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ARTERIAL AND PLASMA CARNITINE METABOLISM

STUDIES OF CARNITINE METABOLISM IN RABBIT ARTERIAL TISSUE AND PLASMA: INFLUENCE OF DIETARY CHOLESTEROL

By

PETER JOHN GILLIES, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

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Doctor of Philosophy

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DOCTOR OF PHILOSOPHY

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Studies of Carnitine Metabolism in Mammalian Arterial Tissue and Plasma: Influence of Dietary Cholesterol

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ABSTRACT

Arterial and plasma carnitine metabolism was investigated in normal and atherosclerotic rabbits. Atherogenic cholesterol-supplemented diets induced hypercholesterolemia in rabbits; hypercholesterolemia was associated with hypercarnitinemia and increased levels of carnitine and acylcarnitines in atherosclerotic aortas. Carnitine derived from the bloodstream started to accumulate in aortas of animals fed cholesterol-supplemented diets before the development of gross atherosclerotic lesions; this response of the aorta to hypercholesterolemia and hypercarnitinemia was not shared by the heart. Carnitine palmitoyltransferase (CPT), a key enzyme in fatty acid metabolism, was also investigated in normal and atherosclerotic aortas. CPT activity was associated with mitochondrial and microsomal fractions isolated from rabbit aortas. The location of CPT activity in the aorta differed from that reported for heart and liver; in these tissues, CPT activity is exclusively mitochondrial. Arterial CPT activity was not affected by the addition of cholesterol to the animal's diet. It is hypothesized that acyl-CoA in atherosclerotic tissue is predominantly esterified to carnitine rather than to glycerol-3-phosphate; supporting this hypothesis was the observation that long-chain acylcarnitines increased in atherosclerotic aortas. The hypothesis offers an explanation for several biochemical changes that occur in fatty acid metabolism in atherosclerosis.

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INTRODUCTION

The concentration of carnitine and activity of carnitine acyltransferase enzymes are important factors in the regulation of many events in fatty acid metabolism. Age, sex, diet, drugs and hormones are well documented factors that influence tissue carnitine concentration and carnitine acyltransferase activity; less well documented are changes in carnitine metabolism associated with pathologic conditions. Since 1973, several human lipid storage myopathies attributable to carnitine deficiency have been reported, including a potentially fatal syndrome termed systemic carnitine deficiency (1). Clearly, a better understanding of carnitine metabolism with respect to disease is needed.

I. Carnitine: Structure-Function Relationships

A. Carnitine

The chemical structure of carnitine (4-trimethylamino-3-hydroxybutyric acid) is presented in Figure 1 below:

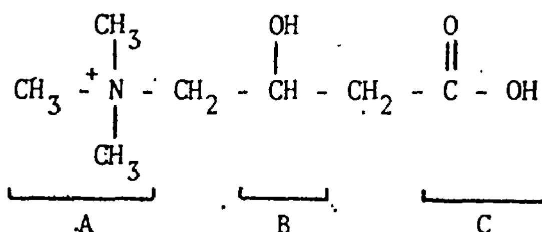


Figure 1. Chemical Structure of Carnitine

The biological activity of carnitine depends on A) a trimethylamino group at the 4-carbon position, B) a hydroxyl group at the 3-carbon position, and C) a carboxyl group (2). The trimethylamino group can be replaced by a dimethylamino group and the hydroxyl group by a thiol group without incurring a major loss in the biological activity of carnitine (3,4). Only the naturally occurring (-)-enantiomer of carnitine is biologically active; (+)-carnitine is generally a competitive inhibitor of (-)-carnitine (5).

B. Acylcarnitines

The O-acyl esters of carnitine comprise a heterogeneous group of compounds. Carnityl esters can be: long or short chain aliphatic compounds (e.g. palmitoylcarnitine or acetylcarnitine) saturated or unsaturated (e.g. stearoylcarnitine or oleoylcarnitine) straight or branched-chain (e.g. butyrylcarnitine or isobutyrylcarnitine) or contain an odd number of carbons (e.g. propionylcarnitine). Analysis of carnityl esters can often provide insight into the biological functions of acylcarnitines. Identification of the branched-chain carnityl esters of valerylcarnitine and isobutyrylcarnitine led to the hypothesis that branched-chain acylcarnitines are involved in amino acid metabolism (6).

Crystal X-ray diffraction analysis of acetylcarnitine and acetylcholine indicates that these compounds have similar conformations (7), this similarity may explain the cholinergic properties of acetylcarnitine (8).

Palmitoylcarnitine is an amphipathic compound and can exist in three configurations i.e. as an inner salt or zwitterion, which are electroneutral, and as a cation (9). The cationic form of palmitoylcarnitine but not acetylcarnitine or carnitine can penetrate artificial phospholipid membranes; this property of palmitoylcarnitine may be important in the translocation of fatty acids, as acylcarnitines, across the inner mitochondrial membrane (9). The zwitterionic form of palmitoylcarnitine is wedge-shaped, some wedge-shaped molecules e.g. lysophosphatidylcholine, are highly lytic molecules (10). The observation that palmitoylcarnitine causes lysis of erythrocytes, mitochondria, and lipoprotein complexes (11,12,13,14) may be related to its wedge-shaped molecular configuration.

Palmitoylcarnitine, like palmitoyl-CoA, forms micelles in aqueous environments. The critical micelle concentration for palmitoylcarnitine (15 μM) is higher than for palmitoyl-CoA (3-4 μM) (15,16).

Acylcarnitines have high acyl-group potentials. The standard free energy change of hydrolysis for carnityl esters of chain length $C_2 - C_{12}$ is - 7.9 kcal/mole; this value is similar to that of palmitoyl-CoA i.e. - 7.7 kcal/mole (17). Consequently, acylcarnitines, like acyl-CoAs, represent an "activated" form of fatty acids.

C. Phosphatidyl Carnitine

Little is known about phosphatidyl carnitine other than the fact that it probably represents what is referred to in the older

carnitine literature as "lipid-bound" carnitine. Dipalmitoyl-phosphatidyl carnitine can be synthesized chemically; it is hydrolyzed by phospholipases A_2 and C but not by phospholipase D (18). Phosphatidyl carnitine has been isolated from developing chick embryo and may have some role in development (18).

II. Concentration and Distribution of Carnitine Compounds

A. Early Analytical Methods and Carnitine Surveys

A major problem in early carnitine research was the analysis of carnitine in biological tissues. For example, carnitine concentrations (μg/g dry weight) reported for rat liver ranged from 100 - 200 using the Tenebrio Bioassay to 400 - 1400 using the Frog Rectus Assay (8). The early carnitine assays lacked specificity and sensitivity for carnitine, these problems were overcome in 1963 with the development of a simple, accurate, enzyme assay involving the use of the chromogenic agent 5,5'-Dithiobis-2-nitrobenzoic acid and carnitine acetyltransferase (19). Despite technical drawbacks of the early carnitine assays, surveys based on these assays revealed that carnitine has a ubiquitous presence in microorganisms, plants, and animals (20).

B. Concentration and Distribution of Carnitine and Acylcarnitine Compounds in Rat Tissues

The data in Table I illustrates the wide variation in the concentration and distribution between carnitine and acylcarnitine

Table I

Concentration and Distribution of Carnitine and Acylcarnitines in Various Tissues from the Rat[a]

Tissue	Net carnitine[b]	Carnitine	Short-chain[c] acylcarnitines	Long-chain[d] acylcarnitines
	Concentration (nmoles/g of frozen tissue)			
Epididymis	6200	5660	540	-
Heart	831	302	477	52
Muscle	627	442	185	-
Liver	307	173	123	11
Brain	50	43	7	-
Epididymal fat pad	16	10	6	-

[a] Adapted from Pearson, D.J., and Tubbs, P.K. (1967). Carnitine derivatives in rat tissues. *Biochemical Journal 105*, 953-963.

[b] Net carnitine = carnitine + short-chain acylcarnitines + long-chain acylcarnitines.

[c] Carnityl esters of chain length C_2-C_{10}.

[d] Carnityl esters of chain length C_{12} and longer.

compounds in different rat tissues. High net carnitine (carnitine + acylcarnitine) concentrations are found in epididymis, heart and skeletal muscle; low net cartinine concentrations are found in brain and epididymal fat pad. In heart, liver and skeletal muscle, 30 - 60% of the net carnitine occurs in acylcarnitine compounds; the major acylcarnitines in these tissues are acetylcarnitine, propionylcarnitine and butyrylcarnitine (21). In contrast, the net carnitine in brain and epididymis occurs primarily as carnitine.

The concentration and distribution of carnitine compounds may reflect their physiological role in a particular tissue. High concentrations of carnitine in heart and other muscle may reflect the utilization of fatty acids by these tissues as a major source of energy; the observation that the carnitine concentration of the brain (which relies primarily on glucose as a source of energy) is 1/16th of that observed in the heart supports this idea (Tables I and II).

Epididymis contains the highest net carnitine concentration of all rat tissues surveyed and nearly 100% of the net epididymal carnitine exists as carnitine. These observations suggest that the function of carnitine in epididymal tissue may be different from that in other tissues. In this regard, a role for carnitine in epididymal sperm maturation has been proposed that is independent of its known effects on fatty acid oxidation. The high concentration of carnitine in the epididymal fluid (63 mM) reduces oxygen uptake and induces changes in the glycoprotein composition on the surface membranes of sperm (22). Although the significance of these changes are poorly understood, they may be important in

maintaining sperm in a resting state during their stay in the epididymis.

Whereas the concentration and distribution of carnitine compounds have been extensively studied at the tissue level, their subcellular concentration and distribution have received little attention. The distribution of carnitine and CoA between cytoplasmic and mitochondrial compartments may be a regulatory factor in heart and liver lipid metabolism. The high ratio of carnitine:CoA (100:1) in the cytoplasm of heart muscle may direct fatty acids toward β-oxidation by promoting acylcarnitine formation; in contrast, the low ratio of carnitine:CoA (17:1) in the cytoplasm of liver may direct fatty acids toward complex lipid biosynthesis (23). The proposed regulatory role for the characteristic carnitine:CoA ratios given above is supported by the fact that the predominant fates of fatty acids in heart and liver are oxidation and esterification, respectively.

C. Concentration of Carnitine in Human Tissues

There is only limited information in the literature concerning carnitine concentrations in human tissue; Table II presents some of this information. High carnitine concentrations are found in heart and skeletal muscle, low carnitine concentrations are found in brain; this pattern is similar to that observed in the rat (Table I).

The concentration of carnitine in human skeletal muscle shows a positive correlation with the enzyme activities of 3-hydroxy-acyl-CoA dehydrogenase and citrate synthetase, this correlation is consistent

Table II

Concentration of Net Carnitine in Various Tissues of Man[a]

Tissue	Net carnitine concentration[b] (µmoles/g wet weight)
Heart	4.8
Pectoral Muscle	3.2
Liver	2.9
Kidney	1.0
Brain	0.3

[a] Adapted from Rudman, D., Sewell, C.W., and Ansley, J.D. (1977). Deficiency of carnitine in cachectic cirrhotic patients. *The Journal of Clinical Investigation 60*, 716-723.

[b] Net carnitine = carnitine + short-chain acylcarnitines + long-chain acylcarnitines.

with a coupled relationship between tissue carnitine concentration and tissue capacity for fatty acid oxidation (24).

III. Factors Influencing the Concentration or Distribution of Carnitine and Acylcarnitines in Tissues and Body Fluids

A. Species Differences

The carnitine concentration of skeletal muscle exhibits marked species differences. Some reported carnitine concentrations (μmoles/g dry weight; based on the same assay) for skeletal muscle are: mice 1.7; rats 3.1; man 16.2; and sheep 50 (25).

Urinary carnitine excretion rates differ between species. Rats and humans excrete 2-3 and 86-175 μmoles of carnitine/day, respectively; this represents 7 and 0.5% of the total carnitine pool in rats and humans (25,26).

Plasma carnitine, in contrast to tissue carnitine, exhibits a similar concentration in several species e.g. rats 38 μM, rabbits 30-40 μM and man 46 μM (25,27,28).

B. Sex

Sex differences in carnitine levels occur in muscle, plasma and urine. Men exhibit slightly higher levels of carnitine (20%) than women with respect to rectus abdominus muscle (e.g. 15.4 *vs* 12.9 μmoles/dry weight, male *vs* female) (29). This sex difference does not occur in all muscles as there are, for example, no differences in the concentration of carnitine in the lower leg muscles of men and women (29).

Plasma carnitine levels are lower in women (47 μM) than men (57 μM) (30), however, this difference has not been observed in all studies (31).

Men excrete twice as much carnitine per day as women e.g. 175 *vs* 86 μmole/day (26). An age factor is probably involved in this sex difference since men and women over 51 years of age excrete similar amounts of carnitine.

Plasma and urine carnitine levels change in women during the menstrual cycle. Plasma and urine carnitine concentrations increase toward ovulation, peak on the day of ovulation and decrease thereafter to pre-ovulation levels (32). These observations suggest that the sex differences in carnitine levels may be attributable, in part, to hormonal differences between men and women.

C. Temperature

Cold acclimated rats exhibit an increase in tissue carnitine. In early studies of the effects of temperature on tissue carnitine levels, rats maintained at 5°C *vs* 25°C for six weeks exhibited an 8-fold increase in body carnitine pool size and a 6-fold increase in the concentration of carnitine plus acylcarnitines in muscle (33). Recent studies confirm an increase in body carnitine pool size in cold-exposed rats but suggest that the magnitude of the change is only of the order of 20-40% (34,35). It is not known how long rats must be exposed to low temperatures before changes in carnitine levels occur but rats that have been exposed to 4°C for 20 days already exhibit a 2-fold increase in carnitine plus acylcarnitine

levels in brown adipose tissue (36). Increased carnitine levels in animals exposed to low temperatures may contribute to the development of non-shivering thermogenesis (36,37).

D. Growth and Development

The concentration of carnitine and acylcarnitines changes during the growth and development of rats and pigs. In neonatal rat liver, at birth the level of carnitine plus acylcarnitines is high but decreases 6-fold during the suckling period and approaches adult levels by the time of weaning. The opposite trend occurs in the heart; at birth the level of carnitine is low but during the suckling period carnitine increases 4-fold and approaches adult levels by the time of weaning (38). In neonatal pig, the concentration of carnitine in the liver doubles within 24 hours postpartum; in contrast to rat liver, the carnitine level remains elevated for several weeks thereafter (39).

The acylation state of carnitine changes with age in some tissues. In the rat, between day 5 and day 45 postpartum, the percentage of net carnitine found as acetylcarnitine decreases 20% in heart and skeletal muscle, while increasing slightly in the liver (37). In embryonic chick liver, acetylcarnitine, which is not detectable prior to the second week of incubation, increases 30% during the third week of incubation (40). Changes in the acylation state of carnitine during growth and development probably reflect changes in the activity of carnitine acyltransferase enzymes.

Plasma carnitine levels increase with age. In man, plasma carnitine concentrations in adults (46 μM) are 35% higher than in children (34 μM) (31). Although there is a positive correlation between age and plasma carnitine levels in adult women, this correlation does not exist in adult men (30).

Maternal-fetal and maternal-neonatal carnitine relationships may be important during early periods of growth. Since it is unlikely that fetal-livers can synthesize sufficient carnitine to meet the needs of the newborn animal (41,42), the major source of carnitine for the neonate is probably the mother's milk which is particularly rich in carnitine (e.g. 0.13 and 0.32 mM in pigs and rats, respectively (38,39). Mother's milk may also be a major source of carnitine for human infants. Little is known concerning the role of carnitine in growth and development, however, it is noteworthy in this regard that administration of carnitine to premature babies and small-for-dates has beneficial effects on appetite and growth (43).

E. Nutrition

Nutritional factors influence the concentration and distribution of carnitine and acylcarnitines in tissues and body fluids. Conditions leading to an increase in the availability of fatty acids to tissues, such as fasting, or high-fat intake, elicit a relatively general response in liver, heart, kidney, and adipose tissue; fasting and high-fat diets increase the concentration of long-chain acylcarnitines and the ratio of long-chain acylcarnitine: carnitine several-fold (44,45).

Carbohydrate-rich diets decrease the ratio of long-chain acylcarnitine:carnitine several fold in heart and liver (44). Similarly, in liver, kidney and adipose tissue, fasting and high-fat diets increase the ratio of short-chain acylcarnitine : carnitine several fold, whereas carbohydrate diets decrease this ratio several fold. Fasting has no effect on the ratio of short-chain acylcarnitine: carnitine in the heart but a high-fat diet decreases this ratio (45). It has been proposed that in liver the carnitine acylation ratio reflects the direction of lipid metabolism i.e. the carnitine acylation ratio increases during increased fatty acid oxidation and decreases during increased fatty acid synthesis (45).

Fasting has a variable effect on urinary carnitine excretion which may be species-dependent. Men fasted for 5 days show a 5-fold increase in urinary carnitine excretion; re-feeding causes urinary carnitine excretion to return to pre-fasting levels (32). In contrast, rats exhibit a slight decrease in urinary carnitine excretion during fasting (35). There may be a diurnal variation in carnitine excretion in man (26) although this has not been a consistent observation (32).

Dietary carnitine can increase tissue carnitine concentration. In the rat, addition of 0.2% carnitine to carnitine-deficient diets results in a 2-3 fold increase in the concentration of carnitine in liver, skeletal muscle and kidney (1). Additions of 0.2% choline or 0.2% lysine to the same carnitine-deficient diet increases tissue

carnitine concentration but only to a limited extent (ca 20%). The greatest increases in tissue carnitine levels occur when the diet is supplemented with a combination of carnitine + choline + lysine (35,36,46). No studies have been reported in which tissue carnitine levels were measured following the addition of carnitine to nutritionally balanced diets.

F. Disease

A number of diseases are associated with changes in the concentration of carnitine in tissue, plasma, or urine. In general, tissue carnitine levels decrease in disease, however, the concentration of carnitine in plasma or urine may either increase or decrease.

In studies of experimentally-induced diphtheria in the guinea pig (47) and experimental ischemia in the dog (40), marked decreases (40% and 600%, respectively) in the carnitine levels of the heart were observed. In the rat, alloxan-induced diabetes is associated with decreases of ca 200% in hepatic carnitine levels (48). In man, carnitine levels decrease 20-fold in some carnitine myopathies (50,51,52). In a fatal case of systemic carnitine deficiency, carnitine levels in the patient's liver and skeletal muscle decreased 7- and 28-fold below normal levels, respectively (1).

Decreased plasma carnitine levels have been reported in the following diseases: Crohn's Disease, malabsorption syndromes, ulcerative colitis, and anorexia nervosa (28). These diseases are associated with a generalized state of malnutrition. It is possible that reduced uptake of carnitine or amino acids essential to

carnitine biosynthesis may be responsible for the observed reduction in plasma carnitine in these diseases and others such as protein-calorie malnutrition due to under-nourishment, Marasmus, or Kwashiorkor, in which decreased plasma carnitine levels of 29%, 69% and 71% respectively, have been reported in affected children (53,54).

Increased plasma carnitine levels have been reported in the following diseases: cirrhosis of the liver, chronic renal failure, and progressive rheumatic heart disease (28,55).

It is possible that changes in plasma carnitine levels may be useful clinical indicators of protein malnutrition or renal insufficiency in man.

Urinary excretion of carnitine is decreased in approximately 50% of the patients suffering from adrenal insufficiency, hypothyroidism and hypopituitarism; hyperthyroid patients exhibit a 2-fold increase in carnitine excretion (56,57).

IV. Carnitine Metabolism

A. Turnover

Early turnover studies in rats identified a number of physiological stress conditions that influence carnitine metabolism; these included pregnancy and cold exposure, which increase carnitine pool size several fold, and choline-deficient diets and alloxan-induced diabetes, both of which decrease carnitine pool size and turnover (33,48,58). Early carnitine turnover studies based on isotopic carnitine die-away curve analysis must be interpreted with caution because of certain technical problems: 1) racemic mixtures

of isotopic carnitine were employed; 2) low radio-specific activity of labelled carnitine available necessitated the injection of large doses of carnitine; 3) turnover studies were conducted for short periods of time, and 4) data analysis was based on a single compartment model. Recent studies of carnitine turnover using (-)-carnitine of high radio-specific activity and computer analysis of radioactive-carnitine die-away curves have overcome many of these earlier problems.

A kinetic model of carnitine metabolism in the rat indicates a carnitine pool size of 57 μmoles (35 μmoles/100 g body weight) which is distributed between two compartments differing in size by a factor of 5 (25). The smaller compartment, consisting of tissues and fluids such as liver, blood and urine, contains 14 μmoles and has an estimated turnover time of 4.6 days. The larger compartment, consisting primarily of muscle,contains 40 - 50 μmoles of carnitine and has an estimated turnover time of 24 days. The average turnover time of both the large and small pools is 15 - 21 days. Daily carnitine biosynthesis and excretion for rats maintained on carnitine-free (lysine and methionine enriched) diets is 2 - 3 μmoles (25).

Tissue carnitine is in equilibrium with blood carnitine through a process of blood-tissue carnitine exchange (59). Liver and kidney exhibit a rapid blood-tissue exchange while that of heart and skeletal muscle is much slower. Some tissues have more than one carnitine pool (59), the physiological nature of these pools is not known although different cell types within an organ and subcellular compartmentalization between organelles are possibilities.

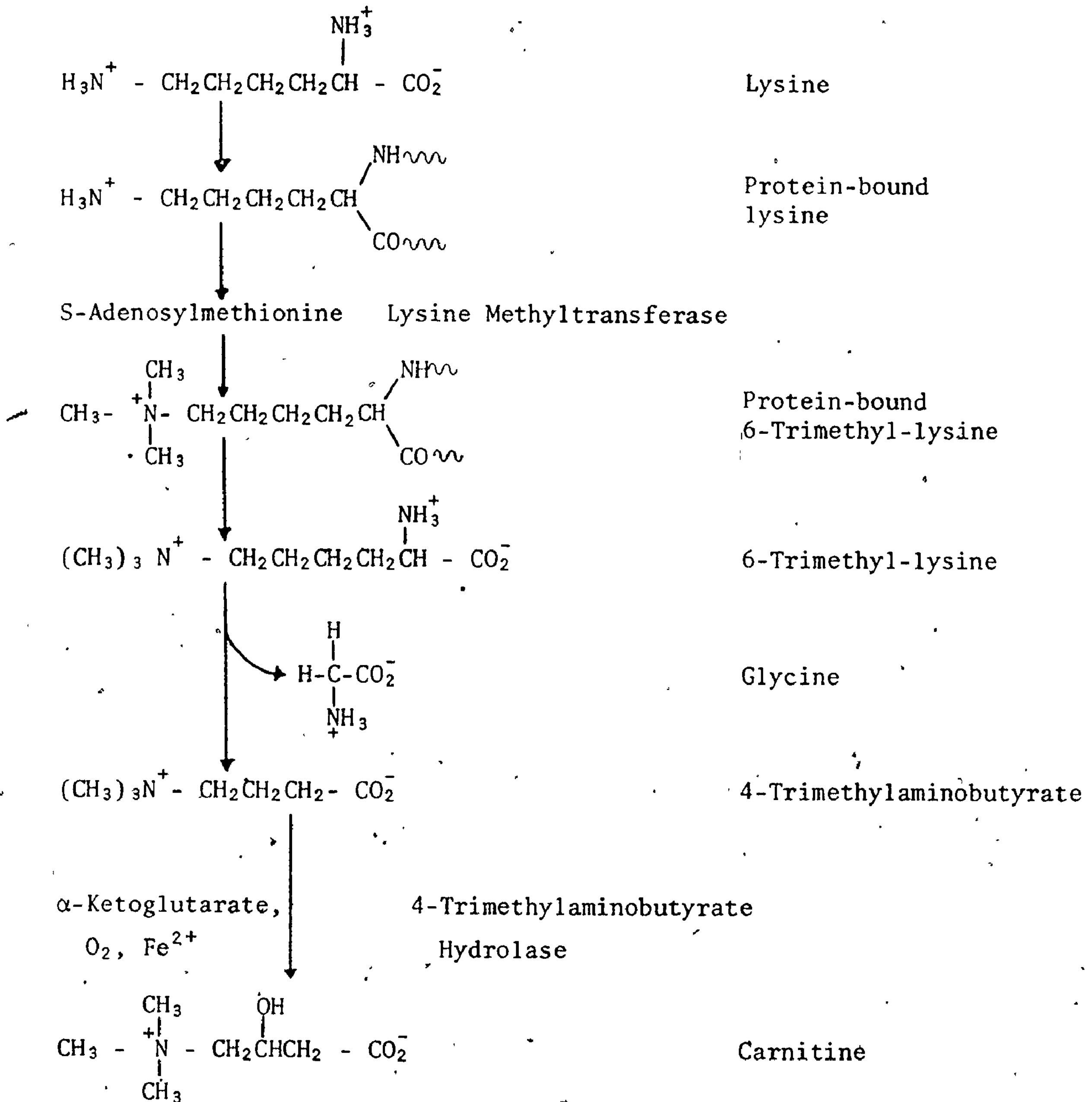
A criticism of carnitine turnover studies to date is the failure to differentiate between carnitine and acylcarnitine compounds in the analysis of tissue. If the concentration of acylcarnitine in tissues were to represent only a small fraction of the net tissue carnitine concentration then the reported studies would provide a first approximation of carnitine turnover; however, acylcarnitines represent 30 to 60% of the total tissue carnitine compounds (Table I). Therefore, it is difficult to draw firm conclusions from existing carnitine turnover data.

B. Biosynthesis

Scheme 1 is a proposed pathway of carnitine biosynthesis in the rat. The butyrate carbon skeleton of carnitine is derived from lysine, the N-methyl groups from methionine (60,61). Lysine, per se, is not the direct precursor of carnitine but is first incorporated into a polypeptide chain where it undergoes successive methylation at the 6-N position to form protein-bound 6-trimethyl-lysine (61). In mammalian systems, 6-trimethyl-lysine originates from the methylation of protein-bound lysine through the action of lysine methyltransferase and S-adenosylmethionine (62). The observation that protein-bound lysine, but not lysine or partially methylated lysine, can be incorporated into carnitine supports the requirement that lysine first be incorporated into a polypeptide chain before participating in carnitine biosynthesis reactions (63). In contrast, in *Neurospora crassa* a pathway exists for direct utilization of lysine and partially methylated lysine for carnitine biosynthesis (60).

Scheme I

Biosynthesis of Carnitine in the Rat[a]



[a] Adapted from : Horne, D.W., and Broquist, H.P. (1973). Role of lysine and ε-N-trimethyllysine in carnitine biosynthesis: Studies in *Neurospora crassa*. *The Journal of Biological Chemistry 248*, 2170-2175.

Hochalter, J.B., and Henderson, L.M. (1976). Carnitine biosynthesis: The formation of glycine from carbons 1 and 2 of 6-N-trimethyl-L-lysine. *Biochemical and Biophysical Research Communications 70*, 364-366.

Cox, R.A., and Hoppel, C.L. (1973). Biosynthesis of carnitine and 4-N-trimethylaminobutyrate from 6-N-trimethyllysine. *136*,

The proteolysis of protein-bound 6-trimethyl-lysine to free 6-trimethyl-lysine is not well understood. It is unlikely, however, that this step is rate limiting in the biosynthesis of carnitine in the normal metabolic steady-state (63). Since free 6-trimethyl-lysine can be converted to carnitine, the proteolytic conversion of protein-bound 6-trimethyl-lysine to free 6-trimethyl-lysine probably occurs prior to the formation of 4-trimethylaminobutyrate.

6-Trimethyl-lysine is converted directly to 4-trimethylaminobutyrate; this direct conversion is supported by the observations that a two-carbon fragment, which is probably glycine, is formed in the reaction (64) and neither 6-trimethylaminohexanoate nor 5-N-trimethylaminopentanoate can be converted to carnitine (65).

4-Trimethylaminobutyrate can be synthesized by most tissues but only the liver, and to a minor extent the testes, are capable of converting it to carnitine (66,67,68,69,70). The hydroxylation of 4-trimethylaminobutyrate to form carnitine is catalyzed by the microsomal enzyme, 4-trimethylaminobutyrate hydroxylase; the reaction requires as co-factors molecular oxygen, ferrous ion, and α-keto-glutarate (71,72). The liver has sufficient 4-trimethylaminobutyrate hydroxylase activity to account for the estimated rates of carnitine biosynthesis in the rat (73).

