THESIS

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CHANGES IN TISSUE AND BODY FLUID ACYLCARNITINES IN RESPONSE TO DIFFERENT PHYSIOLOGICAL STATES

By

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ABSTRACT

CHANGES IN TISSUE AND BODY FLUID ACYLCARNITINES
IN RESPONSE TO DIFFERENT PHYSIOLOGICAL STATES

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Experimentally induced anoxia and hypoxia caused a preferential loss of short-chain acylcarnitines from heart tissue. With hypoxic perfused pig hearts a 24% decrease in free carnitine plus a 38% decrease in short-chain acylcarnitine was found. The acylcarnitines of hypoxic heart showed a 65% decrease in acetylcarnitine while propionyl-, isobutyryl-, and isovaleryl-carnitine increased. Alloxan diabetes elevated the levels of free and short-chain acyl-carnitine approximently 100 fold in sheep liver. Each acyl residue of acylcarnitine increased by about the same magnitude in diabetic sheep livers. In studies with humans, fasting and feeding did not affect the total carnitine concentration or the free-/short-chain acyl-carnitine ratio in blood. The major acylcarnitine of human serum is acetyl with propionyl, butyryl, and valeryl accounting for most of the remainder. When comparing urine samples to serum samples, the ratio of free-/short-chain acyl-carnitine was lower, and the acylcarnitines

contained a lower proportion of acetyl, propionyl, and butyryl residues while isobutyryl and isovaleryl proportions increased. The urine of a patient with a metabolic dysfunction contained high levels of propionylcarnitine. A Fanconi syndrome patient showed high levels of free- and short-chain acyl-carnitine in the urine while the blood carnitine levels were well below controls.

To Mary

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
CAT	Carnitine acetyltransferase
CoA	Coenzyme A
CoASH	Reduced coenzyme A
CPT	Carnitine palmitoyltransferase
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
GC	Gas chromatogram
NADH	Reduced nicotine adenine dinucleotide
NEM	N-ethylmaleimide
TCA	Tricarboxylic acid

LITERATURE REVIEW

Carnitine and Its Role in Fatty Acid Metabolism

Carnitine (the betaine of γ-amino-β-hydroxybutyric acid) was isolated in 1905 by two independent groups (1,2) from mammalian muscle. For forty years its function in metabolism remained unknown. Interest in carnitine increased in 1935 due to its structural similarities to acetylcholine, but no evidence was found to support its physiological role as a neurotransmitter (3,4). In 1948 Fraenkel and Blewett found a compound that was an essential nutrient for growth of the Tenebrio molitor meal worm larva (5). That compound, which was later identified as carnitine (6), was initially named "vitamin B_T" (7). Using the Tenebrio assay method (8) carnitine was found to be widely distributed in tissues of animals, plants, and microorganisms, with the highest levels in the muscles of both vertebrates and invertebrates (9).

The possible function of carnitine in fatty acid metabolism was first observed by Friedman and Fraenkel in 1955 (10). They isolated an enzyme from pigeon liver which catalyzed the transfer of acetyl groups to carnitine. In that same year Fritz (11) showed that a factor in rat liver particulate preparations caused an increase in the oxidation of long chain fatty acids and the factor that causes this increase was found to be carnitine (12). In 1962 both Fritz and Bremer (13,14,15) demonstrated that carnitine catalyzes the mitochondrial oxidation of long-chain acyl-CoAs, and both proposed that activated long-chain fatty

acids are transported across the inner membrane of mitochondria to the matrix, which is the site of β-oxidation, via the conversion of carnitine to palmitoyl-carnitine. The reversible reaction:

palmitoyl-CoA + ℓ-carnitine ⇌ palmitoyl-ℓ-carnitine + CoA

is catalyzed by the enzyme carnitine palmitoyltransferase (CPT). These two investigators (13,15) proposed the following role for carnitine in intermediary metabolism of fatty acids. The enzyme carnitine acyltransferase transfers acyl groups from activated fatty acids to carnitine forming acylcarnitines by the reversible reaction:

acyl CoA + (ℓ)-carnitine ⇌ (ℓ)-acylcarnitine + CoA

these acylcarnitines can now cross the mitochondrial acyl CoA impermeable inner membrane (16), where the above reaction is reversed by an internal carnitine acyltransferase to regenerate acyl CoAs inside the mitochondrial matrix. These acyl CoAs are then used as substrates for β-oxidation (17).

Carnitine Acyltransferases

As stated above Bremer (15) and Fritz (13) both reported enzymatic synthesis of palmitoyl-carnitine by the enzyme carnitine palmitoyltransferase (CPT). Carnitine palmitoyltransferase (CPT) was located on both the outer surface of the inner mitochondrial membrane, and the matrix surface. The role of CPT proposed by Bremer and Fritz is given above. In 1963 Fritz et al. (18) partially purified a carnitine acetyl transferase (CAT) from pig heart mitochondria. Carnitine acetyltransferase (CAT) reacts only with short chain fatty acyl substrates whereas CPT reacts only with long chain fatty acyls, therefore they must be 2 different proteins. The proposed role of CAT,

which is also located on both sides of the mitochondrial inner membrane (102,103), is to facilitate the transport of activated acetyl groups across the inner membrane. Thus CPT in mitochondria can be used to provide substrates for fatty acid oxidation while CAT provides acetyl CoA for the TCA cycle which is also in the matrix of mitochondria. If carnitine and the 2 carnitine acyltransferases (CAT and CPT) only function in metabolism to facilitate the transport of fatty acids to the mitochondrial matrix then the transferases should associate exclusively with mitochondria. Markwell et al. (19) demonstrated that both short-chain and medium-chain carnitine acyltransferase activities are associated with microsomes, peroxisomes, and mitochondria in pig and rat liver. Markwell et al. (20) later partially purified carnitine acetyltransferase proteins from peroxisomes and microsomes, however no carnitine palmitoyltransferase was found in these organelles. Thus the role of carnitine and CPT in long-chain fatty acid metabolism is solely associated with mitochondrial β-oxidation. Carnitine and CAT system role in transfering acyl residues to other organelles and its role in other forms of intermediary metabolism must be more diverse (34).

