rats with a combination of pyruvate (22 g/500 ml of diet), dihydroxyacetone (22 g/500 ml), and riboflavin (2.2 g/500 ml) significantly lowered (56.7%) the triglyceride concentrations (29 ± 3.2 vs 67 ± 7.5 mg/g) in the livers of ethanol-treated rats fed high fat (35% of total calories) diet. Goheen et al. (120) demonstrated the beneficial effect of arachidonate for chronic cases. In their study, administration of ethanol to rats (34% of dietary calories as ethanol; 35%, as fat) increased the hepatic concentrations of triglyceride 10-fold (68.4 ± 6.37 vs 7.01 ± 0.02 mg/g). Replacing part of fat in the diet with arachidonate (7% of the fat, W/V) decreased triglyceride contents by 50% (27.56 ± 4.34 vs 68.4 ± 6.37 mg/g). This effect was also observed in phospholipid contents (19.4 ± 2.4 vs 23.1 ± 1.3 mg/g). Thus, compared to these dietary agents, carnitine is as good a lipotropic agent as these compounds in preventing ethanol-induced fatty liver.

No significant effects of dietary carnitine on changes in lipids in organs other than liver were observed (Tables 12 and 13, pages 88 and 89). These results seem reasonable because metabolism of both ethanol and lipid occurs primarily in the liver. From this phase of the study, it can be concluded that dietary carnitine, and to a lesser extent lysine plus methionine, are effective in preventing accumulation of lipids in the livers of rats chronically fed ethanol. The lipotropic effect of the supplementary carnitine

may be due to increased fatty acid acylation perhaps increased β-oxidation of fatty acids and even elimination of acylcarnitines in urine. It is hypothesized that a state of functional carnitine deficiency exists in chronic ethanol-fed rats.

2. DOSE-RELATED EFFECT OF DIETARY CARNITINE

In order to substantiate the lipotropic effect of supplementary carnitine, it was deemed necessary to establish a dose dependent response in chronic alcoholic rats. To provide this information, two consecutive experiments (Experiment II and III) were carried out. In the first experiment (Experiment II), it was not possible to demonstrate significant effects of dietary carnitine levels on ethanol-induced accumulation of hepatic fat. Although the differences were not statistically significant, there was a definite trend. The concentrations of plasma triglyceride were consistently decreased with increasing levels of carnitine supplementation (Table 15, page 92, and Figure 20, page 93). Similarly, the concentrations of both total lipid and triglyceride in liver were progressively reduced with the increasing levels of supplementary carnitine except for the highest level (2.0%) (Table 16, page 95, and Figure 21, page 96). The 2.0% level of supplemental carnitine showed less lipotropic effect than the 1.0% carnitine level. This established the higher limit of supplementation which will result in effective lipotropic action.

A second dose response experiment (Experiment III) was carried out at range of doses different than those used in the Experiment II with more mature animals and for a longer period of treatment. The reasons for designing this study was based on the assumption that the insignificant effects of carnitine found in Experiment II were due to a shorter experimental period which might be insufficient to cause a significant effect and that the animals were younger in age than those used in other alcohol experiments. The results of this experiment established a definite dose-dependent response of carnitine. Various levels of carnitine progressively and significantly decreased the hepatic and plasma lipid concentrations and were well related with the changes in carnitine pools. Concentrations of lipids (mainly total lipid and triglyceride) were decreased with increasing concentrations of carnitine in both plasma and liver. This relationship (inverse) between concentrations of lipid and carnitines was more obvious when the results were plotted by using the quantities of changes in the values as independent variables instead of the values as shown in Figures 26 and 27. It can be seen from these figures that the peak decreases in both total lipid and triglyceride were associated with peak increases in carnitine concentrations (especially in carnitine in plasma and in total carnitine and AIAC in liver). These peak increases and decreases occurred, primarily, in the group getting 0.8% D,L-carnitine. The highest concentration of hepatic total carnitine was found in the E_3 group