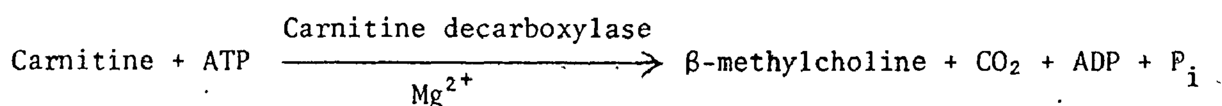
Although the liver has all the enzymes necessary for the complete biosynthesis of carnitine from lysine and methionine it can also use circulating 4-trimethylaminobutyrate (74); the contribution of extra-hepatic 4-trimethylaminobutyrate to hepatic carnitine biosynthesis is not known. Aside from conditions limiting

the exogenous intake of carnitine, lysine or methionine, factors regulating the synthesis of carnitine remain to be elucidated. The need for information in this area of carnitine metabolism is underscored by the recognition that the basic metabolic defect in systemic carnitine deficiency is the patient's inability to synthesize carnitine in sufficient quantity to supplement exogenous carnitine intake to meet body demands (75).

C. Degradation

After injecting methyl-labelled radioactive carnitine into rats, over 90% of the radioactivity excreted in the urine can be found in carnitine; the only other compound containing significant radioactivity is β-methylcholine (76).

β-methylcholine is a decarboxylation product of carnitine and is produced by the enzyme reaction:



$$\text{Carnitine} + \text{ATP} \xrightarrow[\text{Mg}^{2+}]{\text{Carnitine decarboxylase}} \beta\text{-methylcholine} + CO_2 + \text{ADP} + P_i$$

Carnitine decarboxylase is a mitochondrial enzyme found primarily in heart and muscle with lesser amounts in liver and kidney. The purified enzyme, isolated from rat heart, has a K_m of 2.4 X 10^{-4}M for DL-carnitine and a slightly higher value for acetyl- and palmitoylcarnitine (76). The activity of this enzyme increases during conditions of enhanced fatty acid oxidation e.g. choline deficiency, pregnancy, high fat intake, cold exposure and alloxan-induced diabetes in rats (76).

The physiological role of carnitine decarboxylase is not known although it may function to prevent excessive loss of acetyl

groups, as acetylcarnitine, from mitochondria by converting carnitine to β-methylcholine.

V. Carnitine Transport Across Membranes

A. Plasma Membrane Transport

Active transport mechanisms exist in the plasma membrane of the cell that are responsible for the uptake of carnitine from the blood. Active transport of carnitine into cells is supported by the observations that 1) tissue carnitine concentrations are much greater than that of the blood e.g. 60- and 500-fold in heart and epididymis, respectively (77,78), 2) even though tissues *in vivo* are perfused by blood of the same carnitine concentration, there are considerable differences in tissue carnitine concentrations (Section II. B., Table I), and 3) the movement of carnitine through the plasma membrane requires a high activation energy suggesting the presence of a carnitine carrier (79). Active transport of carnitine into isolated liver cells, cultured human heart cells, and fibroblasts has been reported (79,80). Carnitine uptake by these cells exhibited substrate specificity, saturability, transport against a concentration gradient, and inhibition by 2,4-dinitrophenol (78,79).

A common carrier for carnitine and 4-trimethylaminobutyrate exists in heart and liver. In liver, the carrier has a greater affinity for 4-trimethylaminobutyrate (K_m = 0.5 mM) than for carnitine (K_m = 5.6 mM) whereas in heart, the carrier has approximately the same affinity for both carnitine and 4-trimethylaminobutyrate (K_m = 4.8 and 5.7 μM, respectively) (79,80). Differences in K_m

for carnitine and 4-trimethylaminobutyrate in the liver may reflect the physiological role of this tissue in carnitine biosynthesis from circulating 4-trimethylaminobutyrate synthesized by extra-hepatic tissues. Differences in K_m between heart and liver for carnitine may reflect a greater metabolic need for carnitine by the heart to meet the demands of fatty acid oxidation.

An inducible, active, carrier-mediated transport system for carnitine has been described in the bacteria *Pseudomonas aeruginosa* (81). The K_m for carnitine in this system is 0.63 mM. Chloramphenicol inhibits the induction of the transport system by carnitine suggesting the presence of an inducible proteinaceous component of the carrier. Whether or not the carnitine transport system in heart and liver is inducible or constitutional is unknown.

Hormones may be involved in the regulation of carnitine uptake. Carnitine uptake by rat epididymis is enhanced by testosterone and suppressed by estrogen (82). The mechanism of action of hormones on carnitine transport remains to be elucidated.

Information concerning the regulation of carnitine uptake may find clinical application in the treatment of some forms of muscle carnitine deficiency that have as the biochemical lesion impaired carnitine uptake (83,84).

B. Inner Mitochondrial Membrane Transport

The role of carnitine in the translocation of fatty acids, as acylcarnitines, into the mitochondria for β-oxidation requires that carnitine and acylcarnitines penetrate the inner mitochondrial membrane.

In general, however, the inner mitochondrial membrane is impermeable towards low molecular weight compounds including carnitine (85,86,87). The difficulty in moving carnitine through the hydrophobic environment of the inner mitochondrial membrane is indicated by the high energy of activation required for carnitine transport through this membrane (42 kcal/mole) compared with that of the plasma membrane (16 kcal/mole) (79,88).

There are several hypotheses explaining how carnitine and acylcarnitines cross the inner mitochondrial membrane:

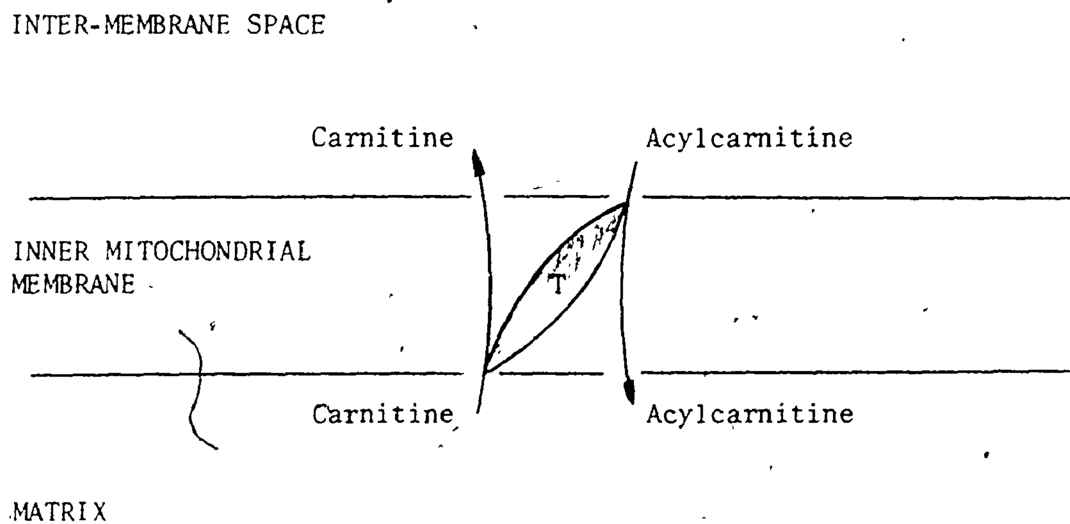
1) Carnitine and acylcarnitines diffuse through the membrane: the anisotropic properties of the carnitine acyltransferase enzymes result in vectorial transport (89).
2) Carnitine acyltransferase enzymes, per se, act as carriers for carnitine and acylcarnitines (90).
3) Acylcarnitines move through the membrane by moving down an electrochemical gradient of hydrogen ions (9).
4) Carnitine and acylcarnitines move through the membrane by a process of exchange-diffusion mediated by a carnitine-acylcarnitine translocase (91,92,93).

The hypothesis most compatible with existing data is the translocase hypothesis.

The role of the carnitine-acylcarnitine translocase system in transporting carnitine and acylcarnitines through the inner mitochondrial membrane is illustrated in Figure 2. According to the hypothesis, there is a mole for mole exchange of carnitine - carnitine or carnitine - acylcarnitine molecules; the exchange is

Figure 2

Carnitine-Acylcarnitine Translocase System in Mitochondria

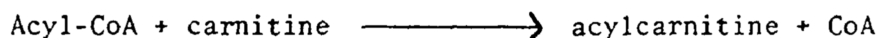


T = Carnitine-acylcarnitine translocase

independent of metabolic energy, independent of carnitine acyltransferase enzymes, and inhibited by N-ethylmaleimide or mersalyl (93). The activity of the translocase system cannot be attributed to other known mitochondrial transport systems such as the tricarboxylate or adenine nucleotide transporters (93).

VI. Carnitine Acyltransferases

Carnitine acyltransferases comprise a family of enzymes that catalyze the reaction:



$$\text{Acyl-CoA} + \text{carnitine} \longrightarrow \text{acylcarnitine} + \text{CoA}$$

On the basis of acyl-group chain length, three carnitine acyltransferases have been identified: carnitine short-chain (C_2- C_4) acyltransferase i.e. acetyl-CoA: carnitine O-acetyltransferase EC 2.3.1.7; carnitine medium-chain (C_6- C_{10}) acyltransferase; and carnitine long-chain (C_{12} and greater) acyltransferase i.e. hexadecanoyl-CoA: carnitine O-acyltransferase EC 2.3.1.21. These enzymes are abbreviated CAT, COT and CPT, respectively.

A. CPT

CPT, in liver and heart preparations, was first reported in 1962-1963 (94,95,96). The subcellular distribution of CPT was controversial, some workers claim that CPT activity is exclusively mitochondrial (97,98) whereas other workers claim the existence of both mitochondrial and microsomal CPT activity (99,100). At least

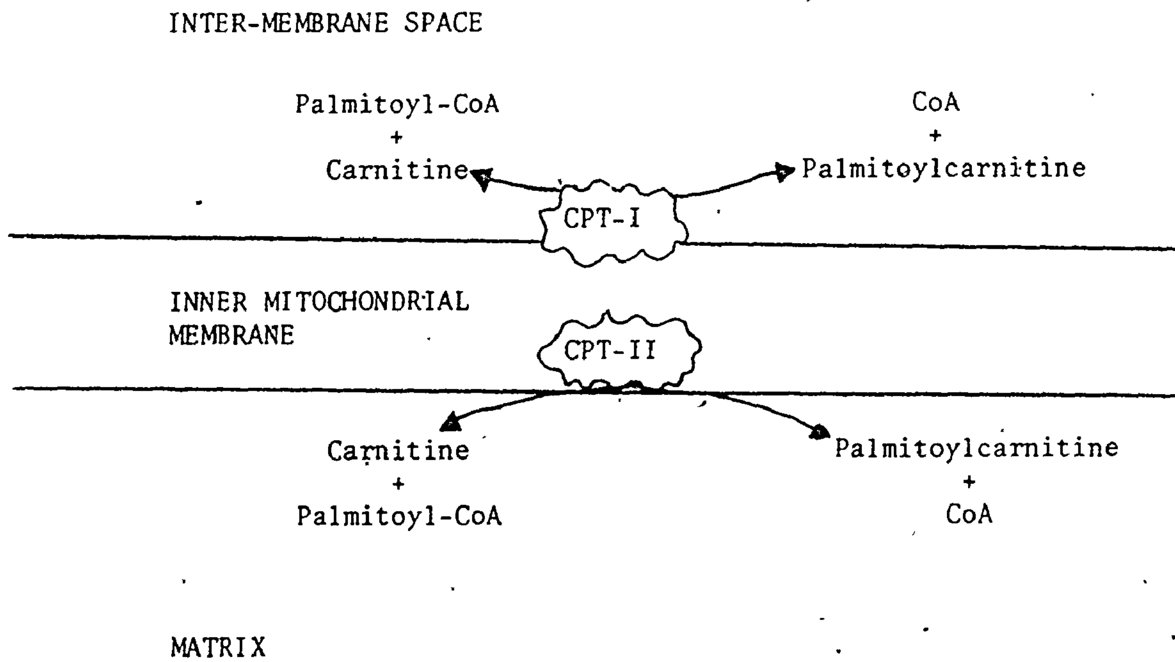
for rat liver, the majority of the studies report that CPT is exclusively mitochondrial.

Topographical studies of CPT indicate that it is located on both the outer and inner surfaces of the inner mitochondrial membrane (97,100,101). The enzyme on the outer surface is easily released by sonication, detergents and digitonin, but is not affected by treatment with trypsin (102). This suggests that although CPT on the outer surface is loosely bound it may be partially buried in the membrane. CPT located on the inner surface of the mitochondrial membrane is not released by digitonin and appears to be tightly membrane-bound (102). CPT located on the outer and inner surfaces of the mitochondrial membrane are designated CPT-I or CPT-A and CPT-II or CPT-B, respectively. Whether or not CPT-I and CPT-A or CPT-II and CPT-B are the same enzymes and whether or not CPT-I and CPT-II are the same enzymes but in different locations remains to be elucidated. The topography of CPT enzymes and reactions they catalyze are presented in Figure 3.

The properties of some purified carnitine palmitoyltransferases are presented in Table III. The conflicting data in this table probably reflect the inherent difficulties in delipidation, solubilization, and purification of membrane-bound enzymes. Although some of the differences in Table III may be attributable to methodological differences during enzyme purification, the possibility of species differences, allotypic behaviour, and the existence of a family of CPT enzymes with different acyl-group specificities can not be excluded.

Figure 3

Topography of Carnitine Palmitoyltransferases in the Inner Mitochondrial Membrane



CPT = Carnitine palmitoyltransferases

Table III

Properties of Some Purified Carnitine Palmitoyltransferases[a]

Preparation	Source	Molecular weight	K_m for Carnitine (mM)	K's (μM)		
				Palmitoylcarnitine	Palmitoyl-CoA	CoA
CPT	calf liver	-	0.25	40	10	50
CPT-"Outer"	ox liver	59,000	0.14	12	0.59	-
CPT-"Inner"	ox liver	65,000	2.60	60	9.0	-
CPT-I	calf liver	150,000	0.25	136	17.6	55
CPT-II	calf liver	150.000	-	-	-	-

[a] Adapted from Hoppel, C.L. (1976). Carnitine palmitoyltransferase and transport of fatty acids. IN: *The Enzymes of Biological Membranes* Vol. 2, *Biosynthesis of Cell Components*. (A. Martonosi, ed.) Plenum Press, New York. pp. 119-143.

Long-chain saturated fatty acids are not the only substrates of CPT, other substrates include unsaturated fatty acids (103), β-substituted fatty acids (104) and dicarboxylic fatty acids (105); the reaction rates of these substrates with CPT are generally less than for long-chain saturated fatty acids.

Drugs, hormones, diet and disease are factors that can alter the activity of CPT enzymes. Factors increasing CPT activity include fasting (100,106), high-fat diets (107), exercise (108), thyroxine (109), clofibrate (110,111), and diabetes (112). Factors decreasing CPT activity include chronic ethanol ingestion (113), myocardial ischemia (49,114), and some forms of carnitine myopathy (115,116,117).

CPT plays an integral role in the translocation of fatty acids from the cytoplasm to the site of β-oxidation in the mitochondria. Although the inner mitochondrial membrane is impermeable to long-chain acyl-CoA, it is permeable to acylcarnitines (102). Through the combined action of CPT and carnitine-acylcarnitine translocase, long-chain acyl-CoA is transported through the inner mitochondrial membrane to the site of fatty acid oxidation in the mitochondrial matrix (Figure 4).

Several lines of evidence indicate that neither CPT nor the translocase system are rate-limiting steps in fatty acid oxidation (113,118,119,120):

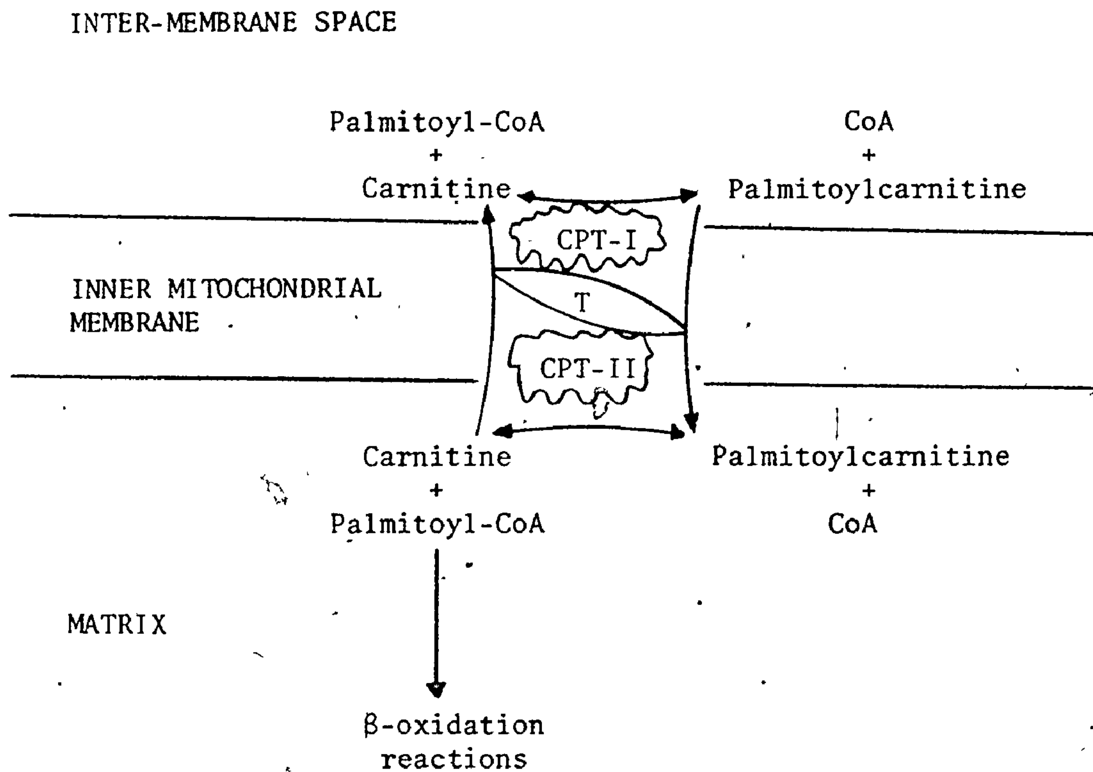
1) Palmitoyl-CoA + carnitine is oxidized at the same rate as palmitoylcarnitine.

Figure 4

Translocation of Acyl-groups into Mitochondria

for

β-oxidation in the Matrix



T = Carnitine-acylcarnitine translocase

CPT = Carnitine palmitoyltransferase

2) CPT activity exceeds the rate of palmitate group oxidation.

3) Palmitoylcarnitine translocation exceeds the formation of the end-products of fatty acid oxidation.

4) CPT catalyzes a reversible reaction.

Although CPT is not rate-limiting in fatty acid oxidation, there is no question of its essentiality to the process; the parallel development of CPT and the enzymes of β-oxidation during growth and development support a close relationship between the two enzyme systems (24,121).

CPT may participate in the regulation of hepatic ketogenesis (121, 122). For maximal ketogenic capacity, there must be activation of both CPT-I and CPT-II, an increase in liver carnitine content, and a decrease in liver glycogen (38,124). CPT-II activity may be under the bihormonal control of insulin and glucagon (123). The interrelationships among these events in the regulation of ketosis is not known.

The physiological functions of extra-mitochondrial CPT are not understood. The presence of CPT in the membranes of erythrocytes and synaptosomes suggests that CPT in these membranes may be involved in fatty acid transport (125,126). The observation that microsomal CPT is influenced to a greater extent than mitochondrial CPT by hormonal and nutritional factors (109) warrants further investigation.

B. CAT

CAT, in extracts of pigeon and sheep livers, was first reported in 1955 (127). Although initially reported to be an exclusively mitochondrial enzyme c.f. CPT, there is now good evidence that CAT

has at least two extra-mitochondrial locations, i.e. the endoplasmic reticulum and peroxisomes (97). The subcellular distribution of CAT is organ specific e.g. the percent distribution of CAT among mitochondria, microsomes, and peroxisomes is 52%, 34% and 14%, in rat liver and 94%, 6% and 0% in rat kidney, respectively (97).

Mitochondrial CAT has a dual location in the inner mitochondrial membrane with 25% being on the outer surface and 75% being on the inner surface (128).

CAT in the endoplasmic reticulum is tightly membrane-bound and evenly distributed between the rough and smooth regions (129). CAT in peroxisomes differs from that of mitochondria and microsomes in that it is not membrane-bound but exists in the soluble space of the peroxisome (130).

The properties of some partially purified carnitine acetyltransferases are presented in Table IV. CAT has not been purified from rat liver, therefore, for comparison purposes data on CAT purified from pigeon breast muscle and pig heart are included in the table. Even though the enzymes are only partially purified, it is apparent from the data in Table IV that microsomal and peroxisomal CAT enzymes might be the same protein, equally apparent is that CAT preparations from pigeon and pig differ from rat preparations.

Although there is little information in the literature regarding factors that alter CAT activity, the following are known to elevate CAT activity: fasting (107); clofibrate (131); di-2-ethylhexyl phthalate (132,133), and possibly testosterone (78,134).

Table IV

Properties of Some Partially Purified Carnitine Acetyltransferases[a]

Enzyme Source	Molecular weight	K_m for Carnitine (μM)	K_m for acetyl-CoA (μM)	pI
Pigeon breast muscle	51,000	120	34	7.9
Pig heart	-	310	41	-
Rat liver microsomes	59,000	150	69	8.3
Rat liver peroxisomes	59,000	143	69	8.3

[a] Adapted from Markwell, M.A.K., Tolbert, N.E., and Bieber, L.L. (1976). Comparison of the carnitine acyltransferase activities from rat liver peroxisomes and microsomes. *Archives of Biochemistry and Biophysics 176*, 479-488.

Several physiological functions have been attributed to CAT. The ability of certain tissues to maintain the equilibrium:

$$K = \frac{[\text{Acetyl-CoA}] \quad [\text{carnitine}]}{[\text{CoA}] \quad [\text{acetylcarnitine}]} = 0.6$$

in different steady-state conditions, indicates that the CAT equilibrium is a mechanism whereby tissues can buffer changes in acetyl-CoA. High CAT activities in heart and sperm suggest that this buffer system may exist in these tissues (135).

CAT may function to transport acetate, as acetylcarnitine, out of the mitochondrial compartment to the cytoplasm for *de novo* fatty acid synthesis (136). The relative contribution of this source of acetate is minor in comparison to that which is provided by the action of the citrate-cleavage enzyme; however, under different conditions or in tissues other than the liver this may not be the case.

Microsomal CAT could provide a mechanism whereby acetate and malonate, as acetylcarnitine and malonylcarnitine, reach sites of acetylation or elongation reactions buried in the membrane of the endoplasmic reticulum. A better understanding of CAT must await topographical studies of CAT in mitochondrial and microsomal membranes.

C. COT

COT was first reported in 1971 (137) and its existence confirmed in 1972 (138). COT has the same subcellular distribution as CAT i.e. in mitochondria, endoplasmic reticulum, and peroxisomes (129,130).

A possible role for COT in amino acid metabolism has been proposed. The degradation of leucine and valine involves their transamination to α-keto-acids in peroxisomes and then decarboxylation to form acyl-CoA in the mitochondria; COT may convert the α-keto-acids to acylcarnitines which can cross the peroxisomal and mitochondrial membranes thereby linking the two compartments involved in amino acid degradation (6).

VII. Carnitine Ester Hydrolases

Carnitine ester hydrolase (CEH) catalyzes the reaction:

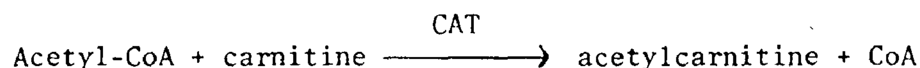
$$\text{Acylcarnitine} + H_2O \xrightarrow{\text{CEH}} \text{carnitine} + \text{fatty acid}$$

CEH located in the microsomal fraction of rat liver has a K_m of 5 mM and catalyzes the hydrolysis of carnityl esters of chain length C_6 - C_{18} (14); CEH located in the outer mitochondrial membrane has a K_m of 2 mM and catalyzes the hydrolysis of carnityl esters of chain length C_2 - C_4 (139).

Microsomal CEH has no established role but may function to prevent the cell from accumulating high concentrations of long-chain acylcarnitines; high concentrations of palmitoylcarnitine e.g. 0.3 mM, inhibit oxygen uptake by mitochondria and cause mitochondrial lysis (14).

Mitochondrial CEH may function to maintain high levels of carnitine for fatty acid transport into mitochondria during times of increased fatty acid oxidation; the observation that mitochondrial CEH activity increases during fasting supports this role (139).

Mitochondrial CEH and CAT are responsible for the hydrolysis of acetyl-CoA to acetate and CoA, a reaction formerly attributed to acetyl-CoA hydrolase. In livers of rats and sheep, acetyl-CoA hydrolase has been shown to be artifactual; its activity is due to the combined action of CAT and CEH in the following reaction sequence (139,140):



$$\text{Acetyl-CoA} + \text{carnitine} \xrightarrow{\text{CAT}} \text{acetylcarnitine} + \text{CoA}$$

$$\text{Acetylcarnitine} + H_2O \xrightarrow{\text{CEH}} \text{acetate} + \text{carnitine}$$

Overall Reaction:

$$\text{Acetyl-CoA} + H_2O \longrightarrow \text{acetate} + \text{CoA}$$

VIII. Carnitine and Arterial Wall Metabolism

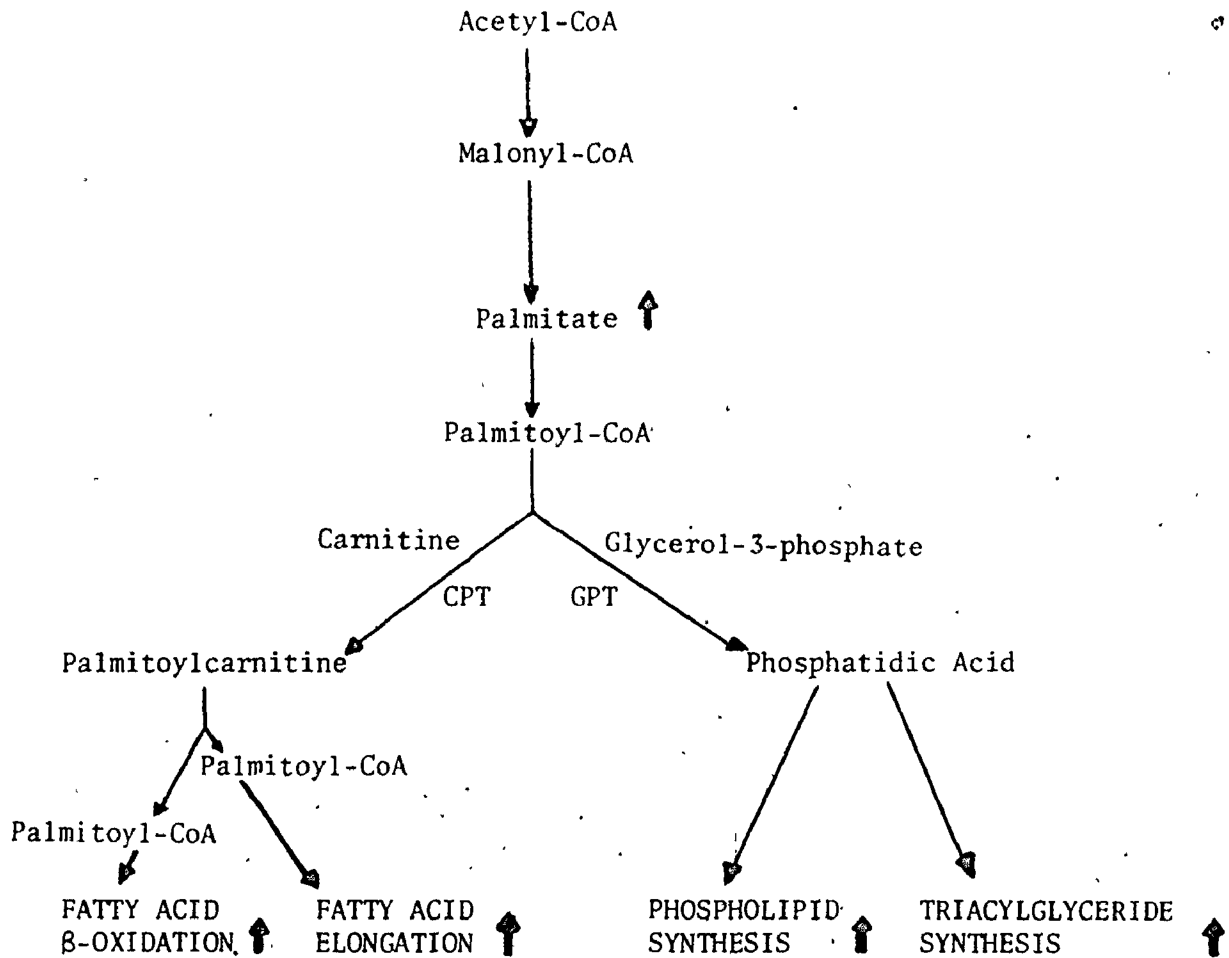
Carnitine and carnitine acyltransferase enzymes are present in arteries and are undoubtedly involved in normal arterial lipid metabolism, and may, in addition, be pertinent to the development of atherosclerosis. Some hypothetical roles for carnitine in long-chain fatty acid metabolism in normal and atherosclerotic arteries are presented in Figure 5.