Role of Carnitine in Branched-Chain Amino Acid Metabolism

In 1970 Solberg and Bremmer (21) showed that mouse and rat tissues produce [^{3}H]-labeled branched-chain acylcarnitines from branched-chain ketoacids and [methyl-^{3}H]-L-carnitine. Bieber et al. (22-27) have demonstrated the presence of branch-chain acylcarnitines and also branched-chain carnitine acyltransferase activities in both rat and beef tissues demonstrating a possible involvement of carnitine in amino acid metabolism. Addition of carnitine stimulates the oxidation of

branched-chain amino acids in both rat skeletal muscle and heart (28) but not in liver (29). Carnitine also stimulates the oxidation of branched-chain ketoacids in rat liver, heart kidney and muscle (30,31). The stimulation of branched-chain ketoacid dehydrogenase activity by carnitine results from removing the inhibitory levels of the branched-chain acyl-CoAs (32) by forming the branched-chain acylcarnitine via carnitine branched-chain acyltransferase activity (30,31,33). Therefore, carnitine stimulates branched-chain amino acid metabolism by forming acylcarnitines from the branched-chain acyl-CoA groups. This will increase the CoASH/acyl CoASH ratio inside the mitochondria and this should activate the branched-chain keto acid dehydrogenase (34).

Carnitine and Myocardium Tissue

Effect of Ischemia on Carnitine and Other Metabolites

Investigations by others have demonstrated that induction of myocardial ischemia or anoxia causes a decrease in tissue levels of both free carnitine and acetylcarnitine and increases the amounts of some other acylcarnitines (35,36,50,51). The extent of these changes is somewhat dependent on the time of and severity of oxygen deficiency. Long-chain acylcoenzyme A esters also accumulate during ischemia (37) and it has been proposed that such esters can reduce mitochondrial energy production (35,37,38,39). Evidence has also been presented that carnitine protects ischemic myocardium (40). Such studies indicate that the levels of carnitine and acylcarnitines are affected by hypoxia and anoxia and that changes in the levels can affect heart viability. It is well established that carnitine has an obligatory role in the oxidation of long-chain fatty acids in both normoxic and ischemic conditions

(41,42). Studies with humans have shown that tissue deficiencies of carnitine or carnitine palmitoyltransferase can cause fatal cardiomyopathies (43,44). Another possible function of carnitine in the heart besides acting as a transport system for fatty acids into the mitochondria is discussed in the next section.

Modulation of the Myocardium Acetyl CoA/CoASH Ratio by Carnitine

Pearson and Tubbs (45) first suggested that carnitine may be involved in regulating the acetyl CoA/CoASH ratio, with acetylcarnitine acting as an acetyl CoA buffer. This was supported by the finding that the ratio of acetylcarnitine to acetyl CoA is high in heart (>20) and by the observation that the reactants of the carnitine acetyltransferase system remain near equilibrium during different metabolic states (46). Neely et al. (46-48) suggested the following function of carnitine and carnitine acetyltransferase in regulating the ratio. Acetyl CoA produced in the matrix of mitochondria, for example by β-oxidation, can be either oxidized or transported out of the matrix by forming acetylcarnitine via carnitine acetyltransferase. The acetylcarnitine can then be converted back to acetyl CoA which can be used as a substrate in other parts of the cell. Therefore carnitine and carnitine acetyltransferase provide a way to remove "excess" intramitochondrial acetyl CoA which is generated during "excess" fatty acid uptake and oxidation. The regeneration of acetyl CoA outside of the matrix can then inhibit fatty acid activation by lowering the amount of extramitochondrial CoASH (49). This process in reversed during the start of work with an increase in citrate synthetase which lowers the levels of intramitochondrial acetyl CoA. This causes carnitine

acetyltransferase to form acetylcarnitine which is transported to the matrix to replace acetyl CoA. Formation of acetylcarnitine will also stimulate fatty acid activation via the regeneration of extra-mitochondrial CoASH (48).

Several groups have shown that excess acetyl CoA in isolated heart mitochondria can also inhibit the myocardial nucleotide translocase system (35-38) and Pande and Blanchaer (35) found that the addition of carnitine could reverse this acetyl CoA induced inhibition. Excess acetyl CoA can affect many other sites of metabolism inside the mitochondria, thus carnitine with carnitine acetyltransferase may provide a pathway for removing excess acetyl residues generated inside the mitochondrial matrix.