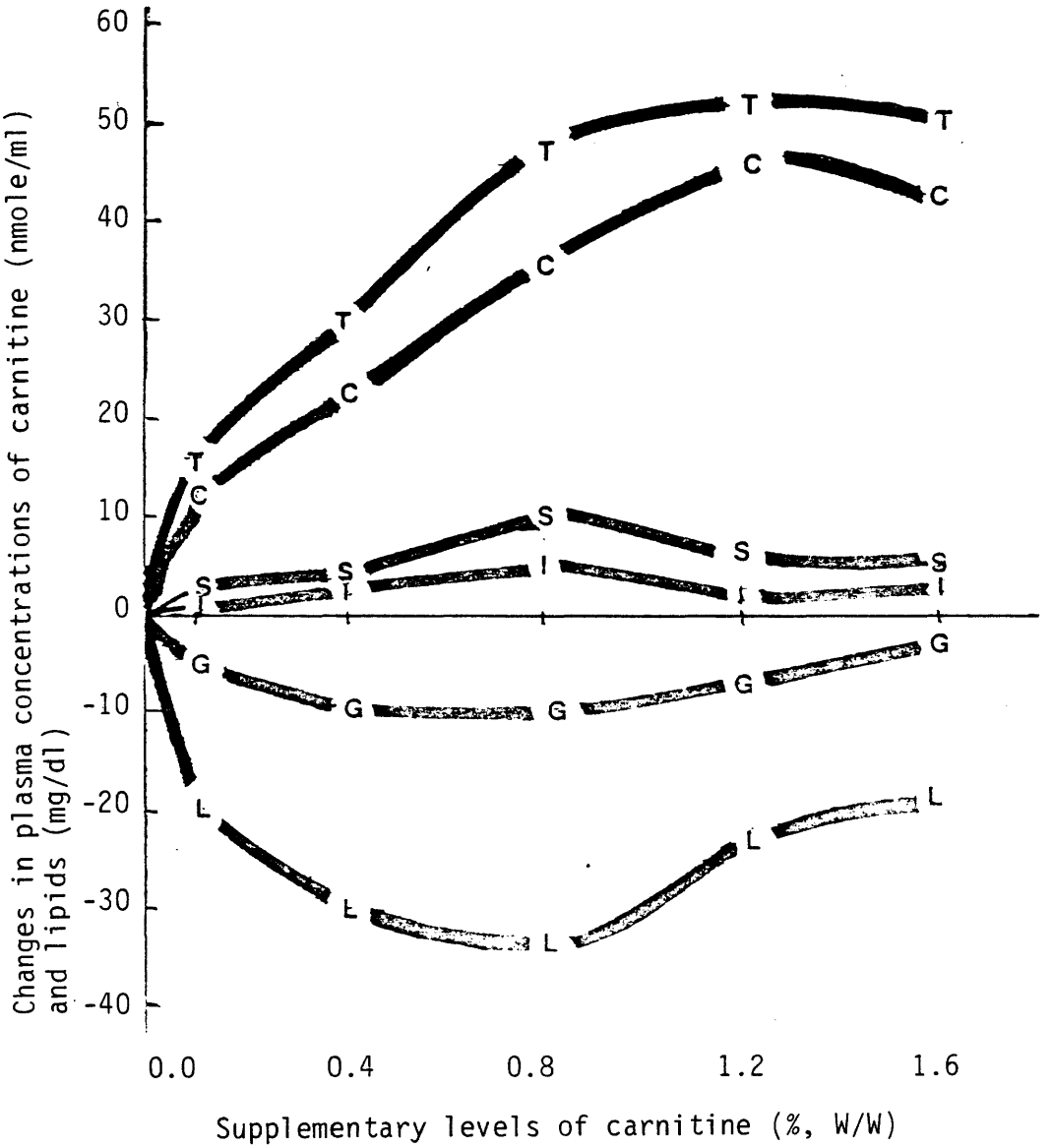

FIGURE 26. Relationship between changes in plasma carnitine species [total carnitine (-T-), carnitine (-C-), ASAC (-S-), and AIAC (-I-)] and lipids [triglyceride (-G-) and total lipid (-L-)].

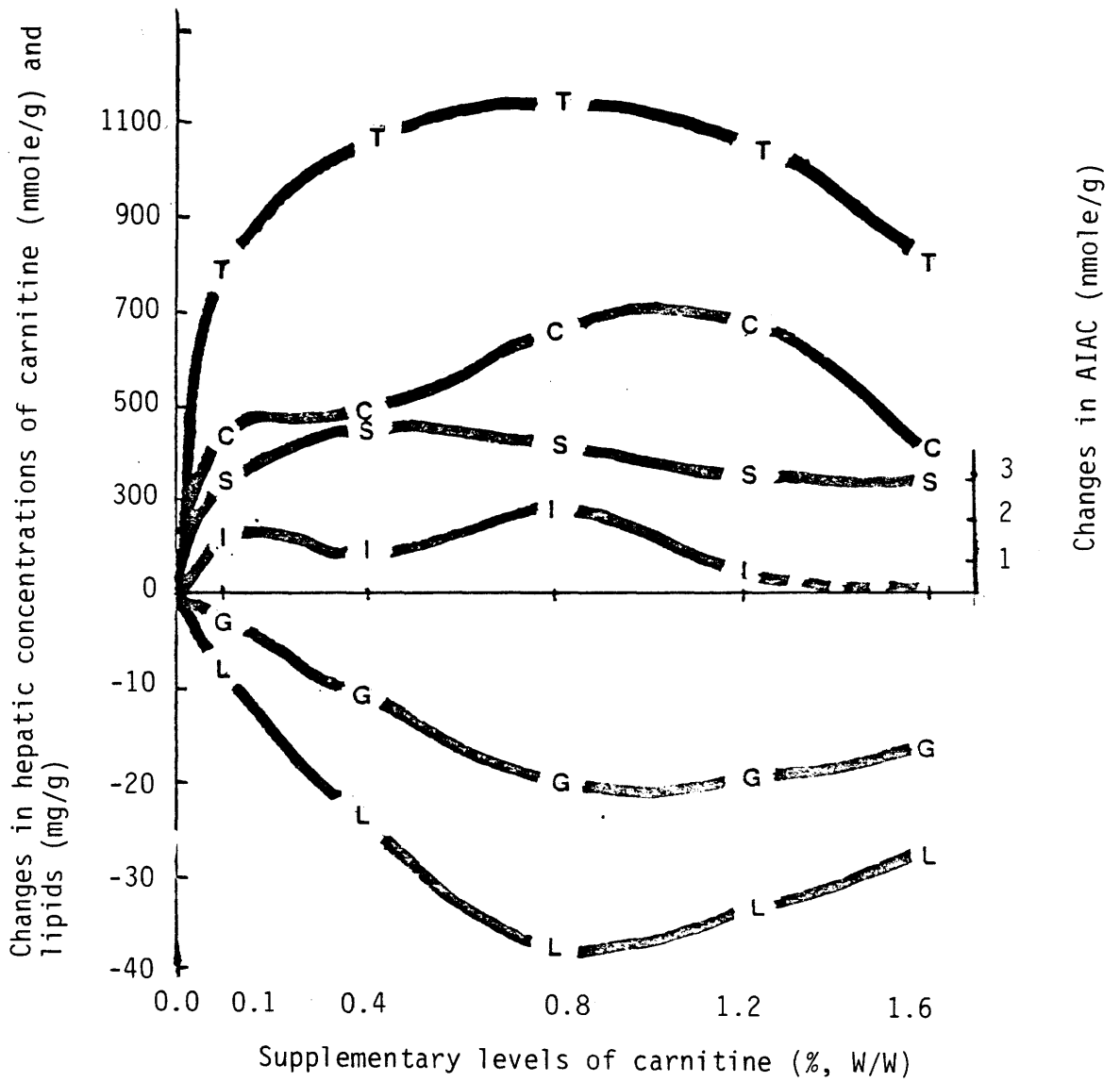

FIGURE 27. Relationship between changes in hepatic carnitine species [total carnitine (-T-), carnitine (-C-), ASAC (-S-), and AIAC (-I-)] and lipids [triglyceride (-G-) and total lipid (-L-)].