A. Fatty Acid Synthesis

Arterial fatty acid synthesis increases in atherosclerosis; both *de novo* synthesis in the cytoplasm and elongation in the mitochondria increase approximately 4-fold (141,142,143,144). *In situ*

Figure. 5

Carnitine and Acyl-CoA Metabolism in the Artery



↑ Indicates reactions that are increased in atherosclerosis

CPT = Carnitine palmitoyltransferase

GPT = Glycerolphosphate acyltransferase

fatty acid synthesis may contribute up to 50% of the fatty acids that accumulate in atherosclerotic arteries (145,146). The mechanisms involved in the stimulation of fatty acid synthesis in atherosclerosis are not known.

Carnitine increases *de novo* fatty acid synthesis in cell-free preparations of rat liver (147,148), and increases the conversion of acetate, glucose, and pyruvate into fatty acids; this suggests that carnitine stimulates an efflux of acetyl-groups from mitochondria to the cytoplasm for fatty acid synthesis. Although in liver the contribution of acetate from acetylcarnitine efflux out of mitochondria is considered to be of minor importance to *de novo* fatty acid synthesis (136), this may not be true in the artery; particularly in the atherosclerotic artery in which the activity of the tricarboxylic acid cycle is markedly depressed and the availability of citrate possibly reduced (149).

In addition to carnitine, acylcarnitines also increase fatty acid synthesis. In 109,000 X g liver supernatant fractions, palmitoylcarnitine increases *de novo* fatty acid synthesis 5- and 10-fold in fed and fasted rats, respectively (150). This increase is observed when acetate, but not malonyl-CoA, is the substrate and suggests that the stimulation of fatty acid synthesis occurs at the level of acetyl-CoA carboxylase, the rate-limiting step in fatty acid biosynthesis. Partially purified preparations of acetyl-CoA carboxylase are stimulated 2-3 fold by palmitoylcarnitine, even after the enzyme has been optimally activated by magnesium and citrate (151). Since palmitoyl-CoA inhibits acetyl-CoA carboxylase,

the ratio of palmitoylcarnitine:palmitoyl-CoA in the vicinity of this enzyme may be a regulatory factor in fatty acid synthesis (150).

The major mechanism of fatty acid synthesis in the aorta is mitochondrial fatty acid elongation (152). This has been demonstrated in arteries from rabbits (153), monkeys (145), chickens (154), and humans (155). Although acyl-CoA is the ultimate substrate for mitochondrial elongation reactions, acyl-CoA is first converted to an acylcarnitine so that it can reach the site of elongation in the inner mitochondrial membrane. Supporting this sequence of events are the observations that acylcarnitines are active primers of mitochondrial fatty acid elongation and acylcarnitines are better primers than acyl-CoA in intact mitochondria but acyl-CoA is the preferred primer in disrupted mitochondria (156,157). Since in the aorta mitochondrial fatty acid elongation is the major mechanism of fatty acid synthesis, the concentration of carnitine and the activity of CPT may be important determinants of fatty acid synthesis in the artery.

B. Phospholipid Synthesis

The phospholipid concentration increases several fold in the arteries of atherosclerotic monkeys, pigeons, rabbits, and man (143, 158). The major phospholipid classes involved in atherosclerotic arteries are phosphatidyl choline and sphingomyelin (159).

Carnitine increases the rate of palmitate incorporation into mitochondrial phospholipids. In guinea pig and beef heart mitochondria, carnitine increases the incorporation of palmitate into phosphatidyl

choline 3-fold; carnitine has no effect on palmitate incorporation into microsomal phospholipids (160). Increased incorporation of acetate into mitochondrial phospholipids occurs in arteries of rabbits fed cholesterol-supplemented diets (161), possibly carnitine is involved in this reaction. In addition, an increase in fatty acid synthesis caused by carnitine or palmitoylcarnitine could increase the supply of fatty acids available for phospholipid synthesis.

C. Fatty Acid Oxidation

In the artery, 40% of the ATP is derived from glycolysis, 60% from oxidative phosphorylation (162). Since only 1-6% of the glucose entering the glycolytic reactions enters the tricarboxylic acid cycle, a major source of acetyl-CoA is from the β-oxidation of fatty acids (143). ATP production from the oxidation of fatty acids is particularly important in the atherosclerotic artery since the activity of many enzymes in the tricarboxylic acid cycle is reduced in atherosclerosis e.g. aconitase - 15%, malate dehydrogenase - 20%, and fumarase - 45% (149). Unimpaired ATP production is essential if the artery is to repair the tissue damage in atherosclerosis.

Oxygen consumption due to fatty acid oxidation increases from 4% in normal arteries to 30% in atherosclerotic arteries (163) and suggests that fatty acid oxidation increases in atherosclerosis. In support of this idea is the observation that atherosclerotic intimal-medial segments exhibit a 15-fold increase in their capacity to oxidize fatty acids to CO_2 compared to normal intimal-medial segments (164). Given the role of carnitine in β-oxidation, changes

in carnitine concentration, CPT activity or perhaps the carnitine-acylcarnitine translocase system may be responsible for the increase in fatty acid oxidation in atherosclerosis.

D. Factors in the Regulation of Oxidation *vs* Esterification Reactions

Whether or not acyl-CoA is esterified to carnitine and directed toward fatty acid oxidation and fatty acid elongation reactions or is esterified to glycerol-3-phosphate and directed toward acylglyceride and phospholipid synthesis, depends on the relative activities of CPT and glycerophosphate acyltransferase (acyl-CoA: sn-glycerol-3-phosphate O-acyltransferase, GPT), the concentration of carnitine, glycerol-3-phosphate, and acyl-CoA.

In rat liver, at low concentrations of acyl-CoA, esterification of acyl-CoA to glycerol-3-phosphate is favoured due to the lower K_m of GPT compared to CPT; at high concentrations of acyl-CoA, esterification of acyl-CoA to carnitine is favoured due to saturation and subsequent inhibition of CPT and concomitant increase in CPT activity (106,165,166). Increasing the concentration of carnitine in hepatocytes results in an increase in the oxidation of acyl-CoA to carbon dioxide and a reduction in triacylglyceride formation but has no effect on phospholipid synthesis; increasing the concentration of glycerol-3-phosphate increases the formation of triacylglycerides only slightly. Although the effects of glycerol-3-phosphate can be overcome by increasing the concentration of carnitine, the effects of carnitine cannot be overcome by increasing the concentration of glycerol-3-phosphate (167).

In atherosclerotic arteries there is increased oxygen consumption (163), increased oxidation of fatty acids (164), increased fatty acid synthesis by mitochondrial elongation (143), and decreased incorporation of fatty acids into triacylglycerides (163). These biochemical changes form the basis of the hypothesis that esterification of acyl-CoA to carnitine rather than to glycerol-3-phosphate is the predominant reaction in atherosclerotic aortas. Evidence will be presented to support this hypothesis.

MATERIALS AND METHODS

MATERIALS

Buffers

Tris-HCl

Tris-(hydroxymethyl) aminomethane was dissolved in distilled water to give a 1.0 M solution; the pH was adjusted to 8.0 using 1.0 N HCl.

Phosphate Buffers

Phosphate buffer used for the preparation of subcellular fractions by differential centrifugation consisted of: 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, 0.5 mM EDTA (Ethylenediaminetetraacetic acid), and 2.0 mM GSH (Glutathione, reduced form); phosphate buffer used for the assay of carnitine palmitoyltransferase activity contained in addition 1.0 mM KCN. Buffers were made up in distilled water and adjusted to pH 7.35 using concentrated HCl.

Chemicals, Acids and Solvents

All dry chemicals, acids and solvents were reagent grade and used without further purification except where indicated otherwise.

Acetylcarnitine (P-L Biochemicals Inc., Milwaukee, WI, U.S.A.)

(-)-Acetylcarnitine chloride, melting point: 188°C, was dissolved in chloroform:methanol (1:1), to give a concentration of 1 mg/ml.

Butyrylcarnitine (P-L Biochemicals Inc.)

(±)-Butyrylcarnitine chloride, melting point: 148°C, was dissolved in chloroform:methanol (1:1) to give a concentration of 1 mg/ml.

Carnitine (Sigma Chemical Co., St. Louis, MO., U.S.A.)

(±)-Carnitine hydrochloride was dissolved in distilled water to give a 2.0 mM standard stock carnitine solution. Carnitine was also dissolved in chloroform:methanol (1:1) to give a concentration of 1.5 mg/ml.

Palmitoylcarnitine (P-L Biochemicals Inc.)

L-Palmitoylcarnitine chloride, melting point: 164°C, was dissolved in chloroform:methanol (1:1) to give a concentration of 1 mg/ml.

Carnitine acetyltransferase (Sigma Chemical Co.)

Acetyl-CoA: carnitine O-acetyltransferase EC 2.3.1.7, had a stated enzyme activity of 90 units/mg of protein; one unit converts 1.0 μmole of acetyl-(-)-carnitine and CoA to (-)-carnitine and acetyl-CoA per minute at pH 8.0 and 25°C.

Acetyl-CoA (Sigma Chemical Co.)

Acetyl-CoA, the lithium salt, purity 90%, was dissolved in 1.0 M Tris-HCl buffer at pH 8.0 to give solutions of the required concentration. Acetyl-CoA solutions were prepared fresh on the day of use and stored at 5°C until use in the carnitine assays.

Palmitoyl-CoA (Sigma Chemical Co.)

Palmitoyl-CoA, the lithium salt, purity 85%, was dissolved in phosphate buffer at pH 7.35 to give solutions of the required concentrations.

Bovine Serum Albumin (Sigma Chemical Co.)

Bovine serum albumin, fraction V powder, was dissolved in distilled water to give a concentration of 2 mg/ml.

Deoxyribonucleic Acid (Sigma Chemical Co.)

Calf thymus DNA standard, sodium salt, was dissolved in distilled water (boiled) to give a concentration of 1 mg/ml.

5,5'-Dithiobis-2-Nitrobenzoic Acid (DTNB) (Sigma Chemical Co.)

DTNB was prepared as a 0.2 mM solution in 2.5% (w/v) $KHCO_3$ in distilled water. DTNB solutions were prepared fresh on the day of use and protected from exposure to light. Re-crystallization of DTNB from glacial acetic acid did not improve the chromogenic properties of DTNB.

Glutathione (GSH) (Sigma Chemical Co.)

GSH, reduced form, was dissolved in 0.1 M phosphate buffer at pH 7.35 to give a 2.0 mM solution.

Sodium Pentobarbital (Diabutal, Diamond Laboratories, Des Moines, IA, U.S.A.)

Sodium pentobarbital was used as obtained at a concentration of 60 mg/ml. Diabutal was slowly infused, i.v., to obtain desired level of anesthesia.

Heparin (The Upjohn Co., Kalamozoo, MI, U.S.A.)

Injectable heparin, sodium salt, USP; 1000 units/ml, isolated from beef lung, was used.

Rhodamine 6 G

Rhodamine 6 G was prepared as a 0.05% solution in 95% ethanol.

O-Phthalaldehyde (Sigma Chemical Co.)

O-Phthalaldehyde was dissolved in glacial acetic acid to give a concentration of 50 mg/dl. Solutions were protected from light and prepared fresh on the day of use.

Liquid Scintillation Counting Fluid (Amersham/Searle Corp., Arlington Heights, IL., U.S.A.)

Scintillation fluid was prepared by dissolving 5.0 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazol-2-yl) benzene in 1.0 liter of toluene. The solution was stored in the dark.

Protosol (New England Nuclear, Boston, MA., U.S.A.)

Protosol, a quaternary ammonium hydroxide tissue solubilizer, was stored at room temperature and out of direct sunlight.

Radioactive Compounds

DL-[methyl ^{14}C] Carnitine Hydrochloride (Amersham/Searle Corp., Arlington Heights, IL., U.S.A.)

Specific activity: 51 - 54 mCi/mmol.

Radiochemical purity: 99%.

DL-[methyl-^{3}H] Carnitine Hydrochloride (Amersham/Searle Corp.)

Specific activity: 1.7 C/mmol.

Radiochemical purity: 98%.

L-[1-^{14}C] Palmitoylcarnitine Chloride (New England Nuclear, Boston, MA., U.S.A.)

Specific activity: 55.26 mCi/mmol.

Radiochemical purity: 99%.

[1-^{14}C] Palmitoyl-CoA (New England Nuclear)

Specific activity: 60.0 mCi/mmol.

Radiochemical purity: 99.4%.

Chromatographic Techniques

Solvent Systems for Thin Layer Chromatography

Solvent System 1

n-butanol:glacial acetic acid:water (120:50:30; v/v/v)

Solvent System 2

chloroform:methanol:50 mM sodium acetate in distilled water (4:4:1, v/v/v).

Solvent System 3

n-hexane:diethyl ether:glacial acetic acid (146:50:4; v/v/v).

Thin Layer Chromatographic Plates (TLC Plates)(EM Laboratories Inc., Elmsford, N.Y., U.S.A.)

Glass TLC-plates, 20 X 20 cm, were coated with cellulose (thickness = 0.01 mm) or silica gel G (thickness = 0.25 mm). Pre-development of plates in solvent systems was not necessary.

METHODS

Animals and Diets

Male New Zealand rabbits were used in all of the studies. Rabbits, initially weighing 2.5 Kg and approximately 12 weeks of age, were individually housed in stainless steel, wire bottomed cages. Food and water were available *ad libitum.*

Blood samples were taken via cardiac puncture using 5 ml heparinized glass syringes with 1 1/2 inch #18 or #20 Luer-Lok needles. Animals were sacrificed by exsanguination, under light sodium pentobarbital anesthesia, by cutting the neck vessels.

Study No. 1

These animals were used in a study designed to investigate the influence of age on arterial carnitine levels. Rabbits, 8 - 24 weeks of age, were fed a low-fat (LF) stock diet of pelleted

rabbit chow (composition of diet given in Table V); 1 week old rabbits were obtained through the McMaster University animal facility on the day of the experiment.

Study No. 2 and No. 3

These animals were used in a study designed to investigate the influence of dietary cholesterol on arterial and plasma carnitine levels. Age-matched rabbits were randomly grouped and maintained on one of the following diets for 15 - 20 weeks: (a) a non-atherogenic high-fat (HF) diet consisting of pelleted rabbit chow supplemented with 5% lard, or (b) an atherogenic high-fat cholesterol (HFC) diet consisting of the HF-diet supplemented with 1% cholesterol. Cholesterol was dissolved in melted lard which was then mixed with the pelleted chow diet.

Study No. 4

These animals were used in a study designed to investigate the influence of dietary cholesterol on the uptake and esterification of intravenously injected radioactive carnitine by aortas and hearts of rabbits. Rabbits were randomly grouped and maintained on one of the following diets: (a) a non-atherogenic control diet consisting of pelleted rabbit chow; or (b) an atherogenic diet consisting of the control diet supplemented with 5% lard and 1% cholesterol. Animals were maintained on the diets for 7 or 17 weeks; upon completion of the dietary regimes all of the animals were approximately 26 weeks of age and weighed on the average 3.7 Kg.

Table V

Composition of Pelleted Rabbit Chow[a]

Component	Amount
Crude protein not less than	16.0%
Crude fat not less than	2.0%
Crude fibre not more than	20.0%
Calcium	1.2%
Phosphorus	0.5%
Salt	0.8%
Vitamin A not less than 2000 I.U./lb.	

[a] Ralston Purina of Canada Ltd.

Each rabbit received via the marginal ear vein an injection of 5 μCi of DL-[methyl-^{14}C] carnitine hydrochloride dissolved in 1.0 ml of a solution of 30 μM (-)-carnitine in saline. At times of either 3,15,30, or 60 minutes post-label injection, blood samples were taken, the animal was then anesthetized, exsanguinated and the heart and aorta rapidly excised. Tissue samples were washed immediately in ice-cold saline and then washed in a solution of 1% carnitine in distilled water.

Study No. 5

These animals were used in a study designed to investigate carnitine palmitoyltransferase activity in normal rabbit aortas. Rabbits, weighing approximately 3.0 Kg, were maintained on a stock diet of pelleted rabbit chow.

Study No. 6

These animals were used in a study designed to investigate the influence of dietary cholesterol on arterial carnitine palmitoyltransferase activity. Age-matched rabbits were randomly grouped and maintained on one of the following diets for 3 - 48 days: (a) a non-atherogenic high-fat (HF) diet consisting of pelleted rabbit chow supplemented with 3% peanut oil; or (b) an atherogenic high-fat cholesterol (HFC) diet consisting of the HF-diet supplemented with 1% cholesterol.

Preparation and Analysis of Tissue and Blood Samples from Animals in Study No. 1, No. 2 and No. 3

Tissue Preparation

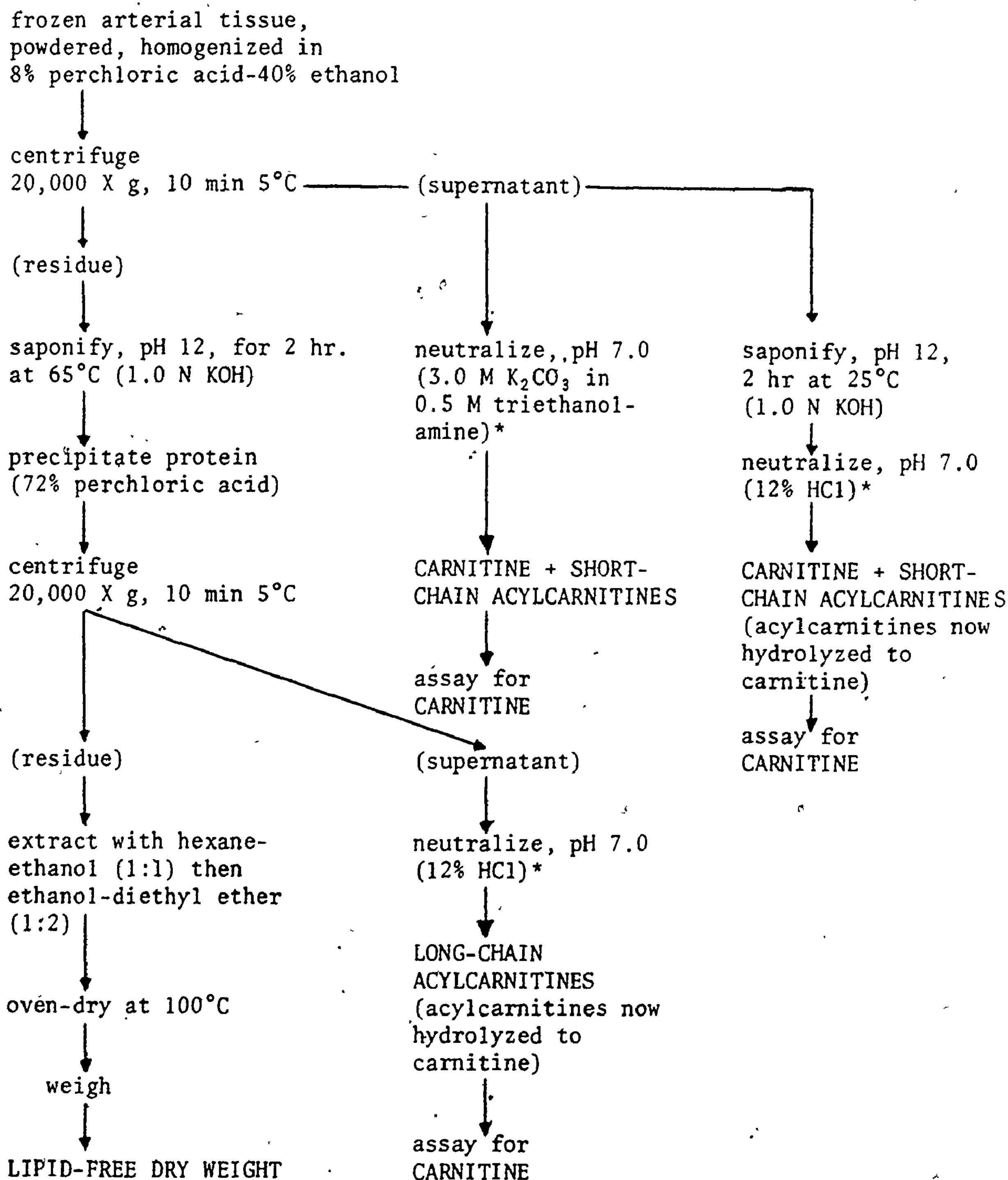
Aortas were rapidly excised, stripped free of adventitial tissue and immediately frozen in dry ice-acetone to reduce potential changes in the acylation state of carnitine (44). The segment of arterial tissue studied was defined anatomically as the aortic arch plus the entire descending aorta to the iliac bifurcation.

Differential Extraction of Carnitine and Acylcarnitine Compounds from Arterial Tissue (Flow Chart 1)

Samples were prepared according to a modified method of Williamson and Corkey (168). Tissue (500 - 1000 mg) was pulverized in a dry ice-cooled stainless steel percussion mortar followed by homogenization in 3.5 volumes of 8% perchloric acid in 40% ethanol; the homogenizers were rinsed with 2.5 volumes of 8% perchloric acid which was added to the homogenate. The homogenization procedure was carried out on crushed dry ice. The homogenates were centrifuged at 20,000 X g (B20 International Centrifuge, rotor no. 873) for 15 minutes at 0°C. The supernatant fluid, containing acid-soluble carnitine compounds i.e. carnitine and short-chain (C_2 - C_{10}) acylcarnitines, was divided into two pools. One pool was neutralized using 3.0 M K_2CO_3 in 0.5 M triethanolamine and assayed for carnitine; the other pool was adjusted to pH 12 using 1.0 N KOH, saponified for 2 hours at 25°C to hydrolyze short-chain

Flow Chart 1

Differential Extraction of Carnitine and Acylcarnitine Compounds from Arterial Tissue



* Centrifugation at 20,000 X g may be necessary to sediment residual $KClO_4$

acylcarnitine compounds and then neutralized to pH 7 using 12% HCl, and assayed for carnitine. The difference in carnitine concentration between the saponified and non-saponified pools provided a measure of short-chain acylcarnitine compounds originally present in the sample. The homogenate residue, containing acid-insoluble carnitine compounds i.e. long-chain (C_{12} and greater) acylcarnitines, was suspended in distilled water (2 ml/g fresh weight of residue) and saponified as described previously for acid-soluble acylcarnitine, however, the samples were incubated at 65°C rather than 25°C. After saponification, the samples were allowed to cool to room temperature. Next, the protein was precipitated with a few drops of 72% perchloric acid and removed by centrifugation. The supernatant fluid was neutralized to pH 7 and assayed for carnitine; this provided a measure of long-chain acylcarnitine compounds originally present in the sample. The proteinaceous residue was washed with 3.0 ml of 5% trichloroacetic acid, extracted with 2 X 3.0 ml n-hexane:ethanol (1:1) then with 2 X 3.0 ml ethanol: diethyl ether (1:2) in a 50°C water bath for 30 minutes. The lipid-free residues were oven dried at 100°C for 1 hour and then weighed.

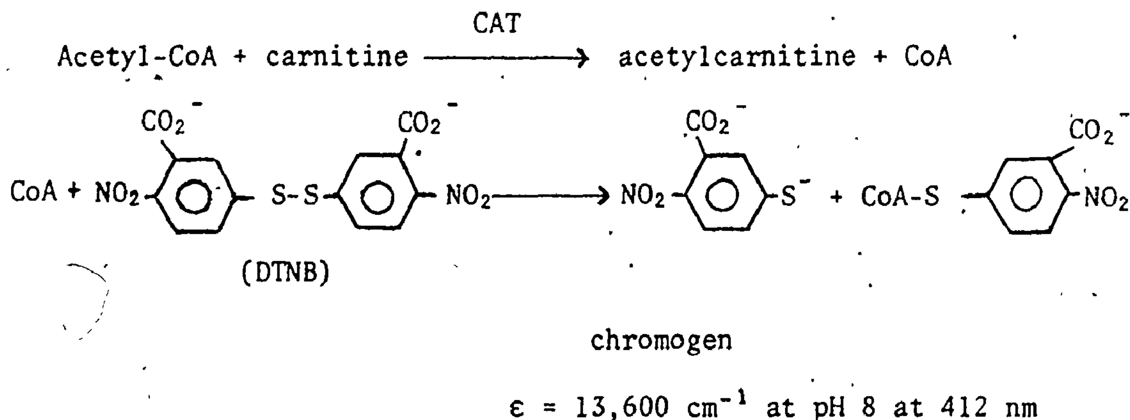
Losses incurred during sample preparation were monitored by adding 0.4 µmoles of (-)-carnitine to half of each sample which was then processed along with other samples. All data were corrected to 100% recovery.

Whole blood was centrifuged at 800 X g (International Equipment Co., Model PR-J) and the supernatant plasma removed.

Plasma protein was precipitated with a few drops of 72% perchloric acid and sedimented by low-speed centrifugation. After adjusting the deproteinated supernatant fluid to pH 7 using 3.0 M K_2CO_3 in 0.5 M triethanolamine, the samples were assayed for carnitine. Total plasma cholesterol i.e. cholesterol + cholesterol esters, and triacylgylcerides were extracted into a suspension of zeolite in isopropyl alcohol and analyzed on a Technicon Autoanalyzer (169).

Enzymatic Determination of Carnitine

Carnitine was assayed spectrophotometrically using the DTNB assay based on the coupled reaction system (19):



Each sample cuvette contained 100 μmoles of Tris-HCl at pH 8, 0.1 μmoles of acetyl-CoA, 0.1 μmoles of DTNB, 0.5 μmoles of neutral EDTA, 0.1 ml of sample and distilled water to give a final volume of 0.5 ml. The reaction was initiated by the addition of 5 μg of carnitine acetyltransferase (CAT). The change in absorbance was followed at 412 nm for 10 minutes using a dual beam Spectronic 20 recording spectrophotometer (Bausch Lomb); all assays were performed at room temperature.

Preparation and Analysis of Tissue and Blood Samples from Animals in Study No. 4

Tissue Preparation

The segment of arterial tissue studied comprised the aortic arch plus 8.0 cm of the descending aorta; aortas were stripped of adventitial tissue, cut longitudinally, and traced on graph paper to permit the calculation of arterial luminal surface area.

Extraction of Carnitine and Acylcarnitine Compounds from Arterial Tissue

The defined segment of aorta, heart (1.0 g wet weight), and blood (1.0 ml) were homogenized in 20 volumes of ice-cold chloroform: methanol (1:1) and allowed to extract overnight at 5°C. Extracts were centrifuged at 800 X g (International Equipment Co., Model PR-J) for 5 minutes to sediment tissue residue and the supernatant fluid removed; tissue residues were re-extracted with 2 X 5.0 ml of chloroform:methanol (2:1). The supernatant fluids were combined then washed according to Folch *et al.* (170). Carnitine and short-chain acylcarnitines were extracted into the aqueous phase of the Folch wash; long-chain acylcarnitines were extracted into the organic phase of the Folch wash. Test tubes containing the aqueous and organic phases were placed in a 45°C water bath and the samples evaporated to near dryness under a gentle stream of nitrogen; 7.0 ml of chloroform:methanol (1:1) was added to each tube.

Analysis of Tissue and Blood Samples

Aliquots (1.0 ml) of the samples containing carnitine and short-chain acylcarnitines were applied to TLC-plates coated with cellulose and chromatographed in two different solvent systems i.e. Solvent System 1 and Solvent System 2, in order to compare and confirm results. Bands on the TLC-plates were visualized with iodine vapour; bands corresponding to carnitine, acetylcarnitine, and butyrylcarnitine were identified with the use of co-chromatographing carnitine and acylcarnitine standards. In Solvent System 1, using cellulose coated TLC-plates, the R_f's for carnitine, acetylcarnitine, and butyrylcarnitine are approximately 0.35, 0.63 and 0.78, respectively; in Solvent System 2, the R_f's are 0.38, 0.53 and 0.74, respectively.