Role of Carnitine in Controlling Ketogenesis in Ruminants

The ratio of free CoA to acetyl-CoA is a major factor in control of hepatic ketogenesis. This ratio in normal sheep and cow liver ranges from 0.2 to 1.0 (52,53), which is much lower than the ratio observed in ketotic rats and guinea pigs (range of 2:1 to 9:1). In starved and alloxan-diabetic sheep livers, Snoswell (54) observed a 4-7 fold increase in carnitine compared to normals, whereas the concentration of carnitine remained constant in similarly treated rat livers. Snoswell (55) stated that these differences between sheep and rat livers are due to carnitine and the carnitine acetyltransferase system exerting an "acetyl buffer" function in sheep liver. The rate that ketone body concentration increased in hepatic venous blood of sheep was found to be inversely related to carnitine concentration following alloxan treatment. Thus carnitine and carnitine acetyltransferase can transfer

acetyl groups from the matrix of mitochondria, where ketogenesis occurs, to the cytoplasm. This will remove acetyl CoA as a substrate for ketogenesis, and provides a mechanism for regulating hepatic ketogenesis.

Carnitine in Humans

Carnitine Concentrations In Humans

In 1978, Mitchell (56,57) reviewed and tabulated the published levels of carnitine in various body tissues and fluids of normal and diseased humans. These articles provide an excellent source for obtaining normal total carnitine levels in human, although Mitchell noted it is difficult to obtain accurate normal values. This is due to several factors: 1) No criterion for age, sex, diet was used by most investigators. 2) No standardized method was used for determining the carnitine concentration. Some of the methods used are described in the carnitine assay section. 3) Many of the carnitine determinations of normal humans tissues were samples from patients who were hospitalized for diseases other than the symptom under study. More recent papers on the total carnitine concentration in human serum and the amount of carnitine in urine extraction have tried to address some of these factors. Tanphaichitr et al. (58) measured plasma and urinary carnitine concentrations in Thai adults living in either a city or village. The village dwellers had lower total carnitine concentrations in both plasma and urine which they attributed to a lower carnitine intake with their diet. So diet may have some affects on carnitine levels. When investigating other factors that may affect carnitine concentrations in humans there are many reports with conflicting data. Carrier and Berthillier (59)

showed that the amount of total carnitine excreted in urine increases with age, while the serum levels of carnitine remain constant in both children and adults. Previously, Cederblad (60) reported that there are significant differences in plasma carnitine concentrations between the sexes and various age groups. Another example of conflicting values is found in carnitine levels during fasting. Maebashi et al. (61) reported an increase in the amount of urinary carnitine excreted during a 6 day fast, while Frohlich et al. (62) showed a decrease in serum and urinary free carnitine levels, but acetylcarnitine increased during a 24-36 hour fast. Recently Hoppel and Genuth (63) measured carnitine levels of normal and obese humans during a fast. There was a rapid increase in the amount of short- and long-chain acylcarnitine in plasma with a delayed decrease in free carnitine observed in both groups. Urinary short-chain acylcarnitines increased parallel to plasma levels while free carnitine levels decreased and then increased slightly. Even though there are conflicting results, the human provides one of the best models for observing other functions for carnitine because metabolic dysfunctions due to carnitine deficiency or CPT deficiency have been reported. Some of these cases are listed below.

Carnitine and Carnitine Palmitoyltransferase Deficiencies

As mentioned earlier, Mitchell (56,57) reviewed the data of some of the cases reported prior to 1978. Briefly, in 1973 Engel and Angelini (64) first described a lipid storage myopathy which was related to a deficiency of serum and muscular carnitine. Since then several cases have been reported (65-74). These studies showed that at least two types of carnitine deficiency syndromes exist, which can be classified

as predominantly myopathic or systemic (104). The systemic syndrome is characterized by a lower than normal content of carnitine in the blood and, presumably, other organs. The other syndrome is a myopathy where serum concentrations are normal but carnitine transport across cell membrane in various tissues such as skeletal muscle, cardiac muscle, Schwann's cell, and leukocytes is impaired, resulting in decreased carnitine content in these tissues. Possible causes of these defficiences included impaired carnitine biosynthesis; excessive carnitine loss from body fluids; impaired active transport of carnitine into cells; excessive release of carnitine from cells, and excess catabolism (104).

Carroll et al. (75) have reported a patient with carnitine deficiency that showed biochemical features of both types of syndromes. Glasgow et al. (76) have reported a case where systemic carnitine deficiency stimulates recurrent Reye's like syndrome. Chapoy et al. (77) reported that systemic carnitine deficiency is a cause of recurrent Reye's-like syndrome, treatable by administering oral carnitine. Pola et al. (78) gave oral carnitine in therapy to hyperlipidemic patients which lowered their serum cholesterol and triglyceride levels.

The first report of carnitine palmitoyltransferase (CPT) deficiency was described by DiMauro and DiMauro (79), in a patient with recurrent paroxysmal myoglobinuria (a classic sign of CPT deficiency) where CPT activity in muscles was < 20% of normal levels. This was apparently due to a lack of the inner CPT activity. Hostetler et al. (80) reported a case of no CPT outer, but normal CPT inner in muscle mitochondria. Two other cases (81,82) of decreased muscle CPT inner activity have been reported. Layzer et al. (83) reported a case where both CPT inner and

outer activities were deficient. DiDonato et al. (84) showed that cultured fibroblasts from a patient deficient in muscle CPT also contained reduced CPT activity and this supports the concept that CPT deficiency may be a systemic rather than just a muscular condition. Ionasescu et al. (85) have linked a combined partial deficiency of muscle carnitine and CPT activity with an autosomal dominant inheritance. These cases demonstrate the various malfunctions that can occur if carnitine content in blood and tissues are lower than normal, and they provide information about other functions of carnitine.