(Table 21, page 107), and conversely, the lowest concentrations of various lipids (total lipids, triglycerides, free cholesterol, total cholesterol, and phospholipids) in liver were found in the same group (Table 20, page 105).

In both Experiment II and III, the lipotropic effect was reduced at levels higher than 1.0% carnitine. The mechanisms involved in the reduced lipotropic effect should be assessed by further research. It may be due to inherent toxicity of carnitine at high levels. This may also be due partly to saturation of carnitine in esterification to form acylcarnitine in connection of β-oxidation. In fact, the concentrations of acylcarnitine in the livers of animals receiving carnitine at levels higher than 1.0% was no greater than those seen in the livers of rats receiving 0.8% of carnitine (Table 20, page 105). It is possible that at higher supplementary levels, there was reduced absorption of exogenous carnitine. No doubt at carnitine supplementary levels higher than 1%, the concentrations of both hepatic and plasma carnitines were decreased compared to those found in the E_3 group (Tables 19 and 21, pages 102 and 107). Also there are some indications (141) that D-carnitine may exert a negative effect at high levels on the availability of exogenous L-carnitine and, therefore, on its function as well.

From the observations, it can be concluded that the lipotropic effect of dietary carnitine in alcoholic rats is dose-related with the optimal level of dietary supplementation being 0.8% carnitine.

The overall conclusion is that dietary carnitine is an effective agent for amelioration of ethanol-induced fatty rat liver, and the effect is dose-related with the optimal level being 0.8% carnitine in the diet.

CHAPTER VI

SUMMARY

The effect of dietary carnitine and its precursors of ethanol-induced fatty liver and hypertriglyceridemia was examined in an animal model. Consistent with the observations in the literature, ethanol fed at 36% of total calories to rats produced a significant increase in hepatic concentrations of total lipids, triglycerides, phospholipids, and free and esterified cholesterol as well as elevated plasma concentrations of triglycerides. The elevation of liver lipids occurred despite increased concentrations of hepatic carnitine which is incongruent with its lipotropic potential. Thus, endogenous carnitine was either quantitatively and/or qualitatively inept in ameliorating fatty liver.

It was only when the ethanol diet was supplemented with D,L-carnitine that there was a significant reduction in the accumulation of lipids in the ethanol-compromised liver. The lipotropic effect of the same magnitude did not occur when the diet was supplemented with lysine and methionine despite the fact that hepatic carnitine concentrations were further elevated. Thus, the lipotropic effect was exerted only by exogenously supplied carnitine. From these results, it appears that a functional carnitine deficiency exists in chronic alcoholic rats and only exogenous carnitine can ameliorate this condition by

perhaps increasing the endogenous pool of carnitine to a state facilitating acylation of fatty acids; thus, removing them by β-oxidation or excretion in the urine. On the other hand, there may be more than one pool of carnitine with dietary carnitine belonging to the functional pool.

Precursors of carnitine, lysine, and methionine were about 82% effective as carnitine in lowering ethanol-induced accumulations of hepatic fat. This effect may be mediated by methionine which is a known lipotropic agent or by lysine which is a promoter of lipoprotein secretion. However, it is unlikely that the observed lipotropic effect was due to new biosynthesis of carnitine from these precursors because the concentrations of carnitine in both plasma and liver were not significantly increased in the Lys and Met supplemented group.

The impaired biosynthesis of carnitine from the precursors may be due to either inadequate supply of cofactors like ascorbic acid, pyridoxal, and niacine, or due to unfavorable competition for the NAD^+ by 4-N-trimethyl-aminobutyraldehyde dehydrogenase against alcohol dehydrogenase, the activity of the latter is usually increased in alcoholic cases. However, definite evidence for impaired carnitine biosynthesis must be followed by further research.

The effect of a combination of carnitine, lysine, and methionine was no greater than that of carnitine alone. The presence of lysine and methionine with carnitine seems even to interfere with the effect of carnitine.

Dietary carnitine was also effective in ameliorating ethanol-induced hypertriglyceridemia. However, like in the case of liver, the hypolipidemic effects of combinations of either lysine and methionine or carnitine, lysine, and methionine were inferior to that of carnitine. Similar to the observations in liver, the presence of lysine and methionine with carnitine seemed to interfere with the hypolipidemic effect of carnitine.

The dose response for dietary carnitine was established in mature rats over a seven week period. The concentrations of total lipid and triglyceride were significantly and progressively reduced and those of carnitine were significantly increased with increasing levels of dietary carnitine supplementation up to 0.8% in both liver and plasma. Thus, there is a definite inverse relationship between the concentrations of lipid and carnitine in both liver and plasma. The highest concentrations of total carnitine and the lowest concentrations of various lipids in liver were found in the animals receiving 0.8% carnitine. The lipotropic effect of carnitine was reduced at levels higher than 1.0% supplementation. This may be due to inherent chemical nature (toxicity) of carnitine at high levels, saturation of the esterification process, reduced intestinal absorption of carnitine, or a negative effect of racemic isomers on availability of L-carnitine. Further research is needed to assess the definite mechanisms involved in the reduced lipotropic effect of carnitine over 1.0% supplementation. From

these observations, it is concluded that carnitine is an effective lipotropic dietary nutrient and its effect is dose dependent.