Aliquots (1.0 ml) of the samples containing long-chain acylcarnitines were applied to TLC-plates coated with silica gel G and chromatographed two times in Solvent System 3, in order to separate long-chain acylcarnitines from neutral lipids (long-chain acylcarnitines do not migrate in this system), and then developed in Solvent System 2. Long-chain acylcarnitines have an approximate R_f of 0.6 when applied on silica gel G-coated TLC-plates developed in Solvent System 2.

Carnitine and acylcarnitine bands were scraped into glass liquid-scintillation counting vials, 15 ml of toluene-based liquid scintillation counting fluid added and the radioactivity measured using an Intertechnique SL-40 liquid scintillation spectrometer (Intertechnique Instruments Inc., Dover, NJ., U.S.A.).

Quench corrections were made using the external standardization method.

Radioactivity in the homogenate residue was determined after dissolving the residue in Protosol.

Total tissue cholesterol, extracted into the organic phase of the Folch wash, was determined by the o-phthalaldehyde assay (171). Tissue DNA was extracted into 5% trichloroacetic acid at 90°C for 15 minutes then determined by the diphenylamine assay (172).

Preparation of Mitochondrial and Microsomal Fractions from Aortas of Animals in Study No. 5 and No. 6

The segment of arterial tissue studied comprised the aortic arch plus 9.0 cm of the descending aorta; aortas were stripped of adventitial tissue and rinsed in ice-cold 0.1 M phosphate buffer at pH 7.35. Arteries were finely diced and transferred to an all-glass homogenizer containing 10 ml of 0.1 M phosphate buffer at pH 7.35, 0.5 mM EDTA, and 2.0 mM GSH. The homogenizer was immersed in an ice-bath while the pestle was slowly rotated mechanically. The homogenate was transferred to ice-cold centrifuge tubes and centrifuged at 900 X g (Beckman Instruments, Inc., Model JC-21) for 10 minutes at 5°C to sediment cellular debris, unbroken cells and nuclei. The supernatant fluid was decanted and centrifuged at 8,500 X g for 10 minutes at 5°C to sediment the heavy mitochondrial fraction; the supernatant fluid was decanted and centrifuged at 15,000 X g for 10 minutes at 5°C

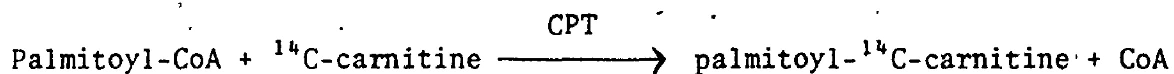
to sediment the light mitochondrial fraction. The light and heavy mitochondrial fractions were combined and washed in 5.0 ml of buffer, re-centrifuged at 15,000 X g and finally suspended in 8 - 10 ml of buffer. Mitochondrial preparations were stored on crushed ice until used in CPT assays. The 15,000 X g supernatant of the light mitochondrial fraction was centrifuged at 40,000 X g for 10 minutes at 5°C, the sediment discarded and the supernatant fluid centrifuged at 110,000 X g (Beckman Instruments Inc., Model L5-65 Ultracentrifuge with a Type 50 Ti rotor) for 1 hour at 5 - 10°C to sediment the microsomal fraction. The microsomal sediment was suspended in 8 - 10 ml of phosphate buffer. The 110,000 X g supernatant fluid was heat inactivated at 100°C for 15 minutes, allowed to cool, and diluted 1:1 with phosphate buffer. Microsomal preparations were stored on crushed ice until used in CPT assays. Some mitochondrial and microsomal preparations were sonicated for 3 X 15 seconds (Biosonik IV, Bronwill VWR Scientific), during the sonication procedure samples were cooled in an ice-bath. In some experiments 3 aortas were pooled and prepared as described but in most experiments the aortas were prepared individually. The protein concentration of the mitochondrial and microsomal preparations was determined by the method of Lowry (173).

Determination of Carnitine Palmitoyltransferase Activity in Mitochondrial and Microsomal Fractions

Assay Conditions

Carnitine palmitoyltransferase activity in mitochondrial

and microsomal suspensions was determined by measuring the incorporation of ^{14}C-carnitine into palmitoyl-^{14}C-carnitine according to the reaction:



$$\text{Palmitoyl-CoA} + {}^{14}\text{C-carnitine} \xrightarrow{\text{CPT}} \text{palmitoyl-}{}^{14}\text{C-carnitine} + \text{CoA}$$

Unless otherwise stated, each sample tube contained 0.11 mmoles of phosphate buffer at pH 7.35, 0.55 μmoles of EDTA, 2.2 μmoles of GSH, 1.1 μmoles of KCN, 2×10^5 dpm of either ^{14}C or ^3H-carnitine, 0.3 mg of heat inactivated 110,000 X g supernatant protein, and 0.2 mg of mitochondrial or microsomal protein in a final volume of 1.1 ml. The reaction was initiated by adding 11 μmoles of palmitoyl-CoA to the samples which were then mixed for 3 seconds. Samples were incubated 6 minutes at 37.5°C in a shaking water bath. The reaction was terminated by adding 2.0 ml of n-butanol to the samples which were then mixed for 3 seconds.

Extraction of Palmitoyl-^{14}C-carnitine (Palmitoyl-^3H-carnitine)

Samples were stored overnight in 5°C to extract palmitoyl-^{14}C-carnitine into the butanol. When necessary, samples were centrifuged at low-speed to induce a phase separation, the upper butanol phase was removed with a pasteur pipette. The aqueous phase was re-extracted with 2 X 1.0 ml of n-butanol and the n-butanol phases combined. Palmitoylcarnitine is quantitatively extracted into the butanol phase by this procedure (174). Test tubes containing the butanol phases were placed in a water bath at 55°C and the butanol evaporated to near dryness under a gentle stream

of nitrogen, 3.0 ml of chloroform:methanol (2:1) was then added to each tube.

Determination of Radioactivity in Palmitoyl-^{14}C-carnitine (Palmitoyl-^{3}H-carnitine)

Aliquots (1.0 ml) of the samples containing palmitoyl-^{14}C-carnitine were applied to TLC-plates coated with silica gel G and chromatographed in Solvent System 2. Radioactivity in the palmitoylcarnitine band was determined as previously described except the palmitoylcarnitine band was visualized with Rhodamine 6 G (rather than I_2) and the radioactivity measured with a Packard Tricarb Liquid Scintillation Spectrometer (Model 3375).

Determination of Acyl-CoA Hydrolase (ACH) Activity in Mitochondrial and Microsomal Fractions

ACH activity in mitochondrial and microsomal fractions prepared from arteries in animals used in Study No. 5 was determined by measuring the release of [1-^{14}C] palmitate from [1-^{14}C] palmitoyl-CoA according to the reaction:

$$[1\text{-}^{14}C]\ \text{palmitoyl-CoA} \xrightarrow{\text{ACH}} [1\text{-}^{14}C]\ \text{palmitate} + \text{CoA}$$

The assay conditions for the ACH assay were identical to those of the CPT assay except the reaction mixture also contained 1.2 X 10^5 dpm [1-^{14}C] palmitoyl-CoA. The reaction was terminated by the addition of 2.0 ml of methanol. The samples were extracted with 20 volumes of chloroform:methanol (2:1) and Folch washed. Test tubes containing the organic phase of the Folch wash, which

contained the [1-^{14}C] palmitate, were placed in a water bath at 30°C and the organic phase evaporated to near dryness under a gentle stream of nitrogen, 3.0 ml of chloroform:methanol (2:1) was then added to each tube. Aliquots (1.0 ml) of the sample were applied to TLC-plates coated with silica gel G, and chromatographed in Solvent System 3. The fatty acid band , as identified by co-chromatographing fatty acid (oleic acid) standard, was visualized with Rhodamine 6 G, scraped into a glass liquid scintillation vial, and the radioactivity in the sample measured as described previously.

Statistical Analyses of Data

Statistical analyses were performed as indicated in footnotes of Tables and legends to Figures. The word "significant" as used throughout the text will refer to the statistical significance between groups based on independent or paired t-tests.

RESULTS AND DISCUSSION

Study No. 1

Influence of Age on Arterial Carnitine Levels in Normal Rabbit Aortas

Introduction

Chemical, enzymatic and morphological changes occur during maturation and aging of the aorta (143,175); consequently, when attempting to identify specific changes in the artery wall that are due to atherogenesis, it is essential to differentiate between pathological changes and those that occur normally with growth and maturation. The purpose of this study was to investigate the utility of the DTNB assay for the determination of carnitine in arterial tissue and to investigate the effects of age on the concentration and distribution of carnitine and acylcarnitine compounds in aortas of growing rabbits.

Methods

Animals were fed the low-fat stock diet of pelleted rabbit chow. Treatment of animals and preparation of arterial tissue for carnitine assays are described in the footnote of Table VI.

Table VI

Effects of Age on the Concentration and Distribution of Carnitine and Acylcarnitines in the Normal Rabbit Aorta[a]

Carnitine concentration (nanomoles per gram of lipid-free dry weight)[b]	Age (weeks)			
	1	8	16	24
	— P < 0.01 —			
Total acid-soluble carnitine	119 ± 30	565 ± 98	486 ± 68	714 ± 83
	— P < 0.02 —	— P < 0.01 —		
Carnitine	46 ± 2	157 ± 35	460 ± 96	281 ± 24
	— P < 0.02 —	— P < 0.05 —	— P < 0.02 —	
Short-chain acylcarnitines	73 ± 31	408 ± 115	118 ± 52	433 ± 100
Acid-insoluble carnitine (long-chain acylcarnitines)	n.d.	n.d.	n.d.	n.d.
		— P < 0.01 —	— P < 0.02 —	
(Acylcarnitines X 10):total acid-soluble carnitine)	5.5 ± 1.0	6.4 ± 1.0	2.0 ± 0.9	5.5 ± 0.9

[a] Male New Zealand rabbits, 8-24 weeks of age, were fed a low-fat stock diet (pelleted Purina Rabbit Chow) and water *ad libitum*; 1-week-old rabbits were obtained directly from the McMaster University animal facility on the day of the experiment. Animals were sacrificed by exsanguination, under light sodium pentobarbital anesthesia, by cutting the neck vessels; aortas were rapidly excised, stripped free of adventitial tissue, and immediately frozen in dry ice-acetone. The frozen tissue was extracted and analyzed as described in detail under Preparation and Analysis of Tissue and Blood Samples from Animals in Studies 1,2 and 3.

[b] Values are the mean of eight animals ± SEM. P values, derived from a two-tailed table of Student's values for t, indicate the significant differences between groups based on an independent t test; only those with $P < 0.05$ are shown. n.d., not detected.

Results

Determination of Arterial Carnitine by the DTNB Assay

The lower limit of detection of (-)-carnitine by the DTNB assay was 3 nmoles, the change in absorbance at 412 nm was linear with respect to carnitine up to 38 nmoles, and the pH optimum of the assay was 8.0.

There was a 20-30% reduction in the concentration of carnitine added as an internal standard to samples during preparation; this reduction was not attributable to inhibition of the DTNB assay by the perchloric acid-ethanol homogenization medium or to limiting concentrations of acetyl-CoA or DTNB.

Arterial carnitine concentrations were within the limits of sensitivity of the DTNB assay.

Effects of Age on the Concentration and Distribution Between Carnitine and Acylcarnitine Compounds

Age-related changes in arterial carnitine concentration were observed in normal rabbits between 1 and 24 weeks of age (Table VI). The concentration of total acid-soluble carnitine increased significantly ($P < 0.01$) between 1 and 8 weeks of age from 119 to 565 nmoles/g of lipid-free dry weight; the increase was reflected in both carnitine and short-chain acylcarnitines, which increased 3- and 5-fold, respectively. The disproportionate increase in carnitine *vs* acylcarnitine was not significant, as indicated by the acylcarnitine:total acid-soluble carnitine ratio (Table VI).

Between 8 and 24 weeks of age, there were no significant changes in the level of total acid-soluble carnitine, although there was a tendency for 24-week values to be elevated. There were, however, significant changes in the distribution between carnitine and acylcarnitines, as shown by the ratio of acylcarnitine:total acid-soluble carnitine (Table VI). The ratio decreased significantly ($P < 0.01$) from 6.4 at 8 weeks to 2.0 at 16 weeks then increased significantly ($P < 0.02$) to 5.5 at 24 weeks of age.

Long-chain acylcarnitine compounds were not detected in aortas of normal rabbits in this study.

Discussion

DTNB Assay

A lower limit of detection of 3 nmoles, a linear relationship between absorbance change and carnitine up to 38 nmoles and a pH optimum of 8.0 are similar to reported values for the DTNB assay (19). DTNB concentrations ranging from 1.0 to 10.0 mM are commonly used in DTNB assays. Since DTNB slowly inactivates carnitine acetyltransferase (176), a low concentration of DTNB (0.2 mM) was used in the present study to minimize this effect. Rather than DTNB, the reagent of choice in future experiments may be 4,4'-dithiobispyridine; this reagent does not inhibit carnitine acetyltransferase and has a greater extinction coefficient than DTNB (19,800 *vs* 13,600 $M^{-1}cm^{-1}$) (91).

The 20-30% reduction in the concentration of carnitine added to the samples as an internal standard during sample

preparation is similar to that reported for chick embryo extracted with perchloric acid-ethanol (40). The reduction in carnitine concentration may be due, in part, to losses incurred during sample preparation or to co-extraction of some inhibitor of the DTNB assay; it is essential, therefore, that a known amount of carnitine be added to samples during preparation in order to calculate an appropriate correction factor.

In conclusion, the DTNB assay, when used with an internal carnitine standard, is suitable for the determination of arterial carnitine.

Influence of Age on Arterial Carnitine

Changes occur in both arterial carnitine concentration and distribution between carnitine and acylcarnitine compounds in the growing rabbit. The net aortic carnitine concentration increased nearly 5-fold (Table VI) during the first 8 weeks post partum; this increase probably represents a developmental phenomenon as the rabbit approaches maturity around 16 - 24 weeks of age. Compared to values at 1, 8 and 24 weeks of age, the concentration of short-chain acylcarnitine compounds showed an unusual variability at 16 weeks with values ranging from 0 - 341 nmoles/g of lipid-free dry weight; there was no evidence to suggest that the variability represented some uncontrolled hydrolysis during sample preparation. Changes in the distribution between carnitine and acylcarnitine between 8 and 24 weeks may reflect variations in the activity of carnitine acyltransferase

enzymes or perhaps some form of biorhythm.

Age-related changes in carnitine concentration and carnitine acyltransferases have been described in developing chick embryo, neonatal liver, and brown adipose tissue in the rat (36,38,40).

The absence of long-chain acylcarnitine compounds in the aorta is consistent with findings of other workers in many tissues of the rat (44). Based on the lower limit of detection of 3 nmoles/sample in the DTNB assay and the lipid-free dry weight of the aorta, if the concentration of arterial long-chain acylcarnitine compounds were less than 36 nmoles/g of lipid-free dry weight, they could not have been detected in this study.

Summary

Arterial carnitine levels increased with age for a period of 8 weeks post partum in rabbits maintained on the commercial low-fat stock diet; the distribution of carnitine and acylcarnitine compounds remained relatively constant during this time. In contrast, during the next 16 weeks, changes in the distribution of carnitine and acylcarnitines occurred, but without a significant increase in the total carnitine concentration. The occurrence of age-related changes in arterial carnitine compounds emphasizes the necessity of performing biochemical studies, particularly lipid metabolic studies subject to the effects of carnitine, on age-matched animals.

Study No. 2

Influence of Dietary Cholesterol on Arterial Carnitine Levels

Introduction

The rabbit is a useful model for studying cholesterol-induced atherosclerosis (177,178). Atheromatous lesions can be induced in rabbit aortas in a relatively short period of time e.g. 8 - 12 weeks, by supplementing the animal's diet with exogenous cholesterol. In this study, cholesterol was dissolved in melted lard and then thoroughly mixed with the low-fat stock diet. Addition of cholesterol to the diet in an oil vehicle ensures an even distribution of cholesterol in the food and promotes the intestinal absorption of cholesterol by the animal. The purpose of this study was to investigate the effects of high-fat (HF) and high-fat plus cholesterol (HFC) supplemented diets on arterial carnitine levels in the rabbit.

Methods

Diets, treatment of animals, and preparation of arterial tissue for carnitine assays are described in the footnote of Table VII.

Results

After 15 - 20 weeks, the intimal surfaces of aortas from animals receiving HFC were extensively involved with atheromatous lesions; no lesions were observed in the HF (control) group.

Table VII

Effects of High-Fat (HF) and High-Fat + Cholesterol (HFC) Supplemented Diets on Arterial Carnitine[a]

Carnitine compound	Aortic carnitine concentration and distribution[b]			
	Nanomoles per aorta		Nanomoles per gram of lipid-free dry weight	
	HF diet (n = 8)	HFC diet (n = 5)	HF diet (n = 8)	HFC diet (n = 5)
Total acid-soluble carnitine	35 ± 4	143 ± 14***	635 ± 58	1068 ± 137**
Carnitine	18 ± 1	58 ± 9***	339 ± 35	432 ± 68
Short-chain acylcarnitines	17 ± 3	85 ± 13***	297 ± 55	636 ± 123*
Acid-insoluble carnitine (long-chain acylcarnitines)	46 ± 7	128 ± 28**	824 ± 83	964 ± 312
Net carnitine[c]	81 ± 10	267 ± 19***	1460 ± 118	2032 ± 332
(Short-chain acylcarnitines X 10): total-acid-soluble carnitine	4.6 ± 0.6	5.9 ± 0.6	—	—
(Long-chain acylcarnitines X 10): long-chain acylcarnitines + carnitine	7.1 ± 0.2	6.6 ± 0.5	—	—

[a] Age-matched male New Zealand rabbits were maintained on a stock diet (pelleted Purina Rabbit Chow) supplemented with either 5% lard (HF) or 5% lard + 1% cholesterol (HFC); food and water were available *ad libitum*. Animals were sacrificed by exsanguination, under light sodium pentobarbital anesthesia, by cutting the neck vessels; aortas were rapidly excised, stripped free of adventitial tissue, and immediately frozen in dry ice-acetone. The frozen tissue was extracted and analyzed as describe in detail under Preparation and Analysis of Tissue and Blood Samples from Animals in Studies 1,2 and 3.

[b] Values are the mean ± SEM; the number of animals per group is given by n in parentheses. P values, derived from a two-tailed table of Student's values for t, indicate the significant differences between groups based on an independent t test.

[c] Net carnitine = total acid-soluble carnitine + acid-insoluble carnitine.

* $P < 0.05$ ** $P < 0.02$ *** $P < 0.001$

The concentration and distribution of arterial carnitine is presented in Table VII. The data in this Table is expressed on both an absolute basis (nanomoles/aorta) and a relative basis (nanomoles/g of lipid-free dry weight); the data is similar when expressed on either basis. However, expression of data on an absolute basis is more meaningful when comparing atherosclerotic *vs* non-atherosclerotic tissue since the atherogenic process is associated with progressive changes in arterial composition such as increased collagen and elastin deposition, and thus one is faced with continuously changing baselines unsuitable for deriving comparative data on a relative basis. For this reason, reference to Table VII will consider data expressed on the absolute basis of nanomoles/aorta.

The data in Table VII indicates that lard and cholesterol are independently capable of modifying arterial carnitine. First, the addition of lard to the diet resulted in the appearance of long-chain acylcarnitines, which were not observed in arteries of similarly aged rabbits fed the stock diet (Tables VI and VII). Second, the addition of cholesterol to the lard-containing diet (HFC) resulted in a 3-fold increase ($P < 0.01$) in net arterial carnitine, which is the sum of total acid-soluble carnitine (carnitine + short-chain acylcarnitines) plus acid-insoluble carnitine (long-chain acylcarnitines), Table VII. The increase in net carnitine in aortas from HFC-diet fed rabbits was reflected in 2- to 3-fold increases in all carnitine fractions. As indicated by the ratio of short-chain acylcarnitine:total

acid-soluble carnitine and the ratio of long-chain acylcarnitine: long-chain acylcarnitine + carnitine, the increases in carnitine and acylcarnitine compounds were not accompanied by changes in distribution.

Discussion

This study demonstrates that carnitine levels in the artery can be altered by diet and disease.

Although arterial long-chain acylcarnitines were not detected in rabbits maintained on the stock diet (Table VI), it is apparent that the rabbit aorta has the enzymatic capacity for long-chain acylcarnitine formation, as the addition of lard to the animal's diet resulted in the appearance of long-chain acylcarnitines representing up to 56% of the net aortic carnitine (Table VII). In this respect, the artery responds to a high-fat diet in a manner similar to organs such as heart, liver and kidney (44,45).

The commercially available lard used in the HF and HFC-diets contained butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) as antioxidants. The total permissible content of BHA + BHT in lard is 0.02%, from this value it can be calculated that the BHA + BHT intake of rabbits in this study would have been approximately 0.3 mg/kg/day. BHA and BHT have no toxic effects at a level of 50 mg/kg/day, some toxic effects occur at higher levels e.g. 500 mg/kg/day (179). It is unlikely, therefore, that antioxidants in the lard used in the present

study would affect the interpretation of data in Table VII.

Aortas from animals fed the stock diet supplemented with 5% lard and 1% cholesterol exhibited increased levels of carnitine and acylcarnitine compounds compared with aortas from control animals receiving the stock diet supplemented with 5% lard (Table VII). The specific increase in the acid-soluble carnitine compounds observed in arteries of rabbits fed the HFC-diet would also have been apparent had the data been expressed on a DNA rather than lipid-free dry weight basis. In our experience with rabbits, arterial DNA and lipid-free dry weight are proportional to one another in both normal and atherosclerotic tissue. This indicates that increased arterial carnitine observed in atherosclerotic arteries does not merely reflect an increase in cellularity.

Although increased carnitine can be observed in aortas from HFC-fed rabbits on either a relative (nanomoles/g lipid-free dry weight) or an absolute (nanomoles/aorta) basis, the absolute basis provides a more consistent mode of expression for comparative analysis between atherosclerotic and non-atherosclerotic tissue. Several recent studies have emphasized the necessity of comparing absolute amounts of tissue components, in identically defined tissue segments, when considering net changes in arterial tissue components (180,181). Expression of data on the relative basis of µg/g wet weight may explain the insignificant differences in arterial carnitine levels between normal and atherosclerotic human vascular tissue (182,183).

The distribution of atherosclerotic lesions in man and experimental animals is focal in nature. This focal distribution of the lesions observed during the early stages of atherosclerosis appears to correlate with focal areas of the arterial wall exhibiting increased permeability toward plasma constituents (184,185). Increased permeability of arteries to plasma albumin and cholesterol has been observed in cholesterol-fed rabbits and pigs (186,187). Although the origin of arterial carnitine is not known, it is possible that part or all of the carnitine accumulating in atherosclerotic arteries is derived from the blood due to increased arterial wall permeability. It may be worthwhile to determine whether carnitine accumulates preferentially in areas of lesion development or whether it is uniformly distributed throughout the entire aorta.

The artery is composed of 3 distinct layers (i.e. tunica adventitia, tunica media and tunica intima) and several cell types (e.g. endothelial cells, smooth muscle cells, and fibroblasts). It is not known in this study in which layer (with the exception of the tunica adventitia which was removed) or cell type carnitine accumulates. It is likely, however, that most of the carnitine accumulates in smooth muscle cells since this cell type is predominant in the arterial tunica media as well as in atherosclerotic lesions.

Summary

Aortas from animals fed the stock diet supplemented with 5% lard and 1% cholesterol exhibited increased levels of carnitine and acylcarnitine compounds when compared to aortas from control

animals receiving the stock diet supplemented with 5% lard. Long-chain acylcarnitines, not detected in aortas of animals fed the stock diet, appeared when the diets were supplemented with 5% lard, accounting for up to 56% of the net arterial carnitine.

Study No. 3

Influence of Dietary Cholesterol on Plasma Carnitine Levels

Introduction

Although carnitine has been found in most tissues, only the liver and the testes seem to have the capacity for complete carnitine biosynthesis (66,68,69,70). These observations suggest that most tissues derive their required carnitine from the blood. Consequently, alteration in the level of circulating carnitine may influence tissue carnitine concentration. The purpose of this study was to investigate the effect of high-fat (HF) and high-fat plus cholesterol (HFC) supplemented diets on plasma carnitine levels.

Methods

Diets, treatment of animals, and preparation of blood samples for carnitine, total cholesterol and triacylglyceride analysis are described in the legends of Figures 6 and 7.

Results

Figure 6 shows that supplementation of the stock diet (LF) with 5% lard (HF) resulted in a significant ($P < 0.001$) 42% lowering of plasma carnitine levels within 1 week. Similar reductions in plasma carnitine levels were observed in low-fat (LF) stock diet fed animals with fasting. Non-fasted values of 3.3 ± 0.3 decreased 42% to 1.9 ± 0.2 µmoles/100 ml of plasma after food deprevation for 18 hours ($P < 0.01$, paired t test, $n = 9$). In contrast to the effect of 5% lard alone, supplementation of the LF-stock diet with 5% lard plus 1% cholesterol significantly ($P < 0.001$) elevated plasma carnitine levels 59% within 1 week. This represented a 4-fold difference in plasma carnitine concentration (1.1 *vs* 4.6 µmoles of carnitine/100 ml of plasma) when comparing HF-to HFC-fed rabbits. No changes in plasma carnitine levels were observed in rabbits fed the non-supplemented stock (LF) diet during this 1-week period.

To investigate further the effect of diet on plasma carnitine, the animals referred to in Figure 6 were maintained on their dietary regimes for an additional time period and then returned to the stock (LF) diet (Figure 7). At regular intervals, plasma carnitine, cholesterol, and triacylglycerides were determined. Cholesterol supplementation of diet increased plasma carnitine to a maximum level within 1 week, after which the level of plasma carnitine remained relatively constant; returning the animals to the stock (LF) diet brought about a steady decrease in plasma carnitine to almost pre-HFC diet levels. Plasma cholesterol levels tended to parallel the changes in plasma carnitine levels although the magnitude of

Figure 6

The effects of low-fat(LF), and high-fat (HF), and high-fat plus cholesterol (HFC) supplemented diets on the level of plasma carnitine. Age-matched male New Zealand rabbits were maintained, for a period of 1 week, on one of the following diets: (a) low-fat stock diet (pelleted rabbit chow) (LF), (b) stock diet (LF) supplemented with 5% lard (HF), or (c) stock diet (LF) supplemented with 5% lard plus 1% cholesterol (HFC); food and water were available *ad libitum*. Blood samples were taken on Days 1 and 8 via cardiac puncture, using heparinized syringes, and plasma carnitine levels were determined. Values are the mean ± SEM of the percent change in plasma carnitine on Day 8 *vs* Day 1. P values, derived from a two-tailed table of Student's values of t, are based upon a paired t test; NS, non statistically significant. The number of animals per group is given by n in parentheses.