Carnitine Assays

The first assay technique for determining the amount of carnitine used was the Tenebrio molitor bioassay (9). Growth of larvae consuming a diet plus the test material was compared to control larvae growing on the diet plus a known amount of carnitine. The problems of all bioassays are inherent in this method, which would affect the determination. Friedman (86) developed a chemical method which involves esterification of carnitine and colorimetric determination, however the determination was non-specific due to interfering quaternary amines. Marquis and Fritz (87) developed an enzymatic assay using carnitine acetyl transferase:

acetyl CoA + carnitine $\rightleftharpoons$ acetyl-ℓ-carnitine + CoASH

The CoASH produced is measured spectrophotometrically by reaction with DTNB. Pearson and Tubbes (45) coupled this reaction to another enzyme reaction and measured citrate and NADH. In 1972 Cederblad and Lindstedt (88) increased the sensitivity down to 20-30 picomoles by using [1-^{14}C]-acetyl CoA and excess acetyl CoA.

Other methods developed recently, include a microbiological method using Torulopsis bovina yeast (89) and a gas chromatographic method (90). Three papers have been published (91,92,93) which have refined the Cederblad and Lindstedt method. These three appear to be the most sensitive methods because they overcome the problem of nonlinearity by using a compound to trap CoASH formed instead of using excess acetyl-CoA, which makes the assay very expensive. These three methods also use CoASH trapping agents which prevent reversibility of the reaction and which do not inhibit the carnitine acetyltransferase enzyme.

STATEMENT OF PROBLEM

As stated earlier, the possibility that carnitine has other roles in intermediary metabolism, besides shuttling activated long-chain fatty acids into the matrix of mitochondria for β-oxidation has been proposed. One role proposed by several investigators is that carnitine and the carnitine acetyltransferase enzyme of the mitochondria modulate the acetyl CoA/CoASH ratio in the matrix of mitochondria by shuttling excess acetyl residues back out of the matrix in the form of acetyl carnitine (42,45,47,54). Recently Bieber, et al. (22,24,34) have suggested that the role of carnitine and the carnitine acyltransferases in regulating the ratio should be expanded to include other short-chain and medium-chain acyl CoAs instead of just acetyl CoA. This would include branched-chain acyl residues, which arise during oxidative degradation of some amino acids. This suggestion correlates with Van Hinsberghs (30) proposed role of carnitine in removing excess branched-chain acyl CoAs which inhibit the keto acid dehydrogenase and regenerates CoASH. Reports that there are both short-chain and medium-chain carnitine acyltransferase activities associated with three organelles, while the long-chain carnitine acyltransferase has so far been found only present in mitochondria, suggest that carnitine may be involved in shuttling short-chain and medium-chain acylcarnitines from one organelle to another.

The goal of my thesis project was to find if quantification of acylcarnitines provide information about the levels of the products

formed and the substrates used by carnitine acyltransferases during various metabolic states. This was investigated by determining changes in the amounts and/or proportion of each specific acyl residue, of acyl carnitine from various tissues, in response to different physiological states. These short-chain acylcarnitines should be the products of the carnitine acyltransferases, which generate the acylcarnitine to maintain the equilibrium with the substrates; acyl CoA. Changes in the proportion of the acyl residues of acylcarnitine may provide some insight of the metabolic pathways that are occurring in the tissue and to the role of carnitine in regulating the acyl CoA/CoASH ratio. Normal and hypoxic myocardium tissue, normal and alloxan diabetic sheep livers, and also human serum and urine were investigated for carnitine levels and the composition of their water-soluble acyl carnitines.

MATERIALS AND METHODS

Materials

Carnitine was a generous gift from Otsuka Pharmaceutical Co. Acetyl-CoA, [1-^{14}C] acetyl CoA were from P-L Biochemicals. DL-(methyl-^{3}H]-carnitine was from Amersham. The Bio-Gel P-2 and Dowex resins were from Bio-Rad. NEM (N-ethylmaleimide), ADP, carnitine acetyltransferase and other compounds were from Sigma. The GC packing material was from Supelco and the fatty acids were from J.T. Baker.

Methods

Sample Acid Extraction Procedure

Pig and rat hearts: The frozen tissues were homogenized in 6% $HClO_4$ (1:6 wt/v) at 4°C. The homogenate was centrifuged at 12,000 x g for 15 minutes and the supernatant fluid collected. The volume of the fluid was recorded, an aliquot was removed for carnitine analysis, and the remaining volume was used for quantitation of the acylcarnitines.

Rat Heart Media: The same procedure was used as above except the ratio of $HClO_4$ to media was 1:3 (v/v).

Human serum: This also used the same procedure except that the ratio of $HClO_4$ to the sample was 1:2 (v/v).

Human Urine: The urine was initially concentrated 10 fold before adding 6% $HClO_4$ at a ratio of 1:2 (v/v). The supernatant fluid was adjusted to pH 6.0 and stored at -80°.

Carnitine Analysis

The method used for carnitine determination is a modified procedure of Cederblad-Lindstedt (88), Parvin-Pande (91) and Bieber-Lewin (94). The assays are based on the reaction:

$$\text{ℓ-carnitine + acetyl CoA} \rightleftharpoons \text{acetyl-ℓ-carnitine + CoASH}$$

The method was used for quantitation of the amounts of free carnitine and water soluble O-acylcarnitines.