LITERATURE CITED

LITERATURE CITED

1. Sorrell, M. F. & Tuma, D. J. (1979) Effects of alcohol on hepatic metabolism: selected aspected. Clin. Sci. 57, 481-489.

2. Lieber, C. S. (1980) Metabolism and metabolic effects of alcohol. Sem. Hematol. 17, 85-99.

3. Mezey, E. (1978) Liver disease and nutrition. Gastroenterol. 74, 770-783.

4. Hoyumpa, Q. M., Jr., Greene, H. L., Dunn, G. D., & Schenker, S. (1975) Fatty liver: biochemical and clinical considerations. Dig. Dis. 20, 1142-1170.

5. Isselbach, K. J. (1977) Metabolic and hepatic effects of alcohol. In: Seminars in Medicine. (Bleich, H. L. and Boro, E. S., eds.). New Eng. J. Med. 17, 612-616.

6. Wisloff, F. & Boman, D. (1979) Acquired stomatosis in alcoholic liver disease. Scan. J. Hematol. 23, 43-50.

7. Gordon, E. R. (1977) ATP metabolism in an ethanol-induced fatty liver. Alcoholism: Clinical and Experimental Res. 1, 21-25.

8. Baraona, E. & Lieber, C. S. (1970) Effects of chronic ethanol feeding on serum lipoprotein metabolism in the rat. J. Clin. Invest. 49, 769-778.

9. Kondrup, J., Bro, B., Dich, J., Grunnet, N., & Thieden, H. I. D. (1980) Fractionation and characterization of rat hepatocytes isolated from ethanol-induced fatty liver. 43, 182-190.

10. DeCarli, L. M. & Lieber, C. S. (1967) Fatty liver in the rat after prolonged intake of ethanol with a nutritionally adequate new liquid diet. J. Nutr. 91, 331-336.

11. Porta, E. A., Hartroft, W. S., & de la Iglesia, F. A. (1965) Hepatic changes associated with chronic alcoholism in rats. Lab. Invest. 14, 1437-1455.

12. Lieber, C. S., Jones, D. P., & DeCarli, L. M. (1965) Effects of prolonged ethanol intake: Production of fatty liver despite adequate diets. J. Clin. Invest. 44, 1009-1021.

13. Klatskin, G. (1961) Alcohol and its relation to liver damage. Gastroterol. 41, 443-457.

14. Lebach, W. K. (1975) Cirrhosis in the alcoholic and its relation to the volume alcohol abuse. Ann. N. Y. Acad. Sci. 252, 85-94.

15. Smith, L. T., Vickers, A. E., Brendel, K., & Gerhart, M. J. (1982) Effects of ethanol diets on cholesterol content and phospholipid acyl composition of rat hepatocytes. Lipids 17, 124-128.

16. Menelson, J. H. & Mello, N. K. (1973) Alcohol-induced hyperlipemia and β-lipoprotein. Science 180, 1371-1374.

17. Lieber, C. S. & DeCarli, L. M. (1977) Metabolic aspects of alcoholism. pp. 31. University Park Press, Baltimore, MD. (Lieber, C. S., ed.).

18. Editorial. (1978) Toward prevention of alcoholic liver disease. The Lancet 12, 353-358.

19. Rubin, E. & Lieber, C. S. (1968) Alcohol-induced hepatic injury in nonalcoholic volunteers. New Engl. J. Med. 278, 869-876.

20. Brown, D. F. (1966) The effect of ethyl α-P.Chlorophenoxyisobutyrate on ethanol-induced hepatic steatosis in the rat. Metabolism 15, 868-873.

21. Koff, R. S., Carter, E. A., Lui, S., & Isselbach, K. J. (1970) Prevention of ethanol-induced fatty liver in the rat by phenobarbital. Gastroenterol. 59, 50-61.

22. Stanko, R. T., Mendelow, H., Shinozuka, H., & Abidi, S. A. (1978) Prevention of alcohol-induced fatty liver by natural metabolites and riboflavin. J. Lab. Clin. Med. 91, 228-235.

23. Rudman, D., Sewel, C. W., & Ansley, J. S. (1970) Deficiency of carnitine in cachetic cirrhotic patients. J. Clin. Invest. 60, 716-723.

24. Hosein, H. A. & Bexton, B. (1975) Protective action of carnitine on liver lipid metabolism after ethanol administration to rats. Biochem. Pharmacol. 24, 1859-1863.

25. Kondrup, J., & Grunnet, N. (1973) The effect of acute and prolonged ethanol treatment of the contents of coenzyme A, carnitine and their derivatives in rat liver. Biochem. J. 132, 373-379.

26. Hoppel, C. L., Genuth, S., Brass, E., Fuller, R., & Hostetler, K. (1980) Carnitine and carnitine palmitoyl transferase in metabolic studies. In: Carnitine Biosynthesis, Metabolism and Functions. (FranKel, A. and McGarry, J. D., eds.). pp.300-301. New York: Academic Press, NY.

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

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

29. Fritz, I. B., Kaplan, E., & Yue, K. T. N. (1962) Specificity of carnitine action on fatty acid oxidation by heart muscle. Amer. J. Physiol. 202, 117-121.

30. Lieber, C. S. & DeCarli, L. M. (1966) Study of agents for the prevention of the fatty liver produced by prolonged alcohol intake. Gastroenterol. 50, 316-322.