77(a)

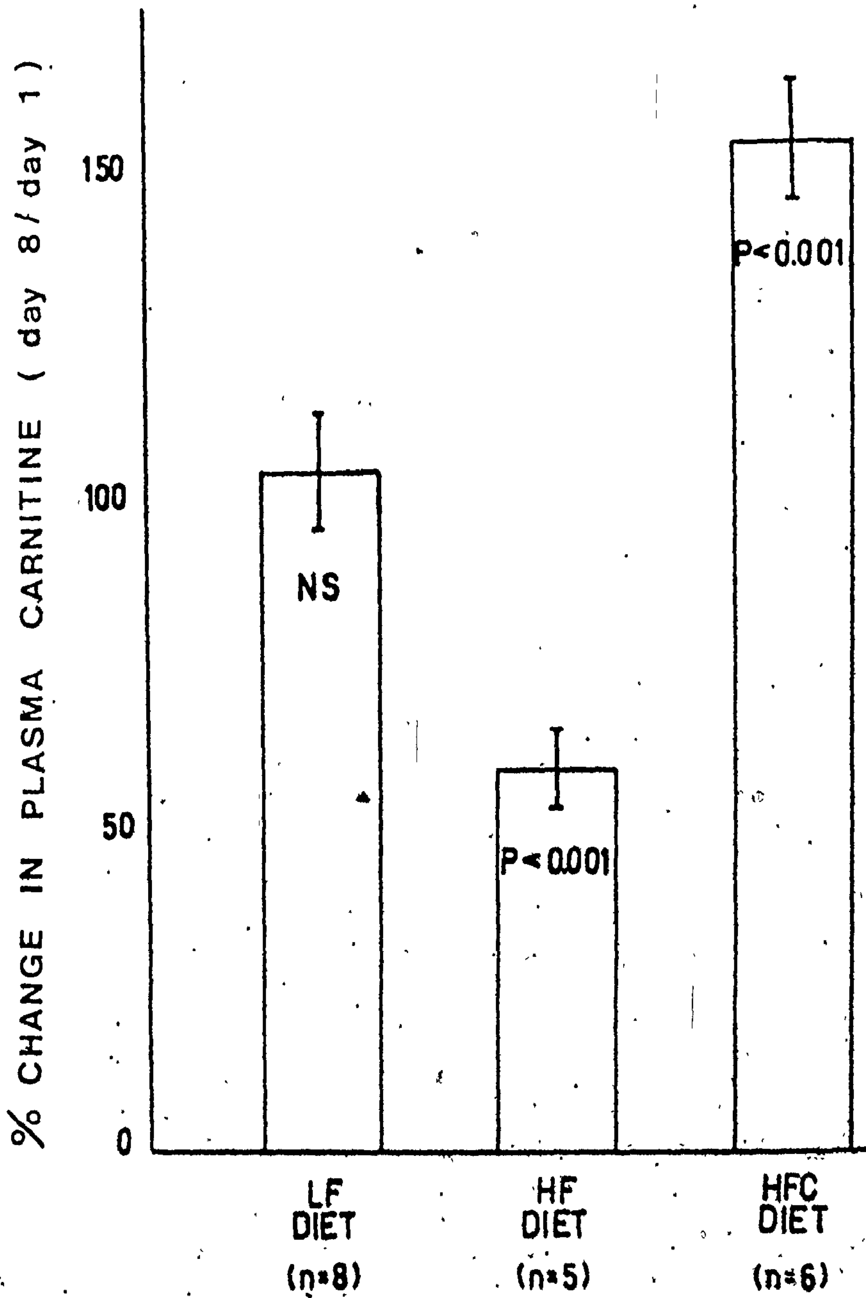
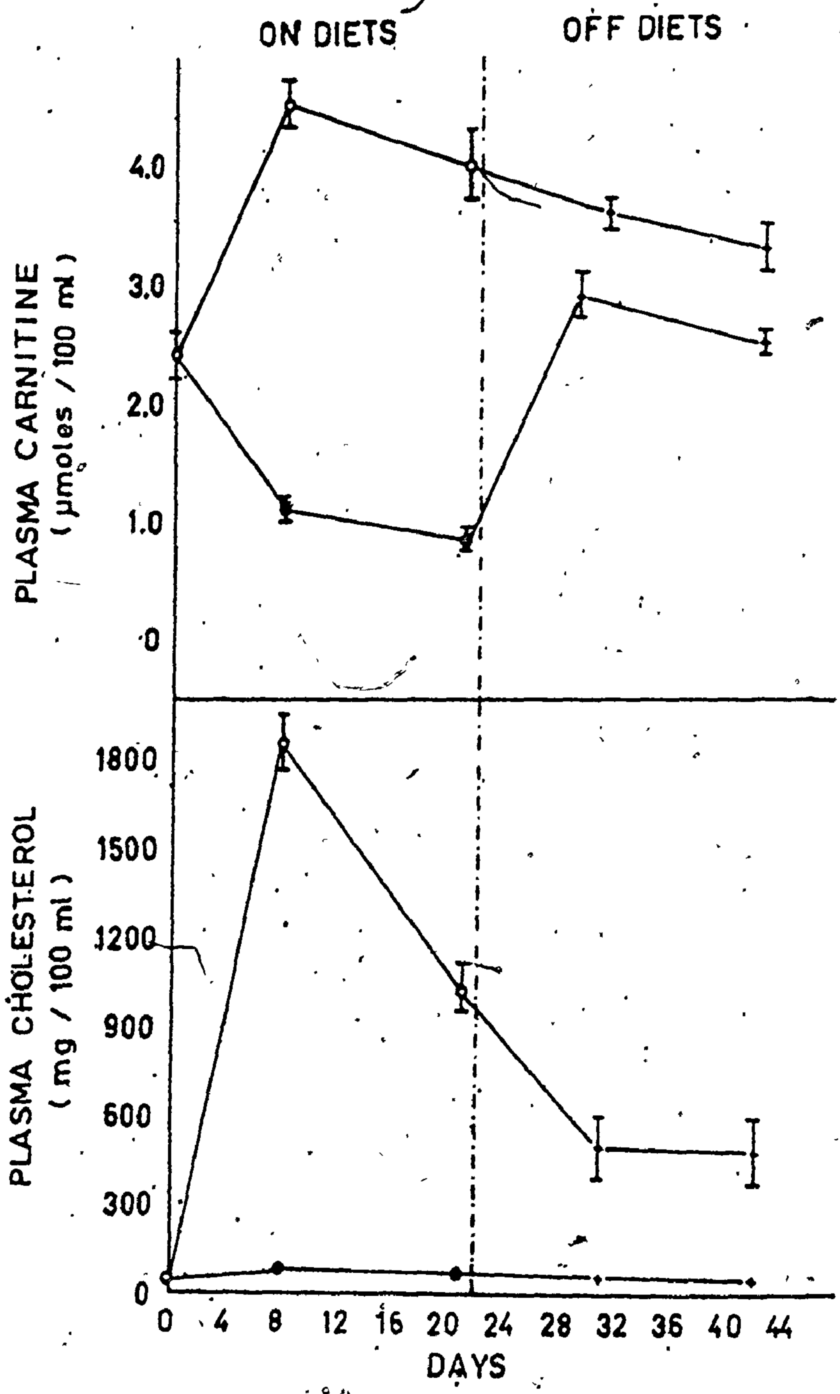


Figure 7

The effects of changing high-fat (HF) or high-fat plus cholesterol (HFC) supplemented diets to a low-fat (LF) stock diet on plasma carnitine and cholesterol. Age-matched male New Zealand rabbits were maintained, for a period of 21 days, on one of the following diets: (a) stock diet (LF) supplemented with 5% lard (HF, ●——●; n = 5) or (b) stock diet (LF) supplemented with 5% lard plus 1% cholesterol (HFC, o——o; n = 6); after 21 days all rabbits were switched to a low-fat stock diet (pelleted rabbit chow, LF, +——+——+) for an additional 21-day period. Food and water were available *ad libitum*. Plasma carnitine and cholesterol were measured in blood samples taken via cardiac puncture using heparinized syringes. Values are the mean ± SEM.


ON DIETS
OFF DIETS
PLASMA CARNITINE
(µmoles / 100 ml)
4.0
3.0
2.0
1.0
0
PLASMA CHOLESTEROL
(mg / 100 ml)
1800
1500
1200
900
600
300
0
0
4
8
12
16
20
24
28
32
36
40
44
DAYS

change exhibited in cholesterol levels was greater. In contrast to the HFC-diet, the addition of lard to the stock diet (HF) decreased plasma carnitine to a minimum level within 1 week. Plasma carnitine levels returned to pre-HF-diet levels within 1 week after changing the animals to the stock (LF) diet. Aside from an elevation in plasma cholesterol from 42 to 85 mg% after 1 week on the HF-diet, plasma cholesterol remained constant over the entire experimental period.

Neither HFC- nor HF-diets resulted in any significant changes in plasma triacylgylcerides during the course of the study (HFC-diet range: 185-200 mg%; HF-diet range: 136-157 mg%).

Although in Figure 7 plasma carnitine and cholesterol levels appear to drop slightly between 8 and 21 days while still on HFC-diet, determination of plasma carnitine and cholesterol in rabbits fed the HFC-diet for 9 weeks (plasma carnitine: 4.0 ± 0.3 μmoles/100 ml of plasma, n = 6; cholesterol: 2,134 ± 397 mg%, n = 6) indicated that these parameters remained elevated as long as the dietary regime was maintained.

Discussion

This study demonstrates that plasma carnitine levels can be modified by diet and nutritional state. Knowledge of factors that increase or decrease plasma carnitine levels may find clinical application in the treatment of diseases involving carnitine deficiency.

The concept that plasma carnitine levels can influence tissue carnitine levels finds support in the *in vitro* studies in which increased uptake of carnitine by isolated heart, liver, and

sperm cells occurred with increased concentrations of carnitine in the suspending medium (79,80,188). Studies No. 2 and No. 3 provide an *in vivo* example of this concept with the finding that elevated plasma carnitine levels associated with cholesterol feeding are accompanied by an accumulation of carnitine in the aorta.

Carnitine participates in the regulation of many metabolic processes, such as gluconeogenesis, fatty acid oxidation, ketogenesis and fatty acid synthesis (189); conceivably, changes in tissue carnitine levels, secondary to altered plasma carnitine levels, can modify intermediary metabolism. In this regard, the findings of the present study may be pertinent to the interpretation of lipid metabolic studies performed with cultured smooth muscle cells exposed to normal and hypercholesterolemic serum. Furthermore, since serum factors that stimulate smooth muscle cell proliferation are of interest to the field of atherosclerosis, it may be noteworthy that carnitine was first identified as a growth factor in insects (20), promotes normal growth and development in human infants (43), and increases the mitotic index of cultured bone cells (190).

Summary

Plasma carnitine levels observed in low-fat stock-diet fed rabbits were decreased by fasting for 18 hours or by addition of 5% lard to the diet; in contrast, addition of 5% lard plus 1% cholesterol to the diet increased plasma carnitine levels.

Study No. 4

Influence of Dietary Cholesterol on the Accumulation and Esterification of Circulating Carnitine by Aorta and Heart: *In Vivo*.

Introduction

The origin of carnitine that accumulates in atherosclerotic aortas and the temporal relationship between arterial carnitine accumulation and atherosclerotic lesion development are unknown. Since carnitine biosynthesis is unlikely in the hypoxic environment of the aorta, the artery probably derives carnitine from the bloodstream. The purpose of this study was to investigate the *in vivo* uptake and esterification of circulating carnitine by aortas of animals maintained on non-atherogenic and atherogenic diets; for comparative purposes a parallel investigation was carried out in the heart.

Methods

Diets, treatment of animals, and preparation of tissue for analysis are described in the footnote of Table VIII.

Results

Effects of Non-atherogenic and Atherogenic Diets on the Composition of Aorta and Heart Tissue

Aortas from rabbits fed the non-atherogenic control diet were entirely free of macroscopically visible atherosclerotic lesions

Table VIII

Effects of Non-atherogenic and Atherogenic Diets on the Composition of Aorta and Heart[a]

	Non-atherogenic diet (n = 8)	Atherogenic diet 7 weeks (n = 8)	Atherogenic diet 17 weeks (n = 8)
Aorta			
DNA (mg/aorta)[b]	0.98 ± 0.02	0.94 ± 0.05	1.76 ± 0.11*
Dry wt. (mg/aorta)	120 ± 6	135 ± 7	209 ±11
Total cholesterol (mg/g lipid-free dry wt.)	7 ± 1	36 ± 7*	137 ±13*
Surface area (cm^2/aorta)	9.6 ± 0.02	9.4 ± 0.2	10.2 ± 0.4
Heart			
DNA (mg/g wet wt.)	1.52 ± 1.0	1.51 ± 0.04	1.18 ± 0.13
Dry wt. (mg/g wet wt.)	186 ± 5	199 ± 9	177 ± 7
Total cholesterol (mg/g lipid-free dry wt.)	14 ± 1	29 ± 2*	23 ± 2*

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[a] Age-matched male New Zealand rabbits were maintained on either a non-atherogenic control diet of pelleted rabbit chow or an atherogenic diet consisting of the control diet supplemented with 5% lard plus 1% cholesterol; food and water were available *ad libitum*. Animals were sacrificed by exsanguination, under light sodium pentobarbital anesthesia, by cutting the neck vessels; the heart and aorta were rapidly excised and washed immediately in ice-cold saline and then washed in a solution of 1% carnitine in distilled water (w/v). Aortas were stripped of adventitial tissue. Samples of heart and aorta were homogenized in ice-cold chloroform:methanol(1:1, v/v) and analyzed as described in detail under Preparation and Analysis of Tissue and Blood Samples from Animals in Study 4.

[b] Values are the mean ± SEM; the number of animals per group is given by n in parentheses. P values, derived from a two-tailed table of Student's values for t, indicate the significant differences between groups based on an independent t test.

* $P < 0.001$.

and had a total cholesterol content (cholesterol + esterified cholesterol) of 7 ± 1 mg/g lipid-free dry weight. Aortas from rabbits fed the atherogenic diet for 7 weeks were essentially lesion-free whereas aortas from rabbits fed the atherogenic diet 17 weeks exhibited extensive atherosclerotic lesion development that covered most (70-90%) of the arterial luminal surface. Feeding the atherogenic diet resulted in a 5-fold increase (P <0.001) in arterial total cholesterol (36 ± 7 mg/g lipid-free dry weight) by 7 weeks and a 20-fold increase (P <0.001) in arterial total cholesterol (137 ± 13 mg/g lipid-free dry weight) by 17 weeks (Table VIII). In addition, arterial lipid-free dry weight and DNA were increased approximately 2-fold (P <0.001) above control values after 17 weeks on the atherogenic diet but were similar to control values in animals fed the atherogenic diet for only 7 weeks (Table VIII). Hereafter, aortas from animals fed the non-atherogenic control diet and atherogenic diet for 7 or 17 weeks will be operationally defines as normal, pre-artherosclerotic and atherosclerotic, respectively.

Heart tissue from animals fed the atherogenic diet 7 or 17 weeks exhibited a 2-fold increase (P <0.001) in total cholesterol (29 ± 2 and 23 ± 2 mg/g lipid-free dry weight). No significant differences were detected in lipid-free dry weight or DNA content of hearts from animals fed the control *vs* atherogenic diet (Table VIII).

<u>Clearance of ^{14}C-Carnitine from the Blood and its Accumulation by Aorta and Heart, *In Vivo*: Effects of Control and Atherogenic Diets</u>

Intravenously injected DL-[methyl-^{14}C] carnitine rapidly disappeared from the bloodstream in animals fed either control or atherogenic diets (Figure 8), however, the clearance rate of ^{14}C-carnitine, calculated from a semi-logarithm plot (Figure 8, insert) of ^{14}C-carnitine blood die-away curves, in rabbits fed the atherogenic diet ($T_{\frac{1}{2}}$ = 29 minutes) was increased approximately 3-fold compared with rabbits fed the non-atherogenic control diet ($T_{\frac{1}{2}}$ = 9.5 minutes).

In general, the accumulation of ^{14}C-carnitine derived from the bloodstream by aortas in each of the three dietary groups was rapid, i.e. detectable within 3 minutes post-label injection, with peak ^{14}C-carnitine tissue activity (dpm/aorta) coinciding with peak plasma activity. In normal aortas, the accumulation of ^{14}C-carnitine activity decreased as the level of blood ^{14}C-carnitine declined (Figures 8 and 9). In contrast, ^{14}C-carnitine activity in pre-atherosclerotic and atherosclerotic aortas remained elevated despite the decline in blood ^{14}C-carnitine; pre-atherosclerotic and atherosclerotic aortas had accumulated 4- and 8-fold as much labelled carnitine as normal aortas (210 ± 36 *vs* 788 ± 249 and 1,695 dpm/aorta) (Figure 9) 60 minutes post-label injection. The accumulation of ^{14}C-carnitine from the blood by aortas of animals fed atherogenic diets was apparent as early as 3 minutes post-label injection and increased with the degree of atherosclerosis as assessed by changes in arterial total cholesterol, lipid-free dry weight, and DNA (Table VIII and Figure 9). Comparison of normal and

Figure 8

Clearance of intravenously injected DL-[methyl-^{14}C] carnitine from the bloodstream of rabbits fed non-atherogenic or atherogenic diets. Twenty age-matched rabbits were fed a non-atherogenic diet (LF) of pelleted rabbit chow (—●—●—; n = 8) or an atherogenic diet (HFC) consisting of the LF-control diet supplemented with 5% lard plus 1% cholesterol for a period of 7 weeks (—□—□—; n = 8) or 17 weeks (—Δ—Δ—; n = 4); food and water were available *ad libitum*. Upon completion of the diets each animal received *via* the marginal ear vein an injection of 5 μCi of DL-[methyl-^{14}C] carnitine hydrochloride. One or two animals were bled and sacrificed at each time interval and the blood analyzed for ^{14}C-carnitine as described under Preparation and Analysis of Tissue and Blood Samples in Study 4. Points representing the mean of 2 animals are shown with their range; other points represent values from individual animals. The insert is a semilog plot of Figure 8; data from animals fed the atherogenic diet for 7 or 17 weeks are combined.

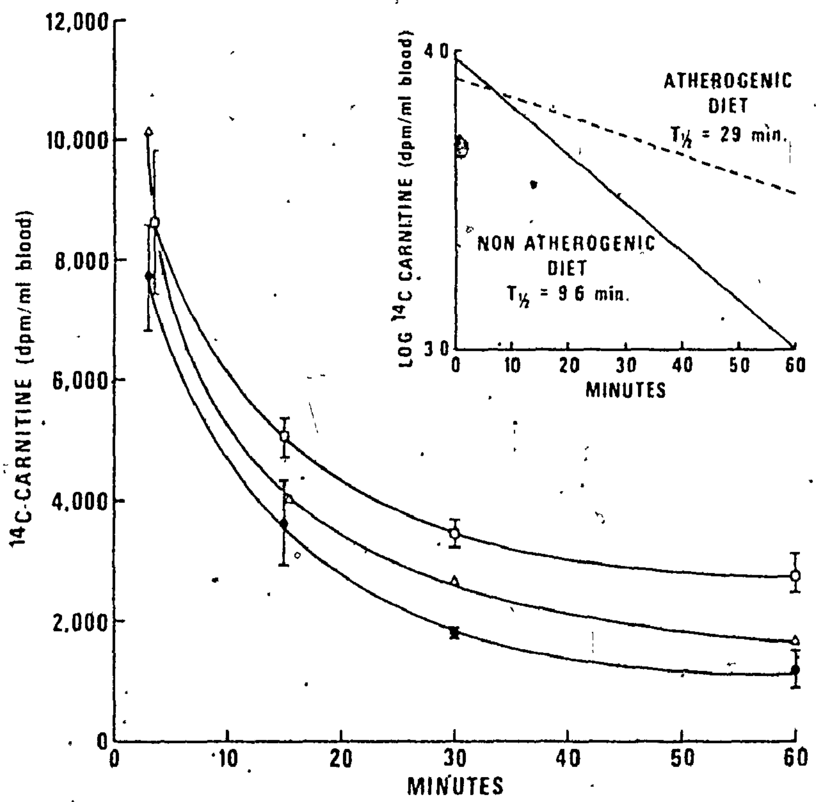
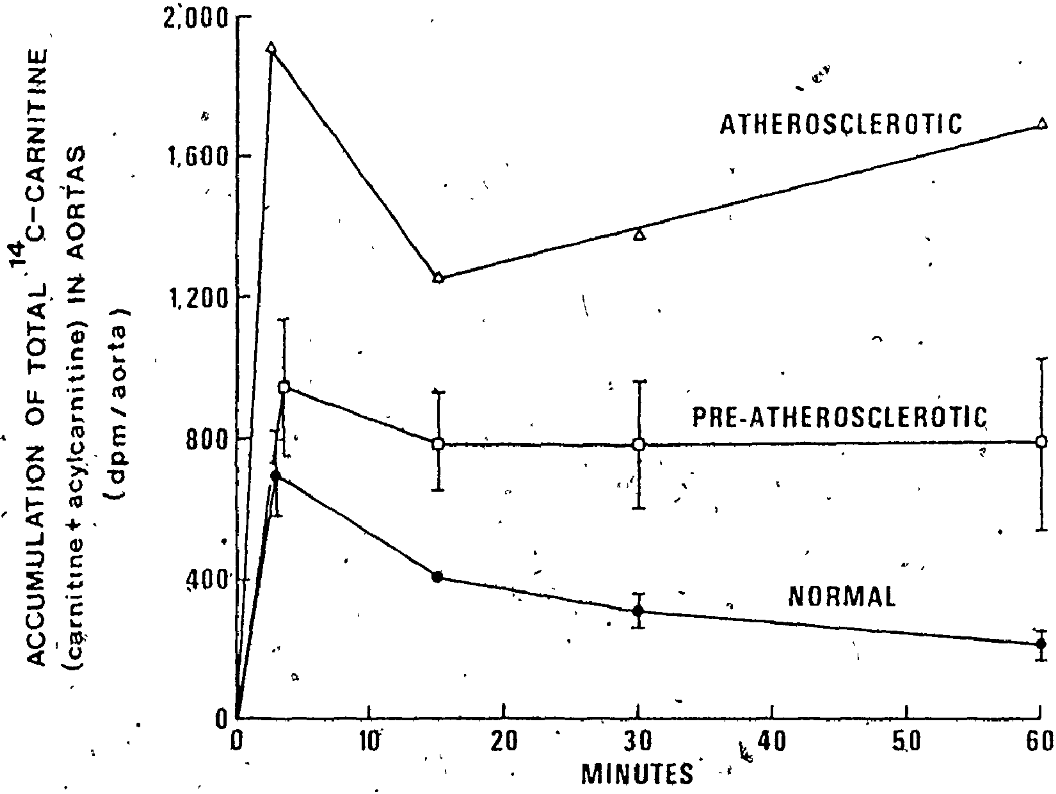

12,000
10,000
8,000
6,000
4,000
2,000
0
^{14}C-CARNITINE (dpm/ml blood)
0
10
20
30
40
50
60
MINUTES
LOG ^{14}C CARNITINE (dpm/ml blood)
4 0
3 0
ATHEROGENIC DIET
T½ = 29 min.
NON ATHEROGENIC DIET
T½ = 9 6 min.
MINUTES

Figure 9

Time course of total ^{14}C-carnitine (carnitine + acylcarnitine) accumulation in aortas after the intravenous injection of labelled carnitine into rabbits maintained on a non-atherogenic control diet (—●—●—; n = 8) or atherogenic diet for 7 (—□—□—; n = 8) or 17 (—Δ—Δ—; n = 4) weeks. Experimental conditions are described in Figure 8. Animals were sacrificed by exsanguination, under light sodium pentobarbital anesthesia, by cutting the neck vessels; aortas were rapidly excised, washed immediately in ice-cold saline, and then washed in a solution of 1% carnitine in distilled water. Aortas were stripped free of adventitial tissue then homogenized in ice-cold chloroform:methanol (1:1); carnitine and acylcarnitines were extracted from the homogenate as described in detail under Preparation and Analysis of Tissue and Blood Samples in Study 4. Points representing the mean of 2 animals are shown with their range; other points represent values from individual animals.


ACCUMULATION OF TOTAL 14C-CARNITINE
(carnitine + acylcarnitine) IN AORTAS
(dpm / aorta)
2,000
1,600
1,200
800
400
0
ATHEROSCLEROTIC
PRE-ATHEROSCLEROTIC
NORMAL
0
10
20
30
40
50
60
MINUTES

atherosclerotic tissue on an absolute basis (dpm/aorta) is preferable to comparison on a relative basis (see page 71 for explanation). Comparison on an absolute basis requires the use of defined arterial segments; the observation that the luminal surface areas of the aortas used in this study were essentially the same (Table VIII) indicates that this requirement was fulfilled.

Accumulation of ^{14}C-carnitine from the blood by the heart was rapid, reaching a maximal level within 3 minutes post-label injection. The activity of ^{14}C-carnitine in heart tissue remained elevated despite the decline in blood ^{14}C-carnitine (Figures 8 and 9). Heart tissue accumulated 6-fold more labelled carnitine than normal aortic tissue (14.3 ± 2.5 *vs* 2.5 ± 0.5 dpm/mg lipid-free dry weight) 60 minutes post-label injection. In contrast to the aorta, accumulation of labelled carnitine from the bloodstream by the heart was not affected by the animal's dietary regime; the accumulation of ^{14}C-carnitine was similar whether animals were fed control or atherogenic diets (Figure 10).

The efficiency of extraction of radioactivity from heart, blood, and aorta was 96, 95, and 85%, respectively.

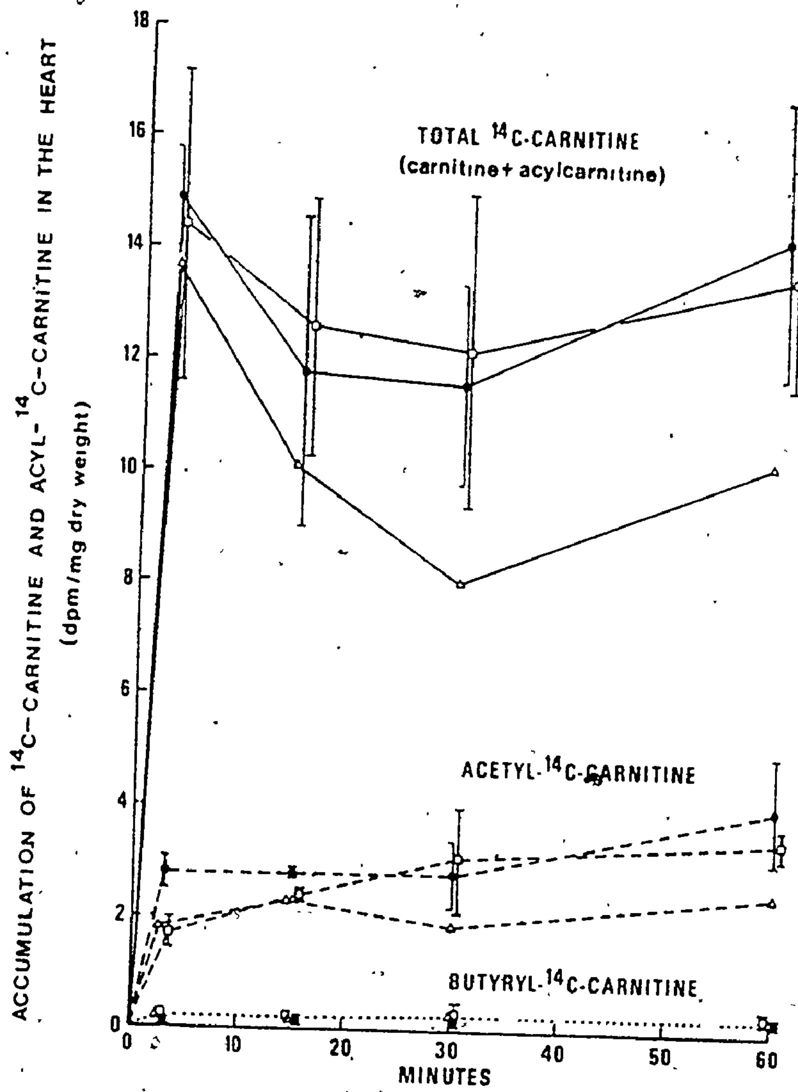
<u>Identification of Labelled Carnitine Compounds in Blood, Aorta and Heart</u>

^{14}C-carnitine was essentially the only labelled carnitine compound detected in the blood. Neither palmitoyl- nor butyryl-^{14}C-carnitine were detected in blood, however, a small amount of

Figure 10

Time course of ^{14}C-carnitine and acyl-^{14}C-carnitine accumulation in hearts after the injection of labelled carnitine into rabbits maintained on a non-atherogenic control diet (—●—●—; n = 8) or atherogenic diet for 7 (—□—□—; n = 8) or 17 (—Δ—Δ—; n = 4) weeks. Experimental conditions are described in Figure 8. Animals were sacrificed by exsanguination, under light sodium pentobarbital anesthesia, by cutting the neck vessels; hearts were rapidly excised, washed immediately in ice-cold saline, and then in a solution of 1% carnitine in distilled water. Heart tissue was homogenized in ice-cold chloroform: methanol (1:1); carnitine and acylcarnitines were extracted from the homogenate as described in detail under Preparation and Analysis of Tissue and Blood Samples in Study 4. Points representing the mean of 2 animals are shown with their range; other points represent values from individual animals.

89(a)



acetyl-^{14}C-carnitine (less than 1% of the total blood ^{14}C-carnitine activity) was detected. There was no evidence for the esterification of ^{14}C-carnitine in the blood.