Aliquots from the perchloric acid extracts, as described in the "Sample Acid Extraction Procedure" section, were neutralized to pH 6.6 with KOH and the precipitated $KClO_4$ was removed by centrifugation at 12,000 x g for 10 minutes. The supernatant fluid was used to determine the amount of free carnitine. 2N KOH was used to bring an aliquot of the pH 6.6 supernatant to 0.2N KOH. These samples were heated to 40°C for 30 minutes to saponify the acyl residues of the acylcarnitines. The fluid was again neutralized to pH 6.6 with $HClO_4$ and centrifuged as previously. Aliquots from each of the two preparations were assayed for carnitine, the former gives the amount of free carnitine and the latter gives the total carnitine (free carnitine plus water soluble short-chain acylcarnitines).

The reaction solution for assaying the carnitine contains in a 0.2 mL volume: 20 µmoles potassium phosphate buffer at pH 7.6, 288 pmoles (1-^{14}C) acetyl coenzyme A (0.0167 µCi), 50 µg NEM, 500 pmoles acetyl-CoA, and the sample. The range of the assay was 10-200

pmoles of ℓ-carnitine. The reaction was initiated by adding 25 ml of carnitine acetyltransferase (0.400 units/assay) and incubated for 30 minutes at 25°C. The reaction was stopped by loading 200 µl onto a 2 x 25 mm Dowex-1 X8 Cl^- form (100-200 mesh) column. The effluent and a 1 mL water wash of the column were collected in a scintillation vial to which 10 ml of scintillation cocktail (4 g of 2,5-diphenyloxazole, 100mg of 1,4-bis [2-(4-methyl-5-phenyloxazolyl)] benzene in 1 liter of toluene and 1 liter of Triton X-100) was added. The amount of ℓ-carnitine per sample was determined by comparing the amount of [1-^{14}C] acetylcarnitine formed during the reaction to the amount formed in an experimentally determined carnitine standard curve.

Quantitation of Short-Chain Acylcarnitines

The method described below provides a method for identifying and quantitating the short-chain acylcarnitines, and removing all other short-chain acyl residues which may be present in the sample. The method used to separate and quantitate the water soluble acylcarnitines is essentially that described previously (94,95) with several modifications that will be elaborated in the multistep procedure described below.

Procedure:

1) The perchloric acid extract, from the "Sample acid extraction procedure" section, is mixed with an internal standard of either crotonylcarnitine or valerylcarnitine (20-30 µg). Valerylcarnitine was the preferred standard because the crotonylcarnitine is unstable in the aqueous phase over an extended period of time, but was used in samples where valerylcarnitine was previously detected.

2) The solution was neutralized to pH 6.6 with 2N KOH and the neutralized solution was placed on ice for 30 minutes to precipitate the $KClO_4$. The sample was then centrifuged at 12,000 x g for 15 minutes. The supernatant was decanted and saved. The pellet was resuspended in 15-20 mL of $HClO_4$ and was recentrifuged as above. The supernatant fluid was combined with the previous supernatant fluid.

3) This step was used only for human urine samples and entails a 1:2 dilution of the supernatant from the previous step with 95% ethanol. The solution was mixed and then set on ice for 30 minutes before centrifugation at 12,000 x g for 15 minutes. The supernatant fluid was collected and the pellet was washed with absolute ethanol and was recentrifuged as above. This supernatant fluid was combined with the first supernatant fluid. This step helps to remove some of the salts.

4) The combined fluids from either step 3 or 4 were concentrated to about 10 mL using a vacuum rotary evaporator. Several drops of octanol were added to urine extracts to prevent foaming during evaporation. Nine volumes of isopropanol were then added to the remaining fluid and it was thoroughly mixed. After standing on ice for 30 minutes the samples were centrifuged at 12,000 x g for 20 minutes and the supernatant was collected. It was then concentrated to approximately 3 mL, being sure to remove all ethanol and isopropanol.

5) To the sample were added a 400 molecular weight marker, 10 µl of 0.2 M ADP, and 0.5 ml of the elution buffer, 0.1 mM KH_2PO_4 at pH 6.6. The sample was then loaded onto a Bio-Gel P-2 column. This 2.5 cm X 45 cm column was used to separate low molecular weight and high molecular weight molecules from carnitine and the water soluble

acylcarnitines. The calibration of this column is described in the Bio-Gel Calibration section. The sample was eluted with the phosphate buffer and the 400-200 molecular weight effluent was collected.

6) The effluent was passed through a 2.5 cm X 13 cm Dowex 1-X8 HCO_3 form (100-200 mesh) column. The total effluent and the 1.5 bed volume water wash of the column were collected. The effluent may be alkaline after passing through the column, so 1.0 ml of 1N HCl was added to the collecting flask prior to the elution. After all of the elutate was collected the pH was adjusted to 6.0, and the volume of the effluent was concentrated to 10 ml with the rotary evaporator.

7) The sample was adjusted to pH 2.0 with 1N HCl and was loaded onto a Dowex 50-X8 H form (100-200) 1.0 cm X 12 cm column. The effluent was discarded and the column was washed with 2 bed volumes of water. The acylcarnitines were eluted with 1.0 N NH_4OH: 95% ethanol (8:2 v/v), and the effluent was collected when the pH became alkaline. Three bed volumes of the effluent, approximately 40 ml, were collected and 2.0 ml of 2N KOH was added, mixed, and incubated for 30 minutes at 40°C to saponify the O-acyl derivatives of carnitine.

8) The sample was concentrated to 2 mL using the rotary evaporator and the pH adjusted to 8.5 with 1N H_2SO_4. The sample was then completely dried either by evaporation or by lyophilization. The evaporation procedure was used for samples that were extracted the same day and the lyophilization procedure was used if the sample was to be stored and extracted at a latter time.