31. Hock, J. B. & Ernster, I. (1974) Mitochondrial transhydrogenase and the regulation of cytolic reducing power. In: Alochol and Aldehyde Metabolizing Systems. (Thurman, R. G., Yonetani, T., Williamson, J. R., and Chance, B., eds.). pp. 351-361. Academic Press, New York.

32. Guynn, R. W., & Pieklik, J. R. (1975) Dependence on dose of the acute effects of ethanol on liver metabolism in vivo. J. Clin. Invest. 56, 1411-1419.

33. Lieber, C. S. & DeCarli, L. M. (1970) Hepatic microsomal ethanol-oxidizing system. In vitro characteristics and adaptive properties in vivo. J. Biol. Chem. 245, 2505-2512.

34. Thurman, R. R. G. (1977) Hepatic alcohol oxidation and metabolic liability. Fed. Proc. 36, 1640-1646.

35. Tottmar, S. O. C., Petterson, H., & Kissling, K. H. (1973) The subcellular distribution and properties of aldehyde dehydrogenases in rat liver. Biochem. J. 135, 577-586.

36. Lundquist, E., Tygstrup, N., Winkler, K., Mellengaard, K., & Munch-Pertersen, S. (1962) Ethanol metabolism and production of free acetate in the human liver. J. Clin. Invest. 41, 955-961.

37. Korsten, M. F., Matsuzaki, S., Feinman, L., & Lieber, C. S. (1975) High blood acetaldehyde levels after ethanol administration. Differences between alcoholic and non-alcohol subjects. New Engl. J. Med. 292, 386-389.

38. Hasumura, Y., Jeschke, R., & Lieber, C. S. (1975) Acetaldehyde oxidation by hepatic mitochondria: Its decrease after chronic ethanol consumption. Science 189, 727-729.

39. Forsander, O. A., Maenpaa, P. H., & Salaspuro, M. P. (1965) Influence of ethanol on the lactate/pyruvate and beta-hydroxybutyrate/acetoacetate ratios in rat liver experiment. Acta. Chem. Scand. 19, 1770-1771.

40. Lieber, C. S., Lefevre, A., & Spritz, N. (1967) Difference in hepatic metabolism of long- and medium-chain fatty acids: The role of fatty acid chain length in the production of the alcoholic fatty liver. J. Clin. Invest. 46, 1451-1460.

41. Ontko, J. A. (1973) Effects of ethanol on the metabolism of free fatty acids in isolated liver cells. J. Lipid Res. 14, 78-86.

42. Williamson, J. R., Scholz, R., & Browning, E. T. (1969) Metabolic effects of ethanol in perfused rat liver. J. Biol. Chem. 244, 5044-5054.

43. Iseri, O. A. & Stenger, R. J. (1964) Ultrastructural alterations in experimental acute hepatic fatty metamorphosis. Lab. Invest. 13, 128-136.

44. Lieber, C. S., Leevy, C. M., & Stein, S. W. (1962) Effect of ethanol on plasma free fatty acids in man. J. Lab. Clin. Med. 59, 826-832.

45. Lieber, C. S., Jones, D. P., & Lowosky, M. S. (1962) Interrelation of uric acid and ethanol metabolism in man. J. Clin. Invest. 41, 1863-1870.

46. Comporti, M., Burdino, E., & Raja, F. (1971) Fatty acids composition of mitochondrial and microsomal lipids of rat liver after acute ethanol intoxication. Life Sci. 10 (part II), 855-866.

47. Johnson, O. (1974) Influence of the blood ethanol concentration on the acute ethanol-induced liver triglycerides accumulation in rats. Scand. J. Gastroenterol. 2, 207-213.

48. Nikkila, E. A. & Ojala, K. (1963) Role of hepatic L-alpha-glycerophosphate and triglyceride synthesis in production of fatty liver by ethanol. Pro. Soc. Exper. Biol. Med. 113, 814-817.

49. Bhagavan, N. V. (1978) Lipids. In: Biochemistry (Bhagavan, N. V., ed.). pp. 807-979. 2nd ed. J. B. Lippincott Co., Philadelphia, PA.

50. Blomstrand, R., Kager, R., & Lantto, O. (1973) Studies on the ethanol-induced decrease of fatty acid oxidation in rat and human liver slices. Life Sci. 13, 1131-1141.

51. Fischel, P. & Oette, K. (1974) Experimentelle Untersuchungen an menschlichen leberpunktaten und Ratten leberschnitten zur Oxidation von Fettsauren mit unterschiedlicher Kettenlange und unterschiedlicher zahl von Doppelbindungen. Res Esp Med (Berl) 163, 1-16.

52. Blomstrand, R. & Kager, L. (1973) The combustion of triolein-1-^{14}C and its inhibition by alcohol in man. Life Sci. 13, 113-123.

53. Lieber, C. S. & Spritz, N. (1966) Effects of prolonged ethanol intake in man: Role of dietary, adipose, and endogenously synthesized fatty acids in the pathogenesis of the alcoholic fatty liver. J. Clin. Invest. 45, 1400-1411.

54. Lieber, C. S., Spritz, N., & DeCarli, L. M. (1966) Role of dietary, adipose, and endogenously synthesized fatty acids in the pathogenesis of the alcoholic fatty liver. J. Clin. Invest. 45, 51-62.

55. Lieber, C. S., Spritz, N., & DeCarli, L. M. (1969) Fatty liver produced by dietary deficiencies: Its pathogenesis and potentiation by ethanol. J. Lipid Res. 10, 283-287.

56. Mendenhall, C. L. (1972) Origin of hepatic triglyceride fatty acids: Quantitative estimation of the relative contributions of linoleic acid by diet and adipose tissue in normal ethanol-fed rats. J. Lipid Res. 13, 177-183.

57. Domschke, S., Domschke, W., & Lieber, C. S. (1974) Hepatic redox state: Attenuation of the acute effects of ethanol induced by chronic ethanol consumption. Life Sci. 15, 1327-1334.