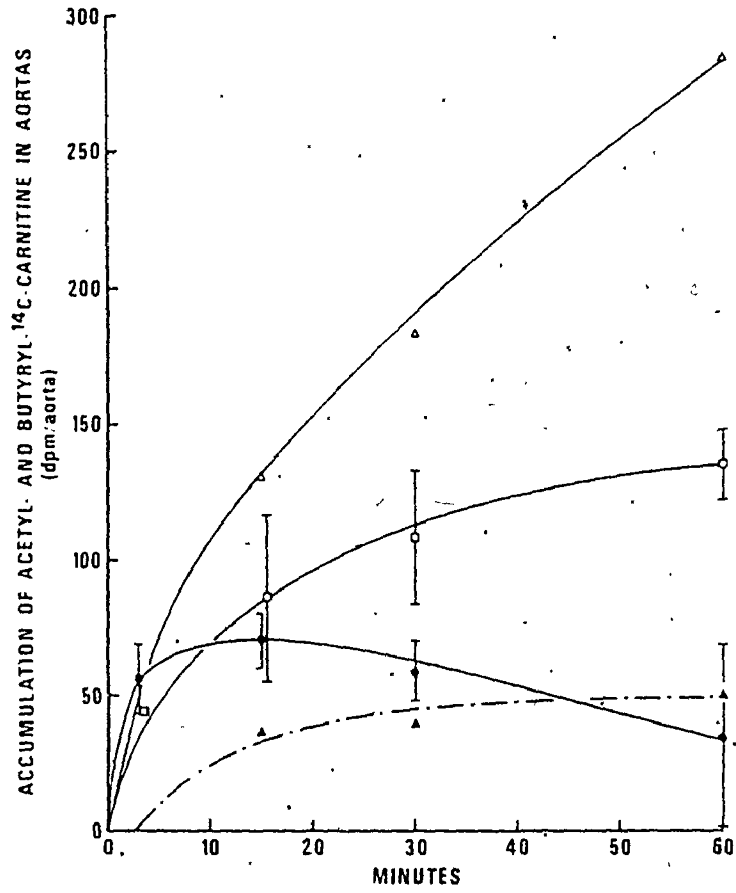
Labelled carnitine taken up by aortas of animals in each of the three dietary groups was recovered as ^{14}C-carnitine and as acyl-^{14}C-carnitine compounds. One hour post-label injection, pre-atherosclerotic aortas contained almost five times as much labelled carnitine as normal aortas (141 ± 36 *vs* 653 ± 326 dpm/aorta, calculated from Figures 9 and 11) and nearly four times as much acetyl-^{14}C-carnitine (34 ± 34 *vs* 135 ± 13 dpm/aorta) (Figure 11). The accumulation of acetyl-^{14}C-carnitine in normal aortas reached a maximum level by 15 minutes post-label injection; in contrast, acetyl-^{14}C-carnitine in pre-atherosclerotic aortas continued to accumulate for at least 60 minutes post-label injection (Figure 11). Atherosclerotic aortas accumulated ^{14}C-carnitine and acetyl-^{14}C-carnitine in even greater amounts than pre-atherosclerotic aortas (Figures 9 and 11) and, in addition, contained butyryl-^{14}C-carnitine an acyl-^{14}C-carnitine not detected in either normal or pre-atherosclerotic arteries (Figure 11).

Labelled carnitine taken up by the heart was recovered as ^{14}C-carnitine and as acyl-^{14}C-carnitines such as acetyl-^{14}C-carnitine butyryl-^{14}C-carnitine, and palmitoyl-^{14}C-carnitine; these acyl-^{14}C-carnitines represented approximately 23% of the total ^{14}C-carnitine activity of the tissue. One hour post-label injection, the heart had accumulated 6- and 12-fold more ^{14}C-carnitine and acetyl-^{14}C-carnitine, respectively, than the normal aorta (^{14}C-carnitine:

Figure 11

Time course of acetyl-^{14}C-carnitine accumulation in aortas after the intravenous injection of labelled carnitine into rabbits maintained on a non-atherogenic control diet (—●—●—; n = 8) or atherogenic diet for 7 (—□—□—; n = 8) or 17 (—Δ — Δ—; n = 4) weeks. Butyryl-^{14}C-carnitine (— ▲.— ▲.—; n = 4) was detected only in the aortas of rabbits fed the atherogenic diet for 17 weeks. Experimental conditions and tissue analysis are described in Figure 9.

91(a)



9.95 ± 1.58 *vs* 1.70 ± 0.05; acetyl-^{14}C-carnitine: 4.07 ± 0.87 *vs* 0.34 ± 0.34 dpm/mg lipid-free dry weight; heart *vs* aorta, respectively). In contrast to the normal aorta, acetyl- and butyryl-^{14}C-carnitine accumulated in the heart for at least one hour post-label injection; the accumulation of acyl-^{14}C-carnitines in the heart was similar whether animals were fed control or atherogenic diets (Figure 10). Acetyl- and butyryl-^{14}C-carnitine were detected in all hearts studied; palmitoyl-^{14}C-carnitine was detected in only 3 of 12 animals.

Discussion

Uptake and esterification of circulating carnitine by aorta and heart was studied, *in vivo*, in rabbits fed non-atherogenic or atherogenic diets.

Since DL-[methyl-^{14}C] carnitine was used in the study, there is the possibility that some of the radioactivity taken up by the aorta was contributed by labelled D-carnitine. However, it is unlikely that D-carnitine entering the aorta would influence the outcome of the experiments or alter the interpretation of the data for the following reasons: (a) only the naturally occurring L-enantiomer could have been converted to the observed labelled acylcarnitine compounds, and (b) D-carnitine would have been diluted to a level of less than 1% of the total blood carnitine pool size at zero time. Transmethylation of labelled methyl groups from carnitine to other compounds would not have been a problem in this study since carnitine does not participate in transmethylation reactions (20).

Effects of Non-atherogenic and Atherogenic Diets on the Accumulation of Blood ^{14}C-Carnitine by Aorta and Heart *In Vivo*

Labelled carnitine was taken up from the bloodstream by both the aorta and heart; intracellular uptake and not merely adsorption was indicated by the conversion of ^{14}C-carnitine to acyl-^{14}C-carnitine compounds (Figures 10 and 11). Accumulation of labelled carnitine in aortas of animals fed atherogenic diets increased with the severity of atherosclerotic lesion development (Figure 9); this increase was not dependent upon gross lesion development since the accumulation of labelled carnitine in aortas of animals fed the atherogenic diet was 4-fold greater than in aortas of animals fed the non-atherogenic control diet even before extensive lesion development occurred (Figure 9).

The differences in carnitine accumulation between normal and atherosclerotic arteries were probably underestimated in this study. If the specific activity of blood carnitine had been calculated, animals on the atherogenic diet would probably have had a lower blood carnitine specific activity than animals fed the control diet due to an increase in plasma carnitine concentration (Study No. 3; Figure 7), yet the accumulation of radioactive carnitine in aortas of rabbits fed the atherogenic diet was greater than that observed in aortas of rabbits fed the control diet.

A decrease in the clearance rate of blood carnitine (Figure 8) and the accumulation of labelled carnitine in atherosclerotic aortas (Figure 9) are consistent with the data from Studies No. 2 and No. 3

in which an elevation of the level of plasma and arterial carnitine was detected by direct chemical quantitation in rabbits fed the HFC-diet (Figure 7, Table VII). It is likely, therefore, that most of the carnitine accumulating in atherosclerotic rabbit aortas is derived from the bloodstream.

The observation that the accumulation of circulating ^{14}C-carnitine in the heart was similar in animals fed control or atherogenic diets indicates that the accumulation of ^{14}C-carnitine in the atherosclerotic aortas is a tissue response characteristic of the aorta and not just a general response of tissues to hypercholesterolemia.

The observation that the heart accumulated 6-fold more ^{14}C-carnitine from the bloodstream than the aorta is consistent with a greater metabolic demand for carnitine by the heart to accommodate the oxidation of fatty acids which represent the major metabolic fuel of the heart.

Effects of Non-atherogenic and Atherogenic Diets on Acyl-^{14}C-carnitine Accumulation by the Aorta and Heart *In Vivo*

Pre-atherosclerotic and atherosclerotic aortas accumulated more acetyl-^{14}C-carnitine than normal aortas (Figure 11). Since the ratio of short-chain acylcarnitine :total acid-soluble carnitine was not significantly increased in atherosclerotic arteries (Study No. 2, Table VII) the activity of carnitine acetyltransferase does not appear to increase in atherosclerosis, therefore, the accumulation of acetyl-^{14}C-carnitine probably represents an increase

in the availability of ^{14}C-carnitine to carnitine acetyltransferase rather than an increase in the activity of this enzyme, per se. The accumulation of ^{14}C-carnitine derived from the bloodstream and conversion to acetyl-^{14}C-carnitine may account for the increased level of short-chain acylcarnitine compounds observed in the aorta of rabbits fed the HFC-diet (Study No. 2, Table VII).

Whether or not acetylcarnitine is present in the blood is controversial. Reported values for circulating acetylcarnitine range from zero (191) to 30% of the total blood carnitine concentration (79). In this study, acetyl-^{14}C-carnitine was detectable in the blood but represented less than 1% of the total blood ^{14}C-carnitine activity. The trace presence of acetyl-^{14}C-carnitine in the blood is unlikely to introduce a problem in tissue analysis since the accumulation of labelled acetylcarnitine in the aorta and heart was far greater than could be accounted for on the basis of blood contamination in the microcirculation of these tissues.

Acyl-^{14}C-carnitine accumulation in the heart differed from the aorta in that acetyl-^{14}C-carnitine accumulation in the heart was essentially the same whether the animals had been fed the control or atherogenic diet. Butyryl-^{14}C-carnitine, which was detected only in atherosclerotic aortas, was commonly detected in the heart (Figure 11). These observations lend further support to the proposition that a greater accumulation and utilization of circulating carnitine by atherosclerotic arteries is a tissue-specific response to hypercholesterolemia.

Summary

The clearance rate of ^{14}C-carnitine from the bloodstream was greater in animals fed the non-atherogenic diet than the atherogenic diet. No evidence was found for carnitine esterification in the blood. ^{14}C-carnitine was taken up and esterified in both the heart and aorta of all animals regardless of diet, however, accumulation of ^{14}C-carnitine from the blood was greater in the heart than in the aorta. Aortas in animals fed the atherogenic diet exhibited an increased accumulation of ^{14}C-carnitine and acetyl-^{14}C-carnitine compared to aortas in animals fed the control diet. Uptake and utilization of circulating ^{14}C-carnitine by heart was not affected by the atherogenic diet. Butyryl-^{14}C-carnitine, although not detected in aortas from animals fed the non-atherogenic diet or atherogenic diet for only 7 weeks, was detected in aortas from animals fed the atherogenic diet for 17 weeks. Butyryl-^{14}C-carnitine was detected in heart tissue regardless of the animal's dietary regime. The increased accumulation and utilization of circulating ^{14}C-carnitine by the atherosclerotic aortas occurred before the development of extensive lesions; this response of the aorta, but not the heart, to atherogenic stimuli indicates that the response of the artery was not a general response to hypercholesterolemia.

Study No. 5

CPT Activity in Subcellular Fractions Isolated from Normal Rabbit Aortas

Introduction

Carnitine palmitoyltransferase (CPT) activity can be assayed by measuring the formation of palmitoyl-^{14}C-carnitine according to the reaction:

$$\text{Palmitoyl-CoA} + {}^{14}\text{C-carnitine} \xrightarrow{\text{CPT}} \text{palmitoyl-}{}^{14}\text{C-carnitine} + \text{CoA}$$

The characteristics of this reaction in the artery wall have not been investigated. The purpose of this study was to investigate CPT activity in subcellular fractions isolated from normal rabbit aortas.

Methods

Animals were fed the low-fat stock diet of pelleted rabbit chow. The isolation of subcellular fractions and enzyme assays are described in the footnotes of Tables and legends of Figures.

Results

Isolation of Arterial Subcellular Fractions

Mitochondrial, microsomal and cytosol fractions were isolated by differential centrifugation from homogenates of defined arterial segments. The protein yields (mg) of these fractions when prepared by the standardized procedure outlined under METHODS were (a) mitochondria - 4.7 ± 0.2, (b) microsomes - 4.7 ± 0.2, and

(c) cytosol - 8.1 ± 0.3 (values are the mean ± SEM of 6 aortas prepared individually).

In this study, the expression of mitochondrial and microsomal CPT activity on a relative activity basis (dpm/mg protein/min) or total activity basis (dpm/aorta/min) provided qualitatively similar information as the recovery of subcellular protein in mitochondrial and microsomal fractions was the same. Since protein sedimented at 40,000 X g during the preparation of microsomes was discarded, total CPT activity in the microsomal fraction would be underestimated; therefore, expression of CPT activity is restricted to relative CPT activity.

CPT Activity in Arterial Subcellular Fractions

CPT activity was associated with particulate fractions isolated from arterial cells; the relative CPT activity was similar in mitochondrial and microsomal fractions (mitochondria: 443 ± 15 dpm/mg protein/min; microsomes: 417 ± 37 dpm/mg protein/min, mean ± SEM, n = 6) (Table IX) and remained associated with these particulate fractions even after repeating washing and resuspension (Table X). Some CPT activity was detected in the 110,000 X g supernatant fluid (designated cytosol in Table IX), this CPT activity was 4-fold less than that detected in either mitochondrial or microsomal fractions.

Effect of Palmitoyl-CoA Concentration on CPT Activity

The effect of palmitoyl-CoA concentration on CPT activity

Table IX

Palmitoyl-^{14}C-Carnitine Formation by Subcellular Fractions Isolated from Normal Rabbit Aortas[a]

Subcellular fraction	Palmitoyl-^{14}C-carnitine formation[b] (dpm/mg protein/min)
Mitochondria	443 ± 15
Microsome	417 ± 37
Cytosol	114 ± 7

[a] Mitochondrial, microsomal and cytosol fractions were isolated by differential centrifugation from aortas of age-matched male rabbits that had been maintained on a stock diet (LF) of pelleted rabbit chow; food and water were available *ad libitum*. Palmitoyl-^{14}C-carnitine formation was assayed in sample tubes containing 0.11 mmoles of phosphate buffer at pH 7.35, 0.55 μmoles of EDTA, 2.2 μmoles of GSH, 1.1 μmoles of KCN, 2 X 10^5 dpm ^{14}C-carnitine, 11 nmoles of palmitoyl-CoA, 0.3 mg of heat-inactivated 110,000 X g supernatant protein, and either 0.2 mg of mitochondrial, microsomal, or cytosol protein, in a final reaction volume of 1.1 ml. Incubations were for 6 minutes at 37.5°C. Palmitoyl-^{14}C-carnitine extracted into n-butanol, identified by TLC, and radioactivity in palmitoyl-^{14}C-carnitine determined as described in detail under Determination of Carnitine Palmitoyltransferase Activity in Mitochondrial and Microsomal Fractions (see text).

[b] Values represent the mean ± SEM of 6 animals; aortas were prepared individually. All assays were performed in duplicate.

Table X

Effect of Repeated Washing and Resuspension of Mitochondrial and Microsomal Fractions on Palmitoyl-^{14}C-Carnitine Formation[a]

Number of washes and resuspensions	Palmitoyl-^{14}C-carnitine formation[b] (dpm/mg protein/min)	
	Mitochondria	Microsomes
First wash and resuspension		
Control	759 ± 29	664 ± 2
Experimental	770 ± 41	717 ± 5
Control / Experimental	0.99	0.93
Second wash and resuspension		
Control	715 ± 24	618 ± 24
Experimental	604 ± 39	515 ± 4
Control / Experimental	1.18	1.20

[a] Preparation of arterial subcellular fractions and assay for palmitoyl-^{14}C-carnitine formation are described in Table IX.

[b] Values are the mean ± SEM of three determinations of mitochondrial and microsomal fractions derived from three pooled aortas. All assays were performed in duplicate.

in mitochondrial and microsomal fractions is shown in Figures 12 and 13. CPT activity was detected at palmitoyl-CoA concentrations as low as 0.1 μM in both mitochondrial and microsomal fractions (153 ± 78 and 67 ± 9 dpm/mg protein/min; mean ± SEM, n = 3, mitochondria and microsomes, respectively). Maximal CPT activity in mitochondrial (1,092 ± 58 dpm/mg protein/min; mean ± SEM, n = 5) and microsomal (1,116 ± 119 dpm/mg protein/min; mean ± SEM, n = 5) fractions occurred between 5 - 25 μM palmitoyl-CoA; at higher concentrations of palmitoyl-CoA, e.g. 50 or 100 μM, CPT activity was reduced 50 and 81%, respectively, in both subcellular fractions (Figures 12 and 13). In the absence of exogenous palmitoyl-CoA, CPT activity represented 2.5% of the maximal activity detected in mitochondrial or microsomal fractions.

Effects of Subcellular Protein and Time on CPT Assays

CPT activity was linear with respect to mitochondrial and microsomal protein between 0.03 - 0.3 mg (Figures 14 and 15) and assay time between 3 - 12 minutes (Figures 16 and 17); no attempt was made to determine the limits of linearity.

Effects of KCN, Sonication, and Storage of CPT Activity

KCN in the incubation medium of CPT assays decreased CPT activity slightly (7%) in both mitochondrial (476 ± 23 *vs* 443 ± 15 dpm/mg protein/min, mean ± SEM, n = 6) and microsomal (417 ± 37 *vs* 443 ± 33 dpm/mg protein/min, mean ± SEM, n = 6) fractions; this decrease was significant ($P < 0.01$) only in the microsomal fraction (Table XI).

Figure 12

Effect of palmitoyl-CoA concentration on mitochondrial palmitoyl-^{14}C-carnitine formation. Mitochondria were isolated by differential centrifugation from aortas of age-matched male rabbits fed a stock diet (LF) of pelleted rabbit chow. Palmitoyl-^{14}C-carnitine formation was assayed in sample tubes containing 0.11 mmoles of phosphate buffer at pH 7.35, 0.55 μmoles of EDTA, 2.2 μmoles of GSH, 1.1 μmoles of KCN, 2 X 10^5 dpm of ^{14}C-carnitine, 0.3 mg of heat-inactivated 110,000 X g supernatant protein, 0.2 mg of mitochondrial protein, and between 0 - 100 μM palmitoyl-CoA. Incubations were 6 minutes at 37.5°C. Palmitoyl-^{14}C-carnitine was extracted into n-butanol, identified by TLC, and radioactivity in palmitoyl-^{14}C-carnitine determined as described in detail under Determination of Carnitine Palmitoyltransferase Activity in Mitochondrial and Microsomal Fractions. Values represent the mean ± SEM of 3 - 5 different mitochondrial preparations; each preparation consisted of mitochondria isolated from a combined homogenate of three aortas. All assays were performed in duplicate.

102(a)

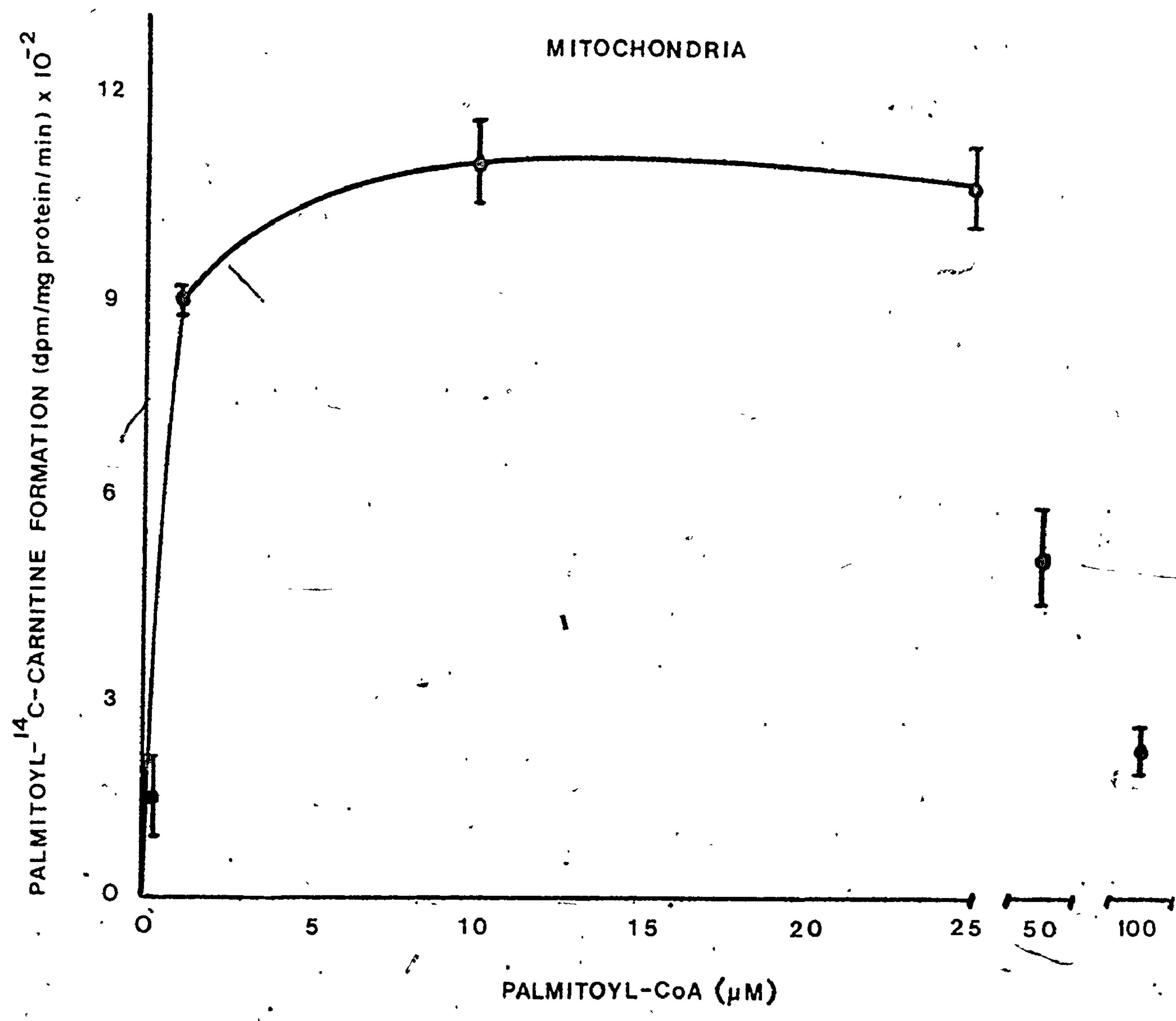


Figure 13

Effect of palmitoyl-CoA concentration on microsomal palmitoyl-^{14}C-carnitine formation. Microsomes were isolated by differential centrifugation from aortas of age-matched male rabbits fed a stock diet (LF) of pelleted rabbit chow. Palmitoyl-^{14}C-carnitine formation was assayed in sample tubes containing 0.11 mmoles of phosphate buffer at pH 7.35, 0.55 μmoles of EDTA, 2.2 μmoles of GSH, 1.1 μmoles of KCN, 2 X 10^5 dpm of ^{14}C-carnitine, 0.3 mg of heat-inactivated 110,000 X g supernatant protein, 0.2 mg of microsomal protein, and between 0 - 100 μM palmitoyl-CoA. Incubations were for 6 minutes at 37.5°C. Palmitoyl-^{14}C-carnitine was extracted into n-butanol, identified by TLC, and radioactivity in palmitoyl-^{14}C-carnitine determined as described in detail under Determination of Carnitine Palmitoyltransferase Activity in Mitochondrial and Microsomal Fractions. Values represent the mean ± SEM of 3 - 5 different microsomal preparations; each preparation consisted of microsomes isolated from a combined homogenate of three aortas. All assays were performed in duplicate.

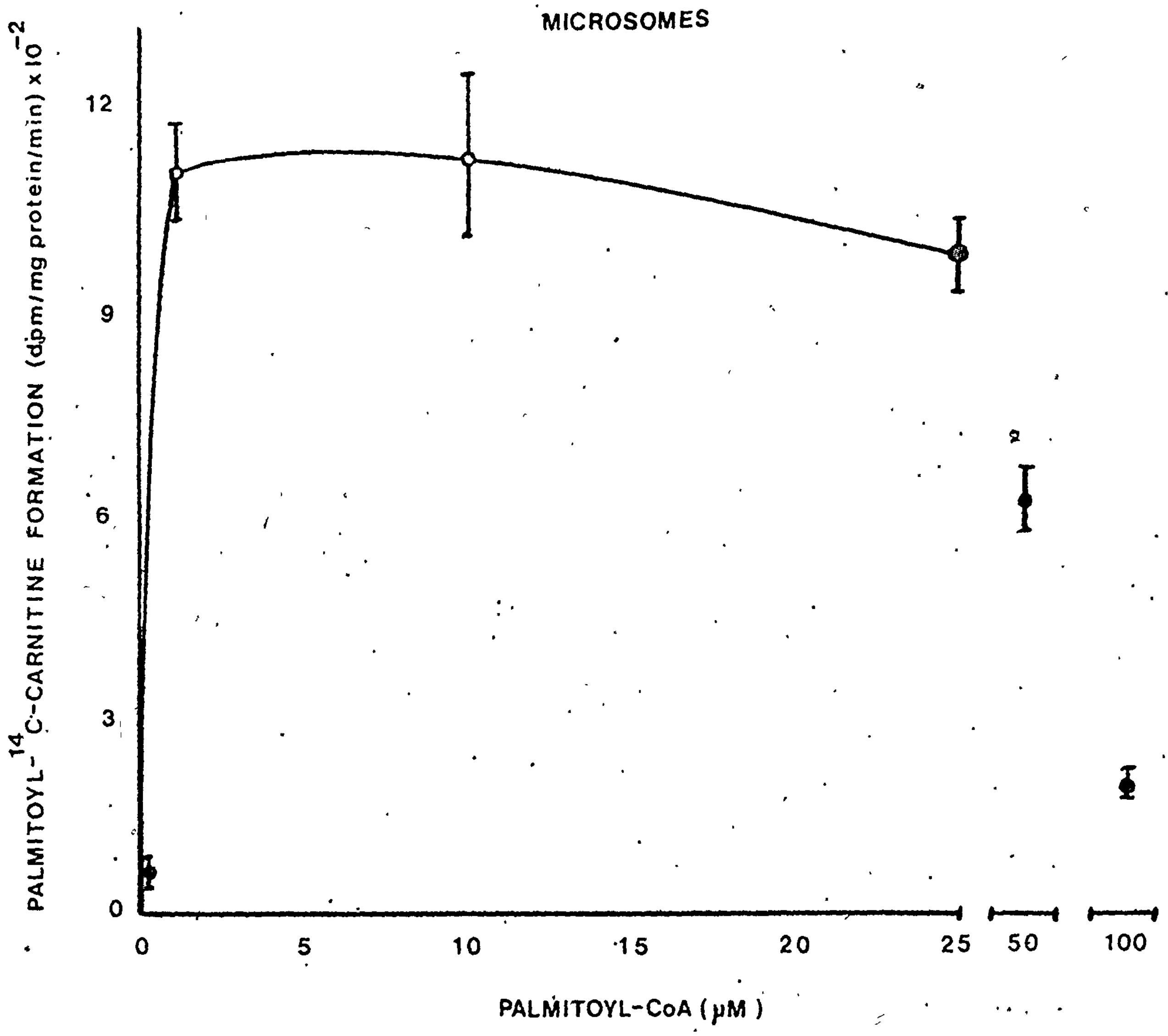

MICROSOMES
PALMITOYL-^{14}C-CARNITINE FORMATION (dpm/mg protein/min) x 10^{-2}
12
9
6
3
0
0
5
10
15
20
25
50
100
PALMITOYL-CoA (μM)

Figure 14

Effect of mitochondrial protein on palmitoyl-^{14}C-carnitine formation. Isolation of mitochondria and assay of palmitoyl-^{14}C-carnitine formation in mitochondrial fractions are described in Figure 12 except that palmitoyl-CoA was 25 μM and the amount of mitochondrial protein ranged from 0 - 0.3 mg. Values represent the mean ± SEM of 5 different mitochondrial preparations. All assays were performed in duplicate.