9) The dry K^+ salts of the volatile fatty acids were resuspended and converted to the free acids by adding a small volume of 25% meta-phosphoric acid (250 g/l of 36% HPO_3). The volume of acid used to

resuspend the fatty acids depended on the initial concentration of acylcarnitines present in the samples. The minimum range required for reproducible results was 3-5 nmoles of fatty acids/µl. Any excess salts in the resuspension were removed by centrifugation at 500 x g for 5 minutes to prevent loading of excess non-volatiles on to GC column packing material.

Identification of the Acyl Residues

Gas chromatography was the method used for the quantitation and identification of short-chain fatty acid residues that resulted from saponification of the acylcarnitines. The fatty acids were separated on a 2mm i.d. x 6 foot GC column, packed with 15% SP-1220 1% H_3PO_4 on 100/120 Chromosorb W AW (Supelco). Due to nonvolatile compounds in the sample preparation a short 6 inch pre-column packed with phosphoric acid treated glass wool was used to collect these components before they contaminated the analytical column. The temperature program for the separation of the fatty acids started at 115° for 5 minutes and then increased to 140°C at a rate of 4°/minute. To calculate the amount of each acyl residue present in a sample, a separate standard solution was prepared which contained a known amount of each fatty acid. The response of the amount of fatty acid/unit of area detected by the flame ionization detector for the standard was then compared to the area obtained for each fatty acid in the sample.

Synthesis of the Acylcarnitines

Crotonyl-, valeryl-, and octanoyl-carnitine were synthesized as described by Bohmer and Bremer (96). [1-^{14}C] acetylcarnitine was

synthesized enzymatically with [1-^{14}C] acetate, CoASH, acetyl CoA synthetase enzyme, ℓ-carnitine, NEM, and carnitine acetyltransferase. The initial reaction volume of 1.0 mL contained 100 mM sodium phosphate buffer, pH 7.6; 1 mg acetyl CoA sythetase; 35 mM ATP; 0.35 mM CoASH; 13,5 mM $MgCl_2$; 0.35 mM [1-^{14}C] Na acetate; 0.35 mM ℓ-carnitine. These components were mixed and stood at room temperature for 3 hours before adding 4.3 mg NEM and 2 units of carnitine acetyltransferase. After 1 hour at room temperature the reaction was terminated by placing in a boiling water bath for 3 minutes. Denatured protein was removed by centrifugation. [1-^{14}C]Acetyl-ℓ-carnitine was separated from [1-^{14}C] acetate or [1-^{14}C] acetyl CoA by passing the solution through a 5 x 35 mm column of Dowex 1-X8 (chloride form, 100-20 mesh). The effluent was tested for any contaminating acetate and the remaining effluent was adjusted to pH 6.0 and was stored at -80°.

Bio-Gel Calibration

The P-2 Bio-gel column was calibrated for the molecular weight range of 400-200. The effluent in this range would contain all of the water soluble short-chain acylcarnitines, and would exclude larger and smaller molecular weight materials. ADP was used as the 400 molecular weight marker in each sample because the elution pattern is similar to that of the largest water soluble acylcarnitines; [1-^{14}C] octanoylcarnitine (95). [^{14}C]Acylcarnitine was used as the lower limit marker. The elution profile is shown in Fig 1. The UV absorbance at 254 nm was monitored until ADP began to elute and 1.8 ml fractions were then collected and 0.1 ml aliquots were counted for the

radiolabels. The volume required to elute ADP to the last of the [^{14}C]-acetylcarnitine was then used for all of the samples.

The elimination of low molecular weight cations and anions would prevent these components from interfering with remaining ion-exchange columns.

Rat Heart Preparations

Prior to sacrificing, 10 mL of modified Krebs/Henseleit bicarbonate buffer containing five millimolar β-hydroxybutyrate was prepared for each heart by equilibrating with either the control gas mixture 95% air:5% CO_2 or the anoxic gas mixture 95% N_2:5% CO_2 in a 50 mL Erlenmeyer flask fitted with an airtight neoprene cap. Two size 20 hypodermic needles were inserted in the cap and the longer one extended into the buffer through which the buffer was flushed with gas.

Hearts from either fasted or fed 200 g male Sprague/Dawley strain of rats were removed rapidly, split and immersed in the incubation buffer. They were incubated in the presence or in the absence of β-hydroxybutyric acid for 30 minutes at 30°C with the gas mixture continuously bubbling into the buffer. The incubation was stopped by freeze clamping the heart tissue and lyophilizing the buffer.

Perfusion of Pig Hearts

This procedure was performed by S. Ely and G. Scott of the Physiology Department at Michigan State University. Poland-China pigs (30-40 kg) were anesthetized with sodium thiamylal (5 mg/lb, i.v.) and maintained with nitrous oxide and supplemental doses of sodium thiamylal. The animals were incubated and ventilated by a positive

pressure respirator (Harvard model 613) on room air with supplemental oxygen, at a 5 cm H_2O end expiratory pressure. Volume and rate of ventilation were adjusted to maintain arterial PO_2, P_{CO_2} and pH within the normal physiological range. Blood anticoagulation was achieved by the intravenous administration of sodium heparin (600 U/kg plus 250 U/kg per hour, i.v.). Esophageal temperature was monitored (Yellow Springs) and maintained at 37°C with a heating pad. All blood pressures were continuously monitored with Statham (low volume displacement) pressure transducers and recorded via inputs into a direct writing oscillograph (Hewlett-Packard). Lead II of the ECG was monitored for determination of heart rate and detection of arrhythmias.