58. Lieber, C. S., deCarli, L. M., & Rubin, E. (1975) Sequential production of fatty liver, hepatitis and cirrhosis in sub-human primates fed ethanol with adequate diets. Proc. Nat. Acad. Sci. U.S.A. 72, 437-441.

59. Grunnet, H. (1973) Oxidation of acelaldehyde by rat-liver mitochondria in relation to ethanol oxidation and the transport of reducing equivalents across the mitochondrial membrane. Eur. J. Biochem. 35, 236-243.

60. Marjanen, L. (1972) Intracellular localization of aldehyde dehydrogenase in rat liver. Biochem. J. 127, 633-639.

61. Cederbaum, A. I., Lieber, C. S., & Rubin, E. (1974) The effect of acetaldehyde on mitochondrial function. Arch. Biochem. Biophys. 161, 26-39.

62. Cederbaum, A. I., Lieber, C. S., & Rubin, E. (1975) Effect of acetaldehyde on fatty acid oxidation and ketogenesis by hepatic mitochondria. Arch. Biochem. Biophys. 169, 29-41.

63. Cederbaum, A. I., Lieber, C. S., & Beattie, D. S. (1975) Effect of chronic ethanol ingestion on fatty acid oxidation by hepatic mitochondria. J. Biol. Chem. 250, 5122-5129.

64. Perin, A., Scalabrino, G., & Sessa, A. (1974) In vitro inhibition of protein synthesis in rat liver as consequence of ethanol metabolism. Biochem. Biophys. Acta. 366, 101-108.

65. Sorrell, M. F., Tuma, D. J., & Schafter, E. C. (1977) Role of actaldehyde in the ethanol-induced impairment of glycoprotein metabolism in rat liver slices. Gastroenterol. 73, 137-144.

66. Feinman, L., Baraona, E., & Matsuzaki, S. (1978) Concentration dependence of ethanol metabolism in vivo rats and man. Alcoholism 2, 381-385.

67. Lieber, C. S. & DeCarli, L. M. (1972) The role of hepatic microsomal ethanol oxidizing system (MEOS) for ethanol metabolism in vivo. J. Pharmacol. exp. Ther. 181, 279-287.

68. Salaspuro, M. & Lieber, C. S. (1977) Non-ADH pathway of alcohol metabolism: Its increase in activity at high ethanol concentrations and after chronic consumption. Gastroenterol. 73, A47/1245.

69. Seitz, H. K., Korsten, M. A., & Lieber, C. S. (1979) Increased oxidation by intestinal microsomes: Increased activity after chronic ethanol administration. Life Sci. 25, 1443-1448.

70. Ohnishi, K. & Lieber, C. S. (1977) Reconstitution of the microsomal ethanol oxidizing system (MEOS): Qualitative and quantitative changes of cytochrome P-450 after chronic ethanol consumption. J. Biol. Chem. 252, 7124-7131.

71. Ishii, H., Jolly, J. G., & Lieber, C. S. (1973) Effect of ethanol on the amount and enzyme activities of hepatic rough and smooth microsomal membranes. Biochemica et Biophysica Acta 291, 411-420.

72. Hasumura, Y., Teschke, R., & Lieber, C. S. (1974) Increased carbon tetrachloride hepatotoxicity, and its mechanism, after chronic ethanol consumption. Gastroenterol. 66, 415-422.

73. Garner, R. C., & McLean, A. E. M. (1969) Increased susceptibility to carbon tetrachloride poisoning in the rat after pretreatment with oral phenobarbitone. Biochem. Pharmacol. 18, 645-650.

74. Rubin, E. & Lieber, C. S. (1968) Hepatic microsomal enzymes in man and rat: Induction and inhibition by ethanol. Sci. 162, 690-691.

75. Cohen, G. M. & Mannering, G. J. (1973) Involvement of a hydrophobic site in the inhibition of the microsomal P-hydroxylation of aniline by alcohols. Molecular Pharmacol. 9, 383-397.

76. Rubin, E., Gang, H., Misra, P. S., & Lieber, C. S. (1970) Inhibition of drug metabolism by acute ethanol intoxication: A hepatic microsomal mechanism. Amer. J. Med. 49, 801-806.

77. Mallov, S. & Baesl, T. J. (1972) Effects of ethanol on rats of elimination and metabolism of zoxazolamine, hexabarbital and warfarin sodium in the rat. Biochem. Pharmacol. 21, 1667-1678.

78. Cinti, D. L., Grundin, R., & Orrenius, S. (1973) The effect of ethanol on drug oxidation in vitro and the significance of ethanol-cytochrome P-450 interaction. Biochem. J. 134, 367-375.

79. Joly, J. G. & Hetu, C. (1975) Effects of chronic ethanol administration in the rat: Relative dependency on dietary lipids- I. Induction of hepatic drug-metabolizing enzymes in vitro. Biochem. Pharmacol. 24, 1475-1480.

80. Joly, J. G., Villeneuve, J. P., & Mavier, P. (1977) Chronic ethanol administration induces a form of cytochrome P-450 with specific spectral and catalytic properties. Alcoholism. Clin. Exper. Res. 1, 17-20.

81. Mallov, S. & Bloch, J. L. (1956) Role of hypophysis and adrenals in fatty infiltration of liver resulting from acute ethanol intoxication. Am. J. Physiol. 184, 29-34.

82. Kalant, H., Khanna, M., Seymour, F., & Loth, J. (1975) Acute alcoholic fatty liver. Metabolism or stress. Biochem. Pharmacol. 24, 431-434.

83. Forbes, J. C. & Duncan, G. M. (1951) The effect of acute alcohol intoxication on the adrenal glands of rats and guinea pigs. Quart. J. Stud. Alcohol. 12, 355-359.