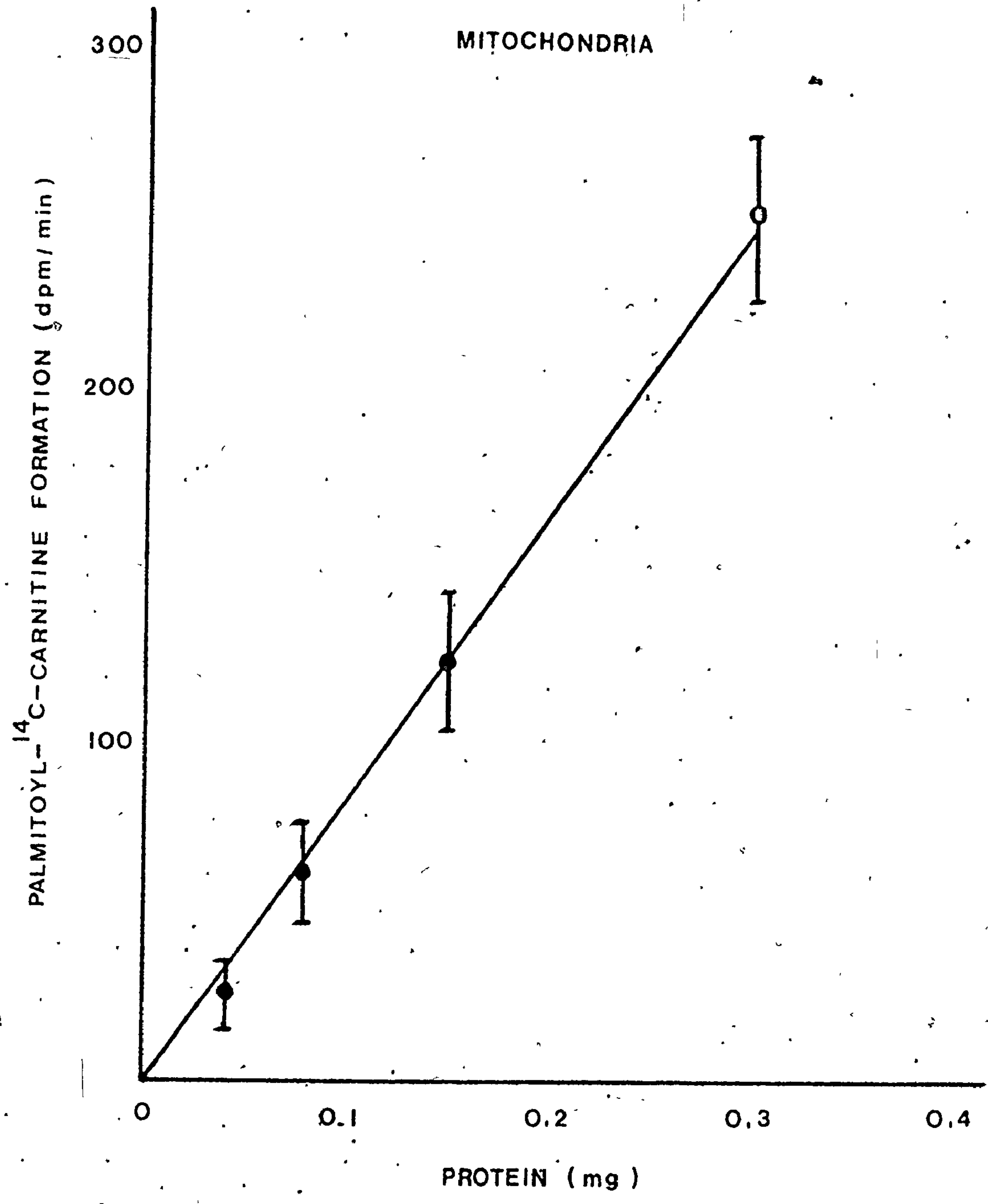

MITOCHONDRIA
PALMITOYL-^{14}C-CARNITINE FORMATION (dpm/min)
300
200
100
0
0.1
0.2
0.3
0.4
PROTEIN (mg)

Figure 15

Effect of microsomal protein on palmitoyl-^{14}C-carnitine formation. Isolation of microsomes and assay of palmitoyl-^{14}C-carnitine formation in microsomal fractions are described in Figure 13 except that palmitoyl-CoA was 25 μM and the amount of microsomal protein in the assay ranged from 0 - 0.3 mg. Values represent the mean ± SEM of 5 different microsomal preparations. All assays were performed in duplicate.

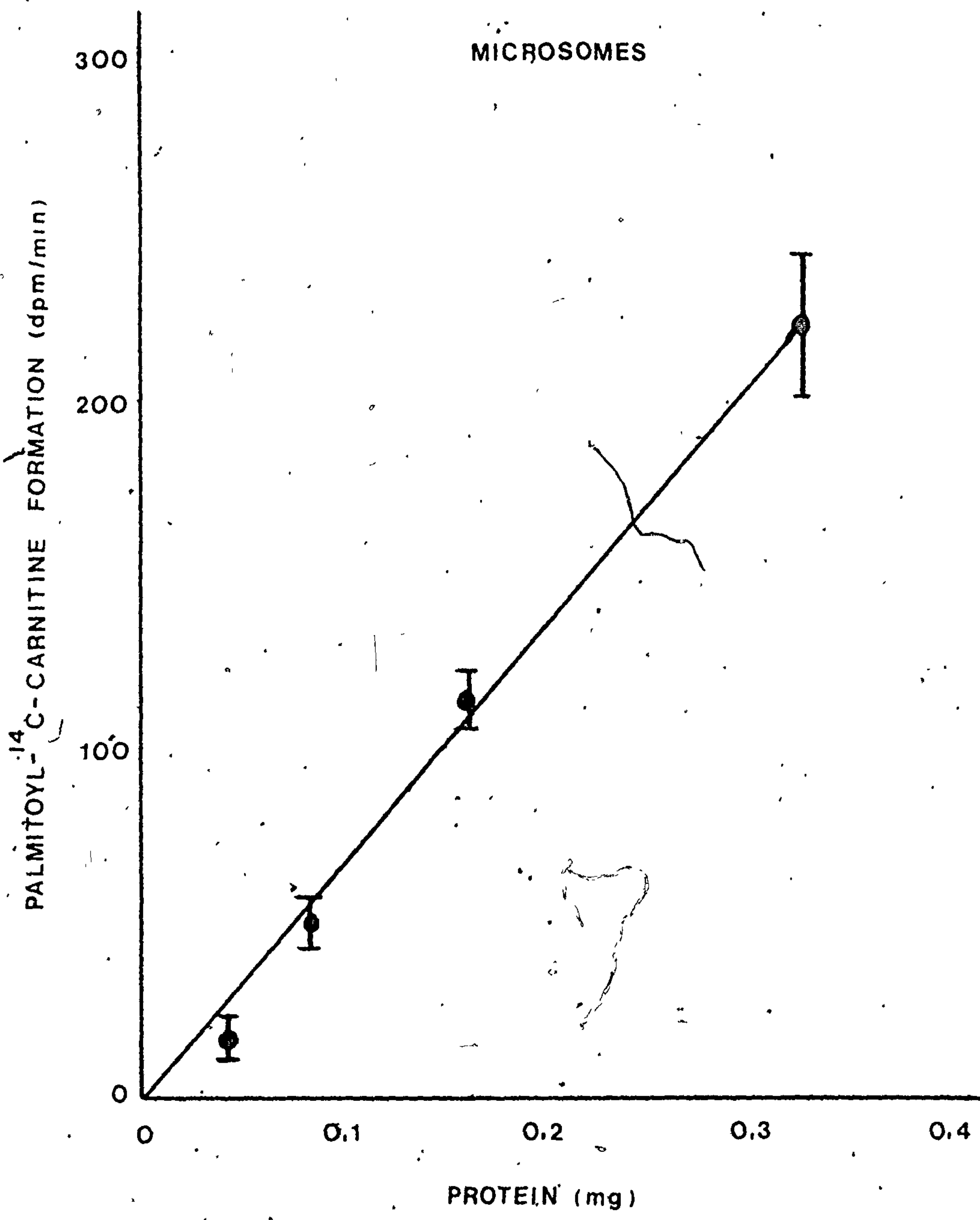

MICROSOMES
PALMITOYL-^{14}C-CARNITINE FORMATION (dpm/min)
300
200
100
0
0
0.1
0.2
0.3
0.4
PROTEIN (mg)

Figure 16

Effect of assay time on mitochondrial palmitoyl-^{14}C-carnitine formation. Isolation of mitochondria and assay of palmitoyl-^{14}C-carnitine formation are described in Figure 12 except that palmitoyl-CoA was 25 μM and assay time ranged from 0 - 12 minutes. Values represent the mean ± SEM of 5 different mitochondrial preparations. All assays were performed in duplicate.

106(a)

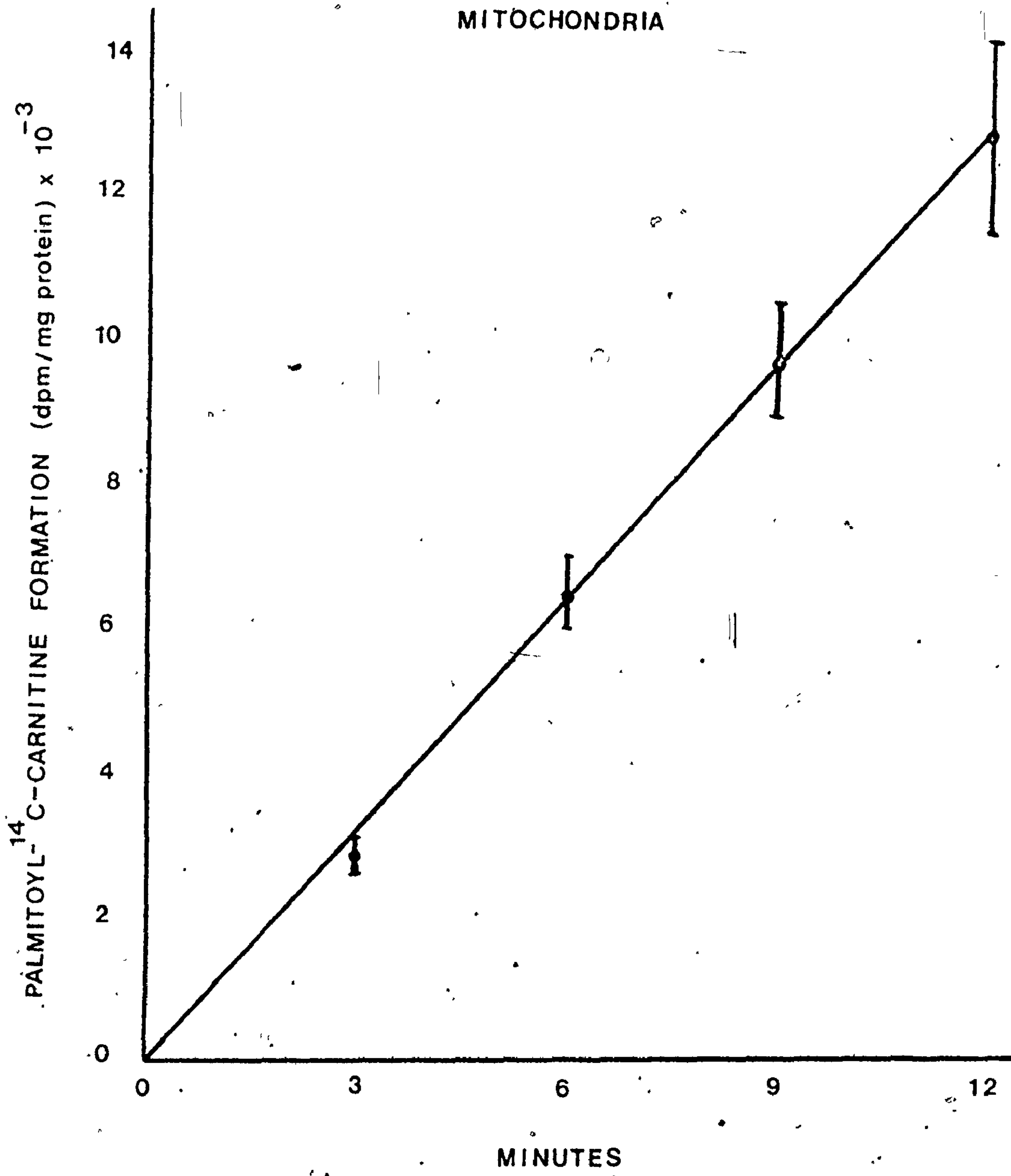


Figure 17

Effect of assay time on microsomal palmitoyl-^{14}C-carnitine formation. Isolation of microsomes and assay of palmitoyl-^{14}C-carnitine formation are described in Figure 13 except that palmitoyl-CoA was 25 μM and assay time ranged from 0 - 12 minutes. Values represent the mean ± SEM of 5 different microsomal preparations. All assays were performed in duplicate.

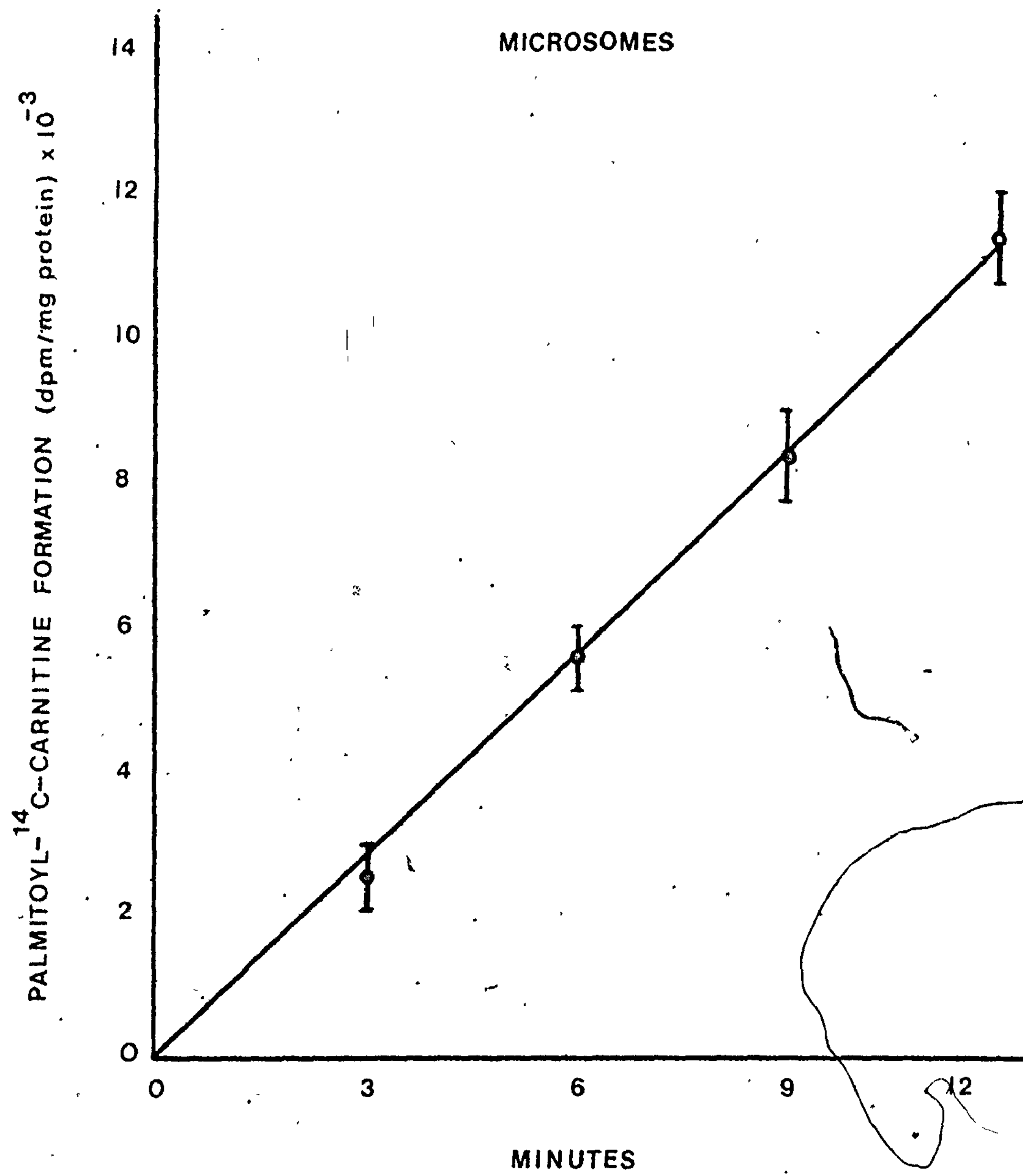

MICROSOMES
PALMITOYL-^{14}C-CARNITINE FORMATION (dpm/mg protein) x 10^{-3}
14
12
10
8
6
4
2
0
0
3
6
9
12
MINUTES

Table XI

Effect of KCN, Sonication and Storage at 5°C, on Palmitoyl-^{14}C-Carnitine Formation by Mitochondria and Microsomes[a]

Experimental conditions	Palmitoyl-^{14}C-carnitine formation[b] (dpm/mg protein/min)	
	Mitochondria	Microsomes
Control	443 ± 15	417 ± 37
minus KCN	476 ± 23	444 ± 33**
sonicated	368 ± 32*	389 ± 27
storage - 5 hours	392 ± 11**	404 ± 24
-17 hours	400 ± 16**	384 ± 28*

[a] Preparation of arterial subcellular fractions and assay for palmitoyl-^{14}C-carnitine formation as described in Table IX.

[b] Values represent the mean ± SEM of 6 animals; aortas were prepared individually. P values, derived from a two-tailed table of Student's values for t, indicate the significant differences between groups based on a paired t test.

* $P < 0.05$, ** $P < 0.01$

Sonication significantly ($P < 0.05$) reduced (17%) the CPT activity in mitochondrial fractions (443 ± 15 *vs* 368 ± 32 dpm/mg protein/min, mean ± SEM, n = 6) but had no significant effect on microsomal fractions (Table XI).

After storage of arterial subcellular fractions for 5 hours at 5°C there was a small (12%) but significant ($P < 0.01$) decrease in mitochondrial CPT activity (443 ± 15 *vs* 392 ± 11 dpm/mg protein/min, mean ± SEM, n = 6), no further decrease occurred after storage for 17 hours at 5°C. Microsomal CPT activity, although unchanged after 5 hours at 5°C, was decreased significantly (8%) ($P < 0.05$) (417 ± 37 *vs* 384 ± 28 dpm/mg protein/min, mean ± SEM, n = 6) after storage at 5°C for 17 hours (Table XI).

Hydrolysis of Palmitoyl-CoA during CPT Assays

The percent hydrolysis of [1-^{14}C] palmitoyl-CoA during the CPT assay was 2.0 ± 0.2%/min (mean ± SEM, n = 6) in both mitochondrial and microsomal fractions; non-enzymatic hydrolysis of [1-^{14}C] palmitoyl-CoA determined in heat inactivated mitochondria and microsomes was 0.1%/min.

Discussion

Preparation of Mitochondrial and Microsomal Fractions

The matrix of collagen, elastin, and glycosaminoglycans in the artery wall makes homogenization of this tough elastic tissue difficult without the introduction of high shear forces; consequently, isolation of homogenous subcellular fractions from

arterial tissue represents a major technical problem in atherosclerosis research.

In this study, mitochondrial and microsomal fractions were isolated and identified on the basis of sedimentation behaviour during differential centrifugation; cross-contamination between mitochondrial and microsomal fractions probably occurred. In an attempt to reduce microsomal contamination in mitochondrial fractions, mitochondria were washed, re-centrifuged, and re-suspended before use in CPT assays. In an attempt to reduce mitochondrial contamination in microsomal fractions, the sediment of the 40,000 X g centrifugation step was discarded before ultracentrifugation of the extra-mitochondrial supernatant fluid at 110,000 X g; this procedure had the drawback of reducing the protein yield of microsomal fractions. No attempt was made to ascertain the extent of contamination between mitochondrial and microsomal fractions; it is recognized, however, that the use of marker enzymes, electron microscopy, or biochemical tests e.g. P:O determination, would be of value in future experiments.

Characteristics of CPT Activity in Mitochondrial and Microsomal Fractions

In this study, palmitoyl-^{14}C-carnitine formation has been considered as a measure of CPT activity; however, palmitoyl-^{14}C-carnitine formation in the mitochondrial CPT assay actually represents the combined activity of CPT-I and CPT-II, future studies could be directed toward the assay of the individual CPT enzymes.

Effects of Protein, Time and Palmitoyl-CoA on CPT Assays

CPT activity in mitochondrial and microsomal fractions was linear with respect to subcellular protein (Figures 14 and 15) and time (Figures 16 and 17); maximal activity was observed between 5 - 25 μM palmitoyl-CoA (Figures 12 and 13). Inhibition of CPT activity occurred in both mitochondrial and microsomal fractions at high concentrations of palmitoyl-CoA e.g. 50 or 100 μM, this may be due to detergent properties of palmitoyl-CoA or to an increase in the K_m for carnitine caused by an increase in the concentration of palmitoyl-CoA (192,193).

Data regarding palmitoyl-CoA concentrations in aqueous media must be interpreted with caution when (i) the concentration of palmitoyl-CoA exceeds its critical micelle concentration i.e. 3 - 4 μM (16), (ii) the surface-volume ratio of the assay conditions exceeds 0.5 cm^2/ml (194) (1.2 cm^2/ml in this study), or (iii) the incubation medium contains protein (0.36 mg/ml in this study); under these conditions the true substrate concentration of palmitoyl-CoA may be less than the added concentration of palmitoyl-CoA.

Effects of KCN, Sonication, and Storage on CPT Activity

KCN was added to the incubation medium of CPT assays in an attempt to decrease the loss of palmitoylcarnitine to β-oxidation reactions. KCN decreased CPT activity slightly (7%) in mitochondrial and microsomal fractions; this effect was significant ($P < 0.01$) only in microsomal fractions (Table XI). Although

KCN could have been omitted from CPT assays in the present study, it was included in anticipation of future CPT assays in athero-sclerotic tissue in which β-oxidation of fatty acids is increased many fold (164).

The activity of some carnitine acyltransferase enzymes e.g. CAT, can be detected only after sonication of the subcellular fraction in which it is found; after sonication of arterial mitochondria and microsomes CPT activity was decreased rather than increased, 17 and 7%, respectively (Table XI). Inhibition of CPT after sonication has been reported for mitochondria isolated from rat liver (106).

CPT activity in subcellular fractions was stable in 0.1 M phosphate buffer at pH 7.35 containing 0.5 mM EDTA and 2.0 mM GSH when stored at 5°C; CPT activity decreased less than 10% (Table XI) after storage for 17 hours.

The differential responses of mitochondrial and microsomal CPT to KCN, sonication, and storage at 5°C support the proposition that both mitochondrial and microsomal CPT activity exists in the artery. This observation contrasts with the exclusive mitochondrial location of CPT in rat liver (97,98). Future experiments could be directed toward assaying CPT activity in microsomes fractionated on a discontinuous Ficoll gradient.

Effects of Acyl-CoA Hydrolase (ACH) on CPT Activity

ACH could interfere with the CPT assay if it were to significantly reduce the concentration of palmitoyl-CoA below 5 μM.

Although ACH could reduce the concentration of palmitoyl-CoA from 10.8 to 9.5 μM during a six minute CPT assay, this would not affect the CPT assay. In atherosclerotic arteries the activity of ACH is reduced, therefore, the assay of CPT activity in atherosclerotic tissue is even less likely to be affected by ACH (195).

Another enzyme that could interfere with the CPT assay is carnitine ester hydrolase. It is unlikely that this enzyme would affect the CPT assay given its high K_m (5 mM) for palmitoylcarnitine (14). The presence of carnitine ester hydrolase has not been reported in the artery.

The observation that mitochondrial and microsomal CPT activity was linear with respect to time and protein (Figures 14,15 16, and 17) argues against either acyl-CoA hydrolase or carnitine ester hydrolase interfering with the CPT assay in this study.

Subcellular Location of CPT Activity

CPT activity was associated with mitochondrial and microsomal fractions isolated from aortas. Some of the microsomal CPT activity might be attributable to mitochondrial contamination, however, it is unlikely that this contamination could account for all of the microsomal CPT activity. Although on a relative basis (dpm/mg protein/min) mitochondrial and microsomal CPT activity was similar, on a total CPT activity basis (dpm/aorta/min) microsomal CPT activity may actually exceed mitochondrial CPT activity.

Although the physiological role of mitochondrial CPT is well established, little is known about microsomal CPT, the artery

may be a good tissue in which to investigate the function of this enzyme. The existence of microsomal CPT activity raises the possibility of a microsomal CPT-II i.e. a counterpart of mitochondrial CPT-II.

Arterial CPT activity introduces some problems in the interpretation of metabolic studies using isotopic palmitoyl-CoA as a substrate. Palmitoylcarnitine and phosphatidyl choline co-chromatograph in the Skipski TLC system commonly used to separate different phospholipid classes (196); similarly, palmitoylcarnitine co-chromatographs with the phospholipid band in the separation of neutral lipid classes using silica gel G and Solvent System 3. Obviously, it is essential when using radioactive palmitoyl-CoA, and perhaps radioactive acetate as well, as a substrate in lipid metabolic studies that radioactivity in palmitoylcarnitine be differentiated from radioactivity in phospholipids in general and phosphatidyl choline in particular. This problem has gone unrecognized in lipid metabolic studies in arterial wall metabolism.

Summary

CPT activity in arterial subcellular fractions can be assayed by measuring palmitoyl-^{14}C-carnitine formation according to the reaction:

$$\text{Palmitoyl-CoA} + {}^{14}\text{C-carnitine} \xrightarrow{\text{CPT}} \text{palmitoyl-}{}^{14}\text{C-carnitine} + \text{CoA}$$

Using this assay, CPT activity was detected in mitochondrial and microsomal fractions isolated from the artery. The CPT reaction

in arterial subcellular fractions was linear with respect to subcellular protein, incubation time and exhibited maximal activity between 5 - 25 μM palmitoyl-CoA. CPT activity in mitochondrial and microsomal fractions was decreased slightly by the presence of KCN in the incubation medium although not enough to affect the assay. Sonication decreased mitochondrial but not microsomal CPT activity. The subcellular fractions were stable with respect to their CPT activity for up to 17 hours when stored at 5°C.

Study No. 6

CPT Activity in Mitochondrial and Microsomal Fractions Isolated From Aortas of Rabbits Fed Cholesterol-Supplemented Diets

Introduction

Atherosclerotic rabbit aortas, compared to normal rabbit aortas, exhibit a 2-fold increase in oxygen consumption (143) a 3-fold increase in long-chain acylcarnitine compounds (Table VIII), and a 15-fold increase in the oxidation of fatty acids to CO_2 (164). Increased β-oxidation of long-chain fatty acids in atherosclerotic aortas could be due to increased CPT activity. The purpose of this study was to investigate CPT activity in mitochondria and microsomes isolated from aortas of animals maintained on the non-atherogenic HF-diet and the atherogenic HFC-diets for 3 - 48 days.

Methods

Diets, treatment of animals, preparation of subcellular fractions and assays are described in the footnotes of Tables and legends of Figures.