The left femoral artery and vein were cannulated (PE 240) for monitoring arterial pressure and intravenous infusions, respectively. The chest was opened by median sternotomy and the pericardium incised and sutured to the inside of the chest wall to form a cradle. An extracorporeal coronary perfusion system was constructed by withdrawing venous blood from the cannulated femoral vein and pumping it (Masterflex roller pump) into the pulmonary artery of an isolated lung obtained from a small pig. Pulmonary venous blood flowed into a large bore cannula tied into the preserved left atrium, passed through a water bath heated at 39°C and was delivered (Holter roller pump) at constant pressure (100 mm Hg) to the cannulated right coronary artery. Pulmonary venous pressure in the isolated lung was monitored from a catheter in the left atrium and maintained at 5 mm Hg with the use of a feedback controller system (Leeds-Northrup) which controlled the speed of the pulmonary arterial inflow pump. The extracorporeal perfusion circuit was primed with cross-matched blood obtained from the donor lung animal. Coronary

perfusion pressure was monitored from the coronary cannula just proximal (3 cm) to its entry into the vessel, and held constant by a second feedback control system. Coronary blood flow was determined from the coronary pump speed which was continuously recorded on an oscillograph. The cyclic variations seen in the coronary blood flow recordings represent oscillations in the servo-feedback control system. At the end of each experiment, the pump was calibrated by timed collections of blood and there was a linear relationship of pump speed to pump flow over the range of flows encountered. The lack of interarterial pump interarterial coronary anastamoses is well documented in the pig (Circulation 1:10-27, 1950; Circulation 27:717-721, 1963). Thus, it is safe to conclude that changes in the gas content of the blood perfusing the right ventricle would not affect gas tensions in the left ventricle.

Ventilation of the isolated perfused lung with various gas mixtures produced changes in blood gas tensions of the right coronary perfusate without affecting systemic gas tensions. Normoxia control was produced with 20% O_2, 5% CO_2, 75% N_2. Hypoxia was produced by ventilating the isolated lung with 5% CO_2, 95% N_2. Blood gases were measured on a radiometer blood gas analysis (Radiometer-Copenhagen).

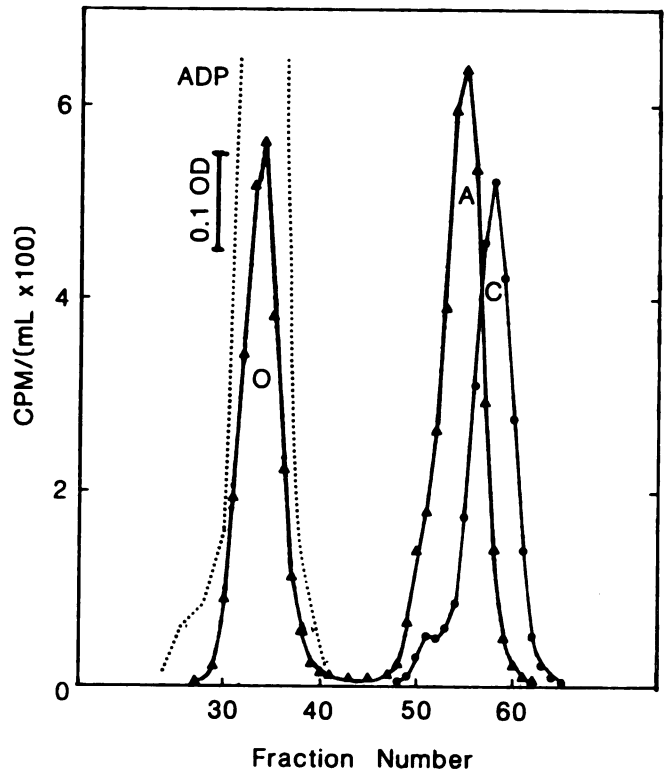

Figure 1. Elution profile of ADP, [1-^{14}C]octanoyl-, [1-^{14}C]acetyl-carnitine, and [^{3}H-methyl] Dl-carnitine from the Bio-Gel P-2 column. [1-^{14}C]Octanoylcarnitine (2.0 x 10^4 cpm), [1-^{14}C] acetylcarnitine (2.5 x 10^4 cpm), [^{3}H-methyl] DL-carnitine (2.5 x 10^4 cpm), and 0.01 mL of 0.2M ADP were combined with 3 mL of the 0.1 mM KH_2PO_4 elution buffer. This was applied to the Bio-Gel P-2 column and the eluate was collected in 1.7 mL fractions, and 0.1 mL aliquots were counted for radioactivity. ADP was monitored continuously by measuring the absorbance at 254 nm.

RESULTS

As described in the introduction myocardium anoxia causes a change in the levels of tissue carnitine. We performed two types of experiments with hearts to test the effect of anoxia and hypoxia on the retention of and changes in the levels of carnitine and its derivatives. The media was analyzed to determine if heart loses short-chain acylcarnitines and to determine if specific acyl derivatives are lost or whether the loss reflects the general short-chain acylcarnitine content of the cardiac tissue.

Affect of Anoxia on Short-Chain Acylcarnitines of Rat Heart.