84. Perman, F. S. (1961) Observations on the effect of ethanol on the urinary excretion of histamine, 5-hydroxy-indolacetic acid, cathecolamine and 17-hydroxycorticosteroids in man. Acta Physiol. Scand. 51, 62-67.

85. Feugelson, E. B., Pfaff, W. W., Karmen, A., & Steinberg, D. (1961) The role of plasma free fatty acids in the development of fatty liver. J. Clin. Invest. 40, 2171-2179.

86. Baraona, E. & Lieber, C. S. (1979) Effects of ethanol on lipid metabolism. J. Lipid Res. 20, 289-315.

87. Baraona, E., Piola, R. C., & Lieber, C. S. (1974) Small intestinal damage and changes in cell population produced by ethanol ingestion in the rat. Gastroenterol. 66, 226-234.

88. Strubelt, O., Sirgers, C. P., & Breining, H. (1972) Die akute hepatotoxische wirkung unterschiedlich kozentrieter Alkohollosungen bei oraller und intraperitonealer application. Arch. Toxical. 29, 129-141.

89. Lieber, C. S., Jones, D. P., Mendelson, J., & DeCarli, L. M. (1963) Fatty liver, hyperlipemia and hyperuricemia produced by prolonged alcohol consumption despite adequate dietary intake. Trans. Assoc. Am. Physicians 76, 289-300.

90. Wiebe, A., Lundquist, A., & Belfrage, P. (1971) Time course of liver fat accumulation in man after a single load of ethanol. Scand. J. Clin. Lab. Invest. 27, 33-36.

91. Mortinore, G. E. & Mondon, C. E. (1970) Inhibition by insulin of valine turnover in the liver. Evidence for a general control of proteolysis. J. Biol. Chem. 245, 2375-2383.

92. Baraona, E., Leo, M. A., Borowsky, S. A., & Lieber, C. S. (1977) Pathogenesis of ethanol-induced accumulation of protein in the liver. J. Clin. Invest. 60, 546-554.

93. Shaw, S. & Lieber, C. S. (1980) Increased hepatic production of α-amino-n-butyricacid after chronic alcohol consumption in rats and baboons. Gastroenterol. 78, 108-113.

94. Patek, A. J., Bowry, S. C., & Sabesin, S. M. (1976) Minimal hepatic changes in rats fed alcohol and a high casein diet. Arch. Pathol. Lab. Med. 100, 19-24.

95. Matsuda, Y., Worner, T., & Lieber, C. S. (1979) Ultrastructure and pathogenesis of perivenular sclerosis (PVS) in alcoholic liver injury. Gastroenterol. 77, A26.

96. Feinman, L. & Lieber, C. S. (1972) Hepatic collagen metabolism: Effect of alcohol consumption in rats and baboons. Sci. 176, 795.

97. Freinkel, N. & Arkey, R. A. (1966) Effects of alcohol on carbohydrate metabolism in man. Psychosom. Med. 23, 551-563.

98. Lieber, C. S. & DeCarli, L. M. (1970) Quantitative relationship between amount of dietary fat and severity of alcoholic fatty liver. Am. J. Clin. Nutr. 23, 474-478.

99. Baraona, E., Les, M. A., Borowsky, S. A., & Lieber, C. S. (1975) Alcoholic hepatomegaly: Accumulation of protein in the liver. Sci. 190, 794-795.

100. Horning, M. G. E. A., Williams, H., Maling, M., B. B. (1960) Depot fat as source of increased liver triglycerides after ethanol. Biochem. Biophys. Res. Commun. 3, 635-640.

101. Abrams, M. A. & Cooper, C. (1976) Quantitative analysis of metabolism of hepatic triglyceride in ethanol-treated rats. Biochem. J. 156, 33-46.

102. Abrams, M. A. & Cooper, C. (1976) Mechanisms of increased hepatic uptake of unesterified fatty acid from serum of ethanol-treated rats. Biochem. J. 156, 47-54.

103. Sheig, R. (1971) Lipid synthesis from ethanol in liver. Gastroenterol. 60, 751-759.

104. Mendenhall, C. L., Brandford, R. H., & Furman, R. H. (1969) Effects of enthanol on glycerolipid metabolism in rat liver. Biochem. Biophys. Acta. 187, 501-509.

105. Lefevre, A. F., DeCarli, L. M., & Lieber, C. S. (1972) Effect of ethanol on cholesterol and bile acid metabolism. J. Lipid Res. 13, 48-55.

106. Mahadevan, S. & Tappel, A. L. (1968) Lysosomal lipases of rat liver and kidney. J. Biol. Chem. 243, 2819-2854.

107. Takeuchi, N., Ito, M., & Yamamura, Y. (1974) Esterification of cholesterol and hydrolysis of cholesteryl ester in alcohol induced fatty liver of rats. Lipid 9, 353-357.

108. Schaourim, R. H., Drummey, G. D., Shimizu, Y., & Isselbach, K. J. (1964) Studies on the pathogenesis of ethanol-induced fatty liver. II. Effect of ethanol on palmitate-1-^{14}C metabolism by isolated perfused rat liver. J. Clin. Invest. 43, 1338-1347.

109. Maddrey, W. C. & Boyer, J. L. (1973) The acute and chronic effects of ethanol administration on bile secretion in the rat. J. Lab. Clin. Med. 82, 215-225.

110. Beaumont, J. L., Carlson, L. A., Cooper, G. R., Fejfar, Z., Fredrikson, D. S., & Strasser, T. (1970) Classification of hyperlipidaemias and hyperlipoproteinaemias. Bull. WHO. 43, 891-915.