Results

Effect of Non-atherogenic HF-Diet and Atherogenic HFC-Diet on Serum and Arterial Total Cholesterol Levels

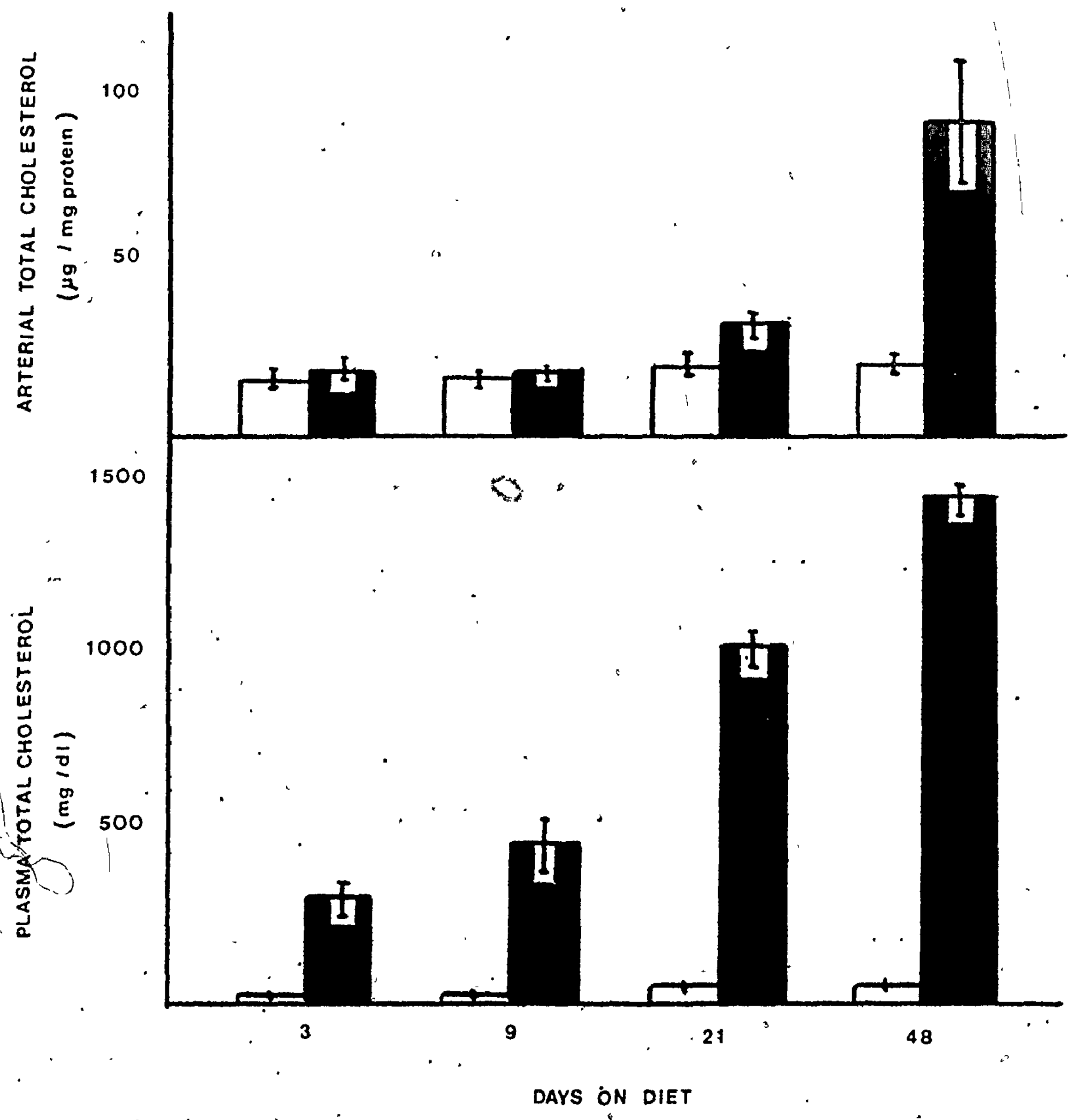
In this study, 3% peanut oil rather than 5% lard was used in the HF- and HFC-supplemented diets. Peanut oil has a higher ratio of polyunsaturated to saturated fatty acids and is less atherogenic in combination with 1% cholesterol than 5% lard plus 1% cholesterol.

Serum cholesterol levels of animals fed HFC-diets were 9-fold (314 ± 45 *vs* 36 ± 5 mg%, HFC *vs* HF; mean ± SEM, n = 4) and 27-fold (1,440 ± 330 *vs* 54 ± 9 mg%, HFC *vs* HF, mean ± SEM, n = 4) greater ($P < 0.001$) than observed in animals fed the HF-diet for 3 and 48 days, respectively (Figure 18).

Arterial cholesterol levels were similar in animals fed either HF- or HFC- diets 3 or 9 days; animals fed the HFC-diet 21 days exhibited slightly higher arterial total cholesterol levels than animals fed the HF-diet 21 days (31 ± 2 *vs* 22 ± 1 µg/mg protein, HFC *vs* HF, mean ± SEM, n = 4) increasing to a 4-fold difference after 48 days (89 ± 20 *vs* 21 ± 1 µg/mg protein, HFC *vs* HF, mean ± SEM, n = 4) (Figure 18). Consistent with the changes in arterial total cholesterol, no visible lesions were observed in aortas from animals fed the HFC-diet 3,9 or 21 days,

Figure 18

Effect of 3% peanut oil or 3% peanut oil plus 1% cholesterol supplemented diets on arterial and plasma total cholesterol levels after 3 - 48 days on diet. Age-matched male rabbits were fed either a non-atherogenic control diet consisting of pelleted rabbit chow supplemented with 3% peanut oil (HF) (open bars) or an atherogenic diet consisting of pelleted rabbit chow supplemented with 3% peanut oil plus 1% cholesterol (HFC) (filled bars); food and water were available *ad libitum*. Arterial total cholesterol (cholesterol plus esterified cholesterol) and plasma total cholesterol were analyzed by the o-phthaldehyde assay described under Preparation and Analysis of Tissue and Blood Samples from Animals in Study 4. Values represent the mean ± SEM of 4 animals; aortas were prepared individually.


ARTERIAL TOTAL CHOLESTEROL
(µg / mg protein)
100
50
PLASMA TOTAL CHOLESTEROL
(mg / dl)
1500
1000
500
3
9
21
48
DAYS ON DIET

however, after 48 days atheromatous lesions covering greater than 60% of the arterial luminal surface were observed. No lesions were observed in arteries from rabbits fed the HF-diet.

Effect of HF- and HFC-Diets on CPT Activity in Mitochondrial and Microsomal Fractions

Mitochondrial CPT activity was similar in aortas of rabbits fed HF- or HFC-diets 3 days (483 ± 31 *vs* 431 ± 59 dpm/mg protein/min, HF *vs* HFC, mean ± SEM, n = 4), 21 days (304 ± 29 *vs* 344 ± 19 dpm/mg protein/min, HF *vs* HFC, mean ± SEM, n = 4) and 48 days (358 ± 24 *vs* 453 ± 53 dpm/mg protein/min, HF *vs* HFC, mean ± SEM, n = 4) (Figure 19). After 9 days on diet, however, there was a transient yet significant ($P < 0.05$) decrease (20%) in CPT activity in mitochondria derived from animals fed the HFC-,but not HF-diets (548 ± 18 *vs* 434 ± 36 dpm/mg protein/min, HF *vs* HFC, mean ± SEM, n = 4). To investigate whether or not this difference was due to some factor(s) in the heat-inactivated 110,000 X g supernatant fluid, heat-inactivated supernatant fluid was interchanged between HF and HFC groups, this did not affect the observed difference in CPT activity (Table XII). Interchanging heat-inactivated supernatant fluid derived from animals fed HF or HFC-diets had no affect on either mitochondrial or microsomal CPT activity at any time between 3 and 48 days on diet (data not shown).

Microsomal CPT activity was essentially unchanged whether the animals were fed HF- or HFC-diets 3 days (440 ± 19 *vs* 554 ± 60 dpm/mg protein/min, HF *vs* HFC, mean ± SEM, n = 4) or 48 days (485 ± 41 *vs* 545 ± 78 dpm/mg protein/min, HF *vs* HFC, mean ± SEM, n = 4)

Figure 19

Effect of 3% peanut oil or 3% peanut oil plus 1% cholesterol supplemented diets on mitochondrial palmitoyl-^{3}H-carnitine formation. Mitochondrial fractions were isolated by differential centrifugation from aortas of age-matched male rabbits fed either a non-atherogenic control diet consisting of pelleted rabbit chow supplemented with 3% peanut oil (HF) (open bars) or an atherogenic diet consisting of pelleted rabbit chow supplemented with 3% peanut oil plus 1% cholesterol (HFC) (filled bars); food and water were available *ad libitum*. Palmitoyl-^{3}H-carnitine formation was measured in sample tubes containing 0.11 mmoles of phosphate buffer at pH 7.35, 0.55 μmoles of EDTA, 2.2 μmoles of GSH, 1.1 μmoles of KCN, 11 nmoles of palmitoyl-CoA, 2 X 10^{5} dpm of ^{3}H-carnitine, 0.3 mg of heat-inactivated 110,000 X g supernatant protein, and 0.2 mg of mitochondrial protein. Incubations were for 6 minutes at 37.5°C. Palmitoyl-^{3}H-carnitine was extracted into n-butanol, identified by TLC, and radioactivity in palmitoyl-^{3}H-carnitine determined as described in Figure 12. Values represent the mean ± SEM of 4 animals; aortas were prepared individually. All assays were performed in duplicate.

119(a)

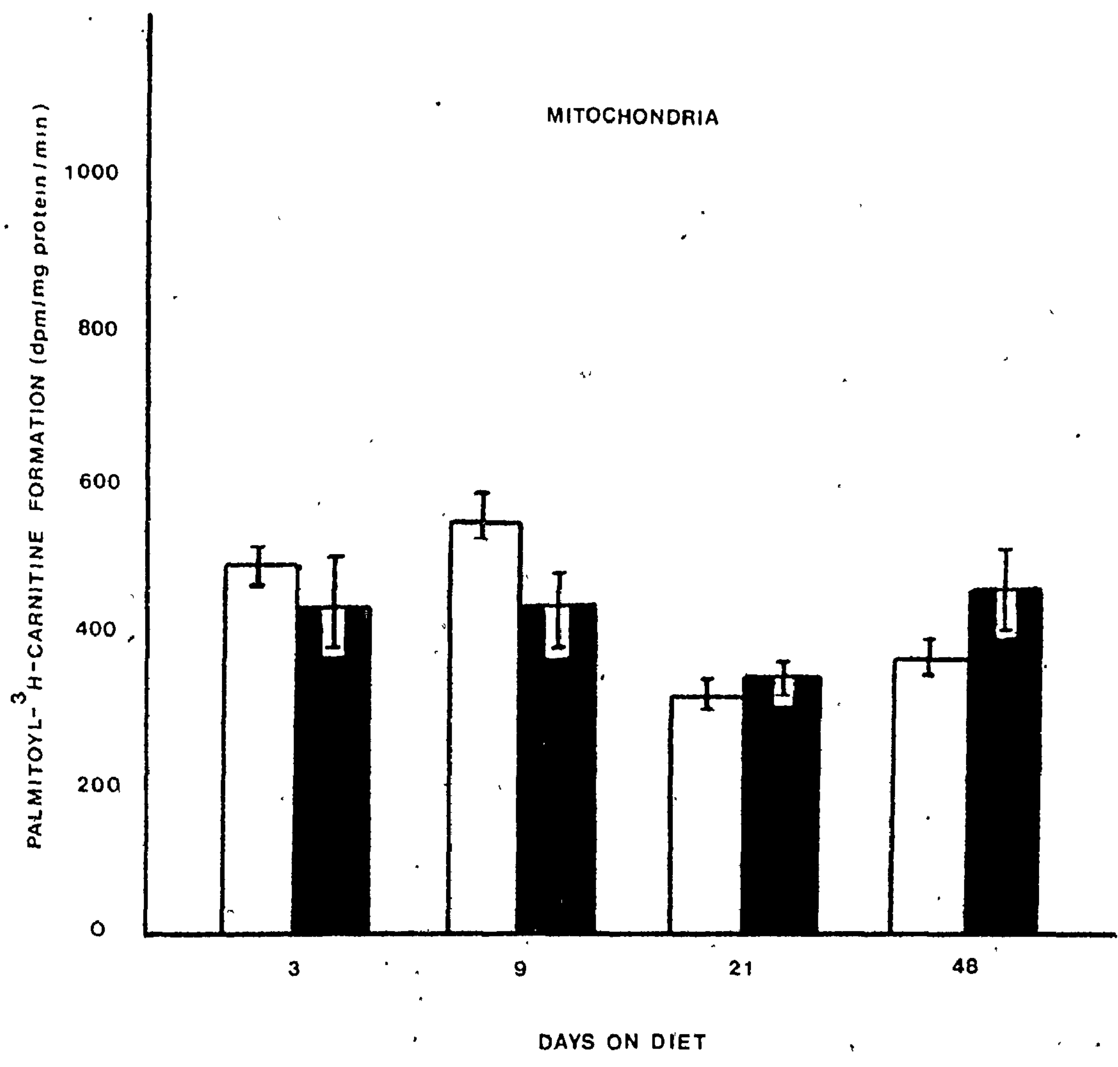


Table XII

Effect of Autologous, Homologous, and Heterologous Heat-Inactivated 110,000 X g Supernatant Fraction on Palmitoyl-^{3}H-Carnitine Formation by Mitochondria Isolated from Aortas of Rabbits Fed 3% Peanut Oil or 3% Peanut Oil plus 1% Cholesterol Supplemented Diets for Nine Days[a]

	Palmitoyl- H-carnitine formation[b] (dpm/mg protein/min)		
	Heat Inactivated 110,000 X g Supernatant Fraction		
	Autologous	Homologous	Heterologous
Mitochondria from rabbits fed 3% peanut oil supplemented diets	548 ± 18	554 ± 56	501 ± 37
Mitochondria from rabbits fed 3% peanut oil plus 1% cholesterol supplemented diets	434 ± 36	368 ± 24	417 ± 44

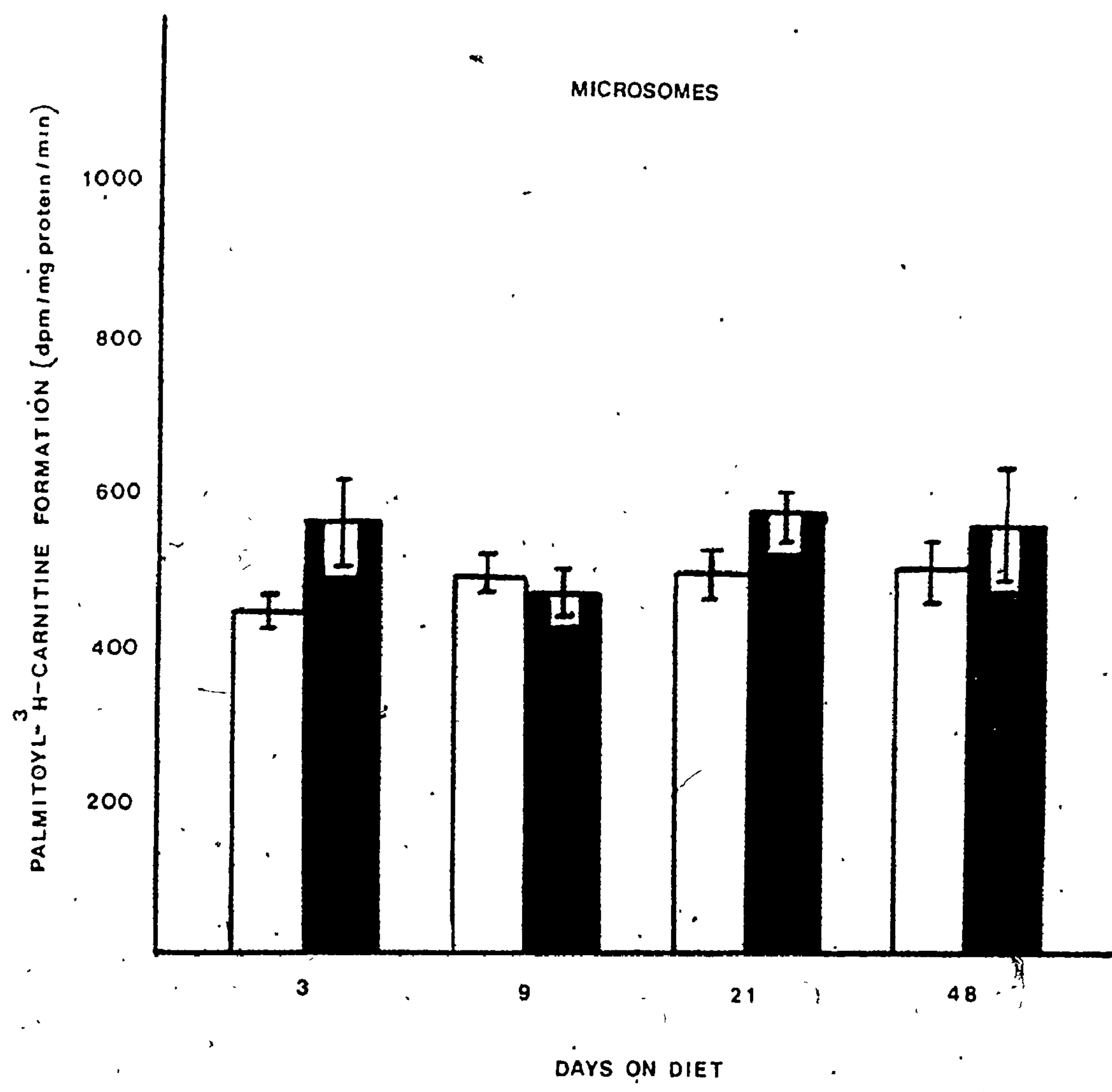
[a] Mitochondrial fractions were isolated by differential centrifugation from aortas of age-matched male rabbits fed either 3% peanut oil or 3% peanut oil plus 1% cholesterol supplemented diets for 9 days. Palmitoyl-^{3}H-carnitine formation was measured in sample tubes containing 0.11 mmoles of phosphate buffer at pH 7.35, 0.55 µmoles of EDTA, 2.2 µmoles of GSH, 1.1 µmoles of KCN, 11 nmoles of palmitoyl-CoA, 2 X 10^{5} dpm of ^{3}H-carnitine, 0.2 mg of mitochondrial protein, and either 0.3 mg of heat-inactivated 110,000 X g supernatant protein from the aorta of the same animal (Autologous), different animal but from the same diet (Homologous), or from an animal of the other diet group (Heterologous). Incubations were for 6 minutes at 37.5°C. Palmitoyl-^{3}H-carnitine was extracted into n-butanol, identified by TLC, and radioactivity in palmitoyl-^{3}H-carnitine determined as described in Table IX.

[b] Values represent the mean ± SEM of 4 animals per group, aortas were prepared individually. All assays were performed in duplicate.

Figure 20

Effect of 3% peanut oil or 3% peanut oil plus 1% cholesterol supplemented diets on microsomal palmitoyl-^{3}H-carnitine formation. Microsomal fractions were isolated by differential centrifugation from aortas of age-matched male rabbits fed either a non-atherogenic control diet consisting of pelleted rabbit chow supplemented with 3% peanut oil (HF) (open bars) or an atherogenic diet consisting of pelleted rabbit chow supplemented with 3% peanut oil plus 1% cholesterol (HFC) (filled bars); food and water were available *ad libitum*. Palmitoyl-^{3}H-carnitine formation was assayed as described in Figure 19 except that 0.2 mg of microsomal protein rather than mitochondrial protein was added to the incubation. Values represent the mean ± SEM of 4 animals; aortas were prepared individually. All assays were performed in duplicate.

121(a)



(Figure 20). The transient decrease in mitochondrial CPT activity observed after 9 days on the HFC-diet was not observed in microsomal CPT activity.

Expression of enzyme activity on a relative basis (dpm/mg/min) or total basis (dpm/aorta/min) would have provided qualitatively similar data since the yield of mitochondrial and microsomal protein (mg) was approximately the same for both diets after 3 days (HF-diet mitochondria = 3.24 ± 0.09, microsomes = 3.49 ± 0.07; HFC-diet: mitochondria = 3.01 ± 0.17, microsomes = 3.58 ± 0.15, mean ± SEM, n = 4) or 48 days (HF-diet: mitochondria = 3.85 ± 0.17, microsomes = 3.88 ± 0.09; HFC-diet: mitochondria = 4.47 ± 0.38, microsomes = 4.09 ± 0.37, mean ± SEM, n = 4).

Discussion

In this study, CPT activity was measured in mitochondrial and microsomal fractions isolated from aortas of rabbits fed HF- or HFC-diets for 3 - 48 days. The atherogenic HFC-diet, but not the control HF-diet, induced biochemical and morphological changes which are commonly associated with the atherogenic process e.g. increased serum and arterial cholesterol (Figure 18) and development of atheromatous lesions; despite these changes, mitochondrial and microsomal CPT activity was not influenced by the addition of cholesterol to the animal's diet (Figures 19 and 20). This finding is consistent with the previous observation that the ratio of long-chain acylcarnitine:long-chain acylcarnitine + carnitine was similar in normal and atherosclerotic aortas (Study No. 2, Table VII).

The transient but significant ($P < 0.05$) decrease (20%) in mitochondrial CPT activity after 9 days on the HFC-diet is similar to the transient decrease in mitochondrial CPT activity observed in ischemic heart tissue in the dog (49,114). It may be that the heart and aorta, both of which rely on fatty acid oxidation for metabolic energy, have regulatory mechanisms which maintain CPT activity at a level that does not limit β-oxidation, regardless of the metabolic state of the tissue.

It is noteworthy that after 9 days on the HFC-diet, microsomal CPT activity did not exhibit a similar decrease in activity as did mitochondrial CPT. This differential response of mitochondrial and microsomal CPT to the cholesterol-supplemented diet supports the existence of microsomal CPT activity in the artery.

The results of this study indicate that increased oxidation of fatty acids in atherosclerotic tissue cannot be attributed to an increase in the activity of arterial CPT.

The possibility should be considered that the results of this study do not reflect CPT activity in the intact artery. Changes in CPT activity may not be detectable in subcellular fractions due to loss of cellular organization i.e. subcellular fractionation may preclude the detection of changes in CPT activity. In support of this possibility is the finding that changes in CPT activity detected in perfused rat livers in response to glucagon and anti-insulin serum cannot be detected in isolated mitochondria (123).

Summary

CPT activity in mitochondrial and microsomal fractions was essentially the same whether the subcellular fractions were isolated from the aortas of animals maintained on either HF- or HFC-diets for 3 - 48 days. The results of this study indicate that the increase in fatty acid oxidation observed in atherosclerotic tissue cannot be attributed to an increase in CPT activity. The transient decrease in mitochondrial but not microsomal CPT activity after 9 days on the HFC-diet adds further support to the proposition that in addition to mitochondrial CPT activity in the aorta, there also exists microsomal CPT activity.

CONCLUDING COMMENTS

Arterial and plasma carnitine metabolism was investigated in normal and atherosclerotic rabbits. Ten major findings emerged from the investigation.

1. Carnitine and short-chain acylcarnitine but not long-chain acylcarnitine compounds were found in aortas of rabbits fed a stock diet of pelleted rabbit chow; long-chain acylcarnitines appeared when the stock-diet was supplemented with 5% lard.

2. Age-related changes occurred in the concentration and distribution of arterial carnitine compounds as rabbits approached maturity around 24 weeks of age. The concentration of carnitine compounds increased several fold during the first 8 weeks post partum; during this time the distribution between carnitine and acylcarnitine compounds remained relatively constant. In contrast, during the next 16 weeks, significant changes occurred in the distribution between carnitine and acylcarnitine compounds but the total concentration of carnitine compounds increased only slightly.

3. Carnitine circulating in the bloodstream was taken up by the heart and aorta and esterified to fatty acids to form acylcarnitines; the heart took up and esterified more carnitine from the bloodstream than the aorta.

4. Plasma carnitine concentration was influenced by diet. A high-fat supplemented diet or fasting decreased the concentration of plasma carnitine, whereas a high-fat plus cholesterol supplemented diet increased the concentration of plasma carnitine.

5. Carnitine, short-chain acylcarnitine and long-chain acylcarnitine compounds increased several fold in aortas of rabbits fed an atherogenic high-fat plus cholesterol supplemented diet; this increase was not observed in rabbits fed a non-atherogenic high-fat supplemented diet.

6. The origin of the carnitine that accumulated in atherosclerotic aortas was carnitine circulating in the bloodstream.

7. Acetylcarnitine formation increased in atherosclerotic and pre-atherosclerotic aortas compared to normal aortas. Butyrylcarnitine formation, not detected in normal or pre-atherosclerotic aortas, was detected in atherosclerotic aortas. Acetylcarnitine and butyrylcarnitine formation in the heart was similar in normal, pre-atherosclerotic and atherosclerotic aortas.

8. Carnitine and acylcarnitine compounds started to accumulate in aortas of rabbits fed atherogenic diets before the development of gross atheromatous lesions.

9. Carnitine palmitoyltransferase activity was associated with particulate fractions isolated from arterial cells e.g. mitochondria and microsomes. The presence of carnitine

palmitoyltransferase activity in arterial mitochondria and microsomes distinguishes the aorta from heart and liver, in these organs carnitine palmitoyltransferase activity is located exclusively in mitochondria.

10. Carnitine palmitoyltransferase activity in arterial mitochondrial and microsomal fractions from rabbit aortas was not influenced by supplementation of the rabbit's diet with cholesterol.

This new information on arterial carnitine metabolism may be integrated with existing knowledge and concepts in arterial wall biochemistry and atherosclerosis; although the process of information integration involves a certain degree of speculation, this same speculation provides a basis for future research.

Atherosclerotic arteries exhibit increased fatty acid β-oxidation (143), increased mitochondrial fatty acid elongation (143), and decreased triacylglyceride synthesis (164). These observations form the basis of the hypothesis that in atherosclerotic aortas, acyl-CoA is predominantly esterified to carnitine rather than to glycerol-3-phosphate. The 3-fold increase in long-chain acylcarnitine compounds detected in atherosclerotic rabbit aortas supports this hypothesis. The increase in long-chain acylcarnitines may be the result of:

A. *An increase in the availability of acyl-CoA.* There is no quantitative information in the literature on arterial acyl-CoA concentrations, however, it is reported that the concentration of CoA is similar in normal and atherosclerotic arteries (149).

A qualitative increase in acyl-CoA in atherosclerotic tissue is suggested by the observations that: (i) the activity of acyl-CoA hydrolase decreases in atherosclerosis (197), (ii) the concentration of fatty acids increase in atherosclerosis (143,164), and (iii) the absolute concentration of long-chain acylcarnitines increase in atherosclerotic tissue without a concomitant increase in CPT activity.

B. *An increase in the ratio of carnitine:glycerol-3-phosphate.* An increase in the ratio of carnitine:glycerol-3-phosphate is directly supported by the observation that carnitine increases 3-fold in atherosclerotic aortas and indirectly supported by the observations that: (i) less than 0.5% of arterial glucose is converted to glycerol-3-phosphate (143) and (ii) the activity of glycerol-3-phosphate dehydrogenase decreases in atherosclerotic tissue (149).

C. *An increase in the enzyme activity ratio of CPT:GPT.* Given the normal arterial carnitine concentration of approximately 0.085 mM (calculated from Table VI), it is unlikely that the K_m for carnitine (0.25 mM, Table III) would be exceeded even in atherosclerotic aortas; consequently, CPT activity may increase due to the accumulation of carnitine and acyl-CoA in atherosclerotic aortas. Concurrent with an increase in acylcarnitine formation, would be a decrease in the acylation of glycerol-3-phosphate due to inhibition of GPT by acyl-CoA.

The metabolic consequences of an increase in long-chain acylcarnitines may be to: (1) direct fatty acids toward β-oxidation reactions, (2) direct fatty acids away from glycerol-3-phosphate acylation reactions, (3) direct fatty acids toward mitochondrial elongation reactions, and (4) stimulate acetyl-CoA carboxylase. Therefore, the hypothesis that in atherosclerotic aortas acyl-CoA is predominantly esterified to carnitine rather than to glycerol-3-phosphate offers an explanation for several biochemical characteristics of atherosclerotic tissue, including: (i) increased oxygen consumption and increased oxidation of fatty acids to CO_2, (ii) decreased triacylglyceride formation, (iii) increased mitochondrial fatty acid elongation, and (iv) increased *de novo* fatty acid synthesis.

An increase in fatty acid oxidation could increase the amount of acetyl-CoA entering the tricarboxylic acid cycle, however, since the activity of many enzymes of the tricarboxylic acid cycle is decreased in atherosclerosis (149), acetyl-CoA may start to accumulate. Arterial CAT may buffer the increase in acetyl-CoA by converting acetyl-CoA to acetylcarnitine; this would account for the increase in short-chain acylcarnitine compounds detected in atherosclerotic rabbit aortas. Acetylcarnitine could represent a source of acetate for mitochondrial fatty acid elongation or *de novo* cytoplasmic fatty acid synthesis; both of these fatty acid synthesizing mechanisms are increased in atherosclerosis (143).

Although it is difficult to ascribe a physiological role to arterial microsomal CPT in the absence of any information on its subcellular location or membrane topography, it is tempting to

speculate that CPT in the endoplasmic reticulum may facilitate the penetration of acyl-CoA substrates to enzymes buried in this membrane e.g. stearoyl-CoA desaturate or fatty acyl-CoA:cholesterol acyltransferase.

Future studies of arterial carnitine metabolism could be directed toward (i) ascertaining the subcellular location of microsomal CPT activity, (ii) elucidating the mechanisms controlling the fate of acylcarnitines in the inner mitochondrial membrane with respect to β-oxidation and elongation reactions, and (iii) verifying the observed changes in arterial carnitine metabolism in atherosclerosis in different animals and using different models for inducing atherosclerosis.

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