111. Avogadro, P. & Gazzolato, G. (1975) Changes in the composition and physico-chemical characteristics of serum lipoproteins during ethanol-induced lipidaemia in alcoholic subjects. Metabolism 24, 1231-1242.

112. Talbott, G. D. & Keating, B. M. (1962) Effects of pre-prandial whisky on postalimentary lipemia. Geriatrics 17, 802-808.

113. Wilson, D. E., Schreibman, P. H., Brewster, A. C., & Arky, R. A. (1970) The enhancement of alimentary lipemia by ethanol in man. J. Lab. Clin. Med. 75, 264-274.

114. Seakins, A. & Robinson, D. S. (1964) Changes associated with the production of fatty livers by white phosphorus and by ethanol in the rat. Biochem. J. 92, 308-312.

115. Glomset, J. A. (1970) Physiological role of lecithincholesterol acyltransferase. Am. J. Clin. Nutr. 23, 1129-1136.

116. Marzo, A., Ghirardi, P., Sardini, D., Prandini, B. D., & Albertini, A. (1970) Serum lipids and total fatty acids in chronic alcohol liver disease at different stages of cell damage. Klin. Sochenschr. 48, 949-950.

117. Borowsky, S. A., Perlow, S., Baraona, E., & Lieber, C. S. (1976) Disappearance of alcoholic hyperlipemia as a sign of advancing liver damage. Gastrenterol. 70, 987.

118. Jabbari, M. & Leevy, C. M. (1967) Protein anabolism and fatty liver of the alcoholic. Medicine 46, 131-137.

119. Porta, E. A., Koch, O. R., & Gomez-Dumm, C. L. A. (1968) Effects of dietary protein on the liver of rats in experimental chronic alcoholism. J. Nutr. 94, 437-441.

120. Goheen, S. C., Larkin, E. C., Mamix, M. & Rao, G. A. (1980) Dietary arachidonic acid reduces fatty liver, increase diet consumption and weight gain in ethanol-fed rat. Lipids 15, 328-336.

121. Friedman, S. & Fraenkel, G. S. (1972) Carnitine. In: The vitamins. (Sebrell, W. H. and Harris, R. B., eds. 2nd ed.). pp. 329-355. New York Academic Press, New York.

122. Broquist, H. P. & Boru, P. R. (1982) Carnitine Biosynthesis. Nutritional implications: In: Advanced Nutritional Research. (Draper, H. H., ed.). Vol. 4, pp. 181-200. Plantum Press, New York.

123. Cederblad, G. & Lindstedt, S. (1972) A method for the determination of carnitine in the picomole range. Clin. Chem. Acta. 37, 235-243.

124. Sachan, D. S., Rhew, T. H., & Ruark, R. (1984) Ameliorating effects of carnitine and its precursors on alcohol-induced fatty liver. Am. J. Clin. Nutr. 39, 738-744.

125. Gornall, A. G., Bardwill, C. J., & David, M. M. (1949) Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177, 751-766.

126. Elleston, R. D. & Caraway, W. T. (1976) Lipids and liproteins. In: Fundamental of Clinical Chemistry. (Tietz, N. W., ed.). pp. 492-494. W. B. Saunder Co., Philadelphia, PA.

127. Giegel, J. L., Ham, A. B., & Glema, W. (1975) Manual and semi-automated procedures for measurement of triglyceride in serum. Clin. Chem. 21, 1575-1581.

128. Ceriotti, G. J. (1952) A microchemical determination of deoxiribonucleic acid. J. Biol. Chem. 198, 297-303.

129. Sardesai, V. M. & Manning, J. A. (1968) The determination of triglycerides in plasma and tissues. Clin. Chem. 14, 156-161.

130. Searcy, R. L. & Berquist, L. M. (1960) A new color reaction for the quantitation of serum cholesterol. Clin. Chem. 5, 192-199.

131. Connerty, H. V., Briggs, A. R., & Eaton, E. H., Jr. (1961) Simplified determination of the lipid components of blood serum. Clin. Chem. 7, 37-53.

132. Novak, M. (1965) Colorimetric ultramicro method for the determination of free fatty acids. J. Lipid Res. 6, 431-433.

133. Frings, C. S.& Dunn, R. T. (1970) A colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reaction. Am. J. Clin. Path. 53, 89-91.

134. Itaya, K. & Ui, M. (1965). Colorimetric determination of free fatty acids in biolofical fluids. J. Lipid Res. 6, 16-20.

135. Dole, V. P. (1956) A relation between non-esterified fatty acids in plasma and the metabolism of glucose. J. Clin. Invest. 35, 150-154.

136. Steel, R. G. D. & Torrie, J. H. (1960) Principles and procedures of statistics. pp. 67-87. New York, NY, McGraw Hill Book Co.

137. Folch, J., Lees, M., & Stanley, H. S. (1957) A simple method for the isolation and purification of total lipids from animal tissue. J. Biol. Chem. 226, 497-501.

138. Tanphaichitr, V., Zaklama, M. S., & Broquist, H. P. (1976) Dietary lysine and carnitine: Relation to growth and fatty livers in rats. J. Nutr. 106, 111-117.

139. Israel, Y., Salazar, I., & Rosenmann, E. (1968) Inhibiting effects of alcohol on intestinal amino acid transport in vivo and in vitro. J. Nutr. 96, 499-504.

140. Askew, E. W., Dohm, G. L., Weiser, P. C., Huston, R. L., & Doub, W. H., Jr (1980) Supplemental dietary carnitine and lipid metabolism in excercising rats. Nutr. Metab. 24, 32-42.

141. Rebouche, C. J. (1983) Effect of dietary carnitine isomers and γ-butyrobetaine on L-carnitine biosynthesis and metabolism in the rat. J. Nutr. 113, 1906-1913.

VITA

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