A series of experiments were performed in which hearts from fed and 48 hour fasted rats were incubated in the presence of air/CO_2 or in the presence of N_2/CO_2 with and without β-hydroxybutyric acid. The results of these experiments are shown in Table I. 1) Acylcarnitines and carnitine present in the buffer were derived solely from the heart tissue. Fasting decreased the ratio of free carnitine to short-chain acylcarnitines in both heart and incubation media. 2) After 30 minutes the incubation buffer contained a lower ratio of free carnitine to short-chain acylcarnitines than the tissue. Thus, rat hearts incubated with either normoxic conditions and or anoxic conditions appear to preferentially lose short-chain acylcarnitines or selectively retain free carnitine. 3) Within the experimental conditions used, there was

little difference between the loss of carnitine and acylcarnitines from normoxic tissue and anoxic tissue. It should be noted that in these experiments the hearts were not perfused, but rather were rapidly removed from the animal, split open and incubated in the bicarbonate buffer.

Since the loss of short-chain acylcarnitines plus free carnitine was less than 10% of the total carnitine content of heart and our anoxic conditions did not increase the amount of acylcarnitines lost, the time course for loss of free carnitine and short-chain acylcarnitines was done to determine if the loss of carnitine was a continuous process. The results of such an experiment are shown in Figure 2. The data show that the release of both free carnitine and short-chain acylcarnitines was linear for 30 minutes and, as with the data in Table I, anoxia did not enhance the release of carnitine or acylcarnitines at the levels reported by Shug et al. (35,36,50,51).

The composition of the individual short-chain acylcarnitines from normoxic and anoxic experiments was determined because the rat heart appeared to selectively release short-chain acylcarnitines into the buffer. In addition, fasted animals released proportionately larger amounts of short-chain acylcarnitines. These data are shown in Table II for the tissue and buffer from 10 combined individual experiments. The samples were combined because inordinately large amounts of time are required for doing each individual acylcarnitine analysis and because of the low concentration of acylcarnitines in individual samples. The data show that with both control (normoxic) and anoxic tissue, the short-chain acylcarnitines lost to the buffer reflect the short-chain acylcarnitine content of the tissue. However, the relative distribution of specific acylcarnitines was different even though the major short

chain acylcarnitine was acetylcarnitine in all samples. During normoxic conditions, the heart and buffer contained relatively large amounts of propionyl- and butyryl-carnitine, both of which might be derived from β-oxidation of long-chain fatty acids. In contrast under anoxic conditions, the amount of propionyl carnitine in both tissue and media decreased and the isobutyryl- and caproyl-carnitine amount increased.

Affect of Hypoxia on the Carnitine Concentration of Perfused Pig Hearts.

Although the studies with rat hearts show that the hearts selectively lost short-chain acylcarnitines under both normoxic and anoxic conditions, anoxia did not appear to enhance the loss of carnitine or short-chain acylcarnitines. These were non-perfused samples and the total carnitine loss was relatively small, less than 10% in 30 minutes. Thus another heart model was tested. This model was *in situ* perfused pig heart in which the left ventricle served as the control and the right ventricle was the hypoxic treated sample. In these experiments, the right ventricle was made hypoxic for 10 minutes by ventilating the isolated lung with a gas mixture containing 5%, 95% N_2. This ventilation mixture dropped the PO_2 in the coronary perfusate from 144 to 12 mm Hg with little change in pH (7.27-7.34). After sacrificing the animal, the tissue samples were removed and freeze clamped and subsequently analyzed for acylcarnitines. Representative samples were also taken from non-perfused pig hearts, both the left and right ventricle, to serve as controls for the free carnitine and short-chain acylcarnitine analyses. The carnitine content of the blood before and after passage through the heart was not analyzed because of

the difficulty in accurately measuring the small changes in large fluid volumes.

The results of these experiments are summarized in Table III and they show that perfusion caused a reduction of or loss of short-chain acylcarnitines compared to non-perfused ventricles. There was a 37% decrease in short-chain acylcarnitines and a 24% decrease in free carnitine; compare the non-perfused heart values with the left ventricle values for the perfused heart [note the left ventricles were perfused with normoxic conditions]. When the heart was perfused with hypoxic conditions, the loss of carnitine, especially short-chain acylcarnitines, was greater than when the tissues were perfused under normoxic conditions. Compare 173 nmoles/g short-chain acylcarnitine in the left ventricle and 108 nmoles/g in the right ventricle. The value of short-chain acylcarnitines in non-perfused heart was 268 ± 43 nmoles/g, this was reduced to 108 ± 9 nmoles/g by hypoxic perfusion, a 60% reduction in short-chain acylcarnitines. Similar, but not as large of a reduction in free carnitine was found. In contrast, a small increase in the total amount of long-chain acylcarnitine was obtained by hypoxic perfusion.

Since the heart lost large amounts of short-chain acylcarnitines during hypoxic perfusion, the short-chain acylcarnitines of perfused control ventricles, left ventricle, and perfused hypoxic ventricles, right ventricle, were determined. These data are given in Table IV and show that hypoxia caused a major decrease in acetylcarnitine (approximately 65%) and an increase in propionyl-,isobutyryl-, butyryl- and valeryl-carnitine. The large decrease in acetylcarnitine could indicate a decrease in β-oxidation due to hypoxia or a selective loss of acetylcarnitine to the blood, or both.

Figure 2. The effect of anoxia on the rate of release of carnitine and short-chain acylcarnitines from rat hearts. The incubations contained 5 mM β-hydroxybutyric acid. Hearts from fed animals were used and analyses were performed as described for Table I.

★Free carnitine (air-CO_2).
☆Free carnitine (N_2-CO_2).
●Free + Short-chain acylcarnitines (air-CO_2).
✪Free + Short-chain acylcarnitines (N_2-CO_2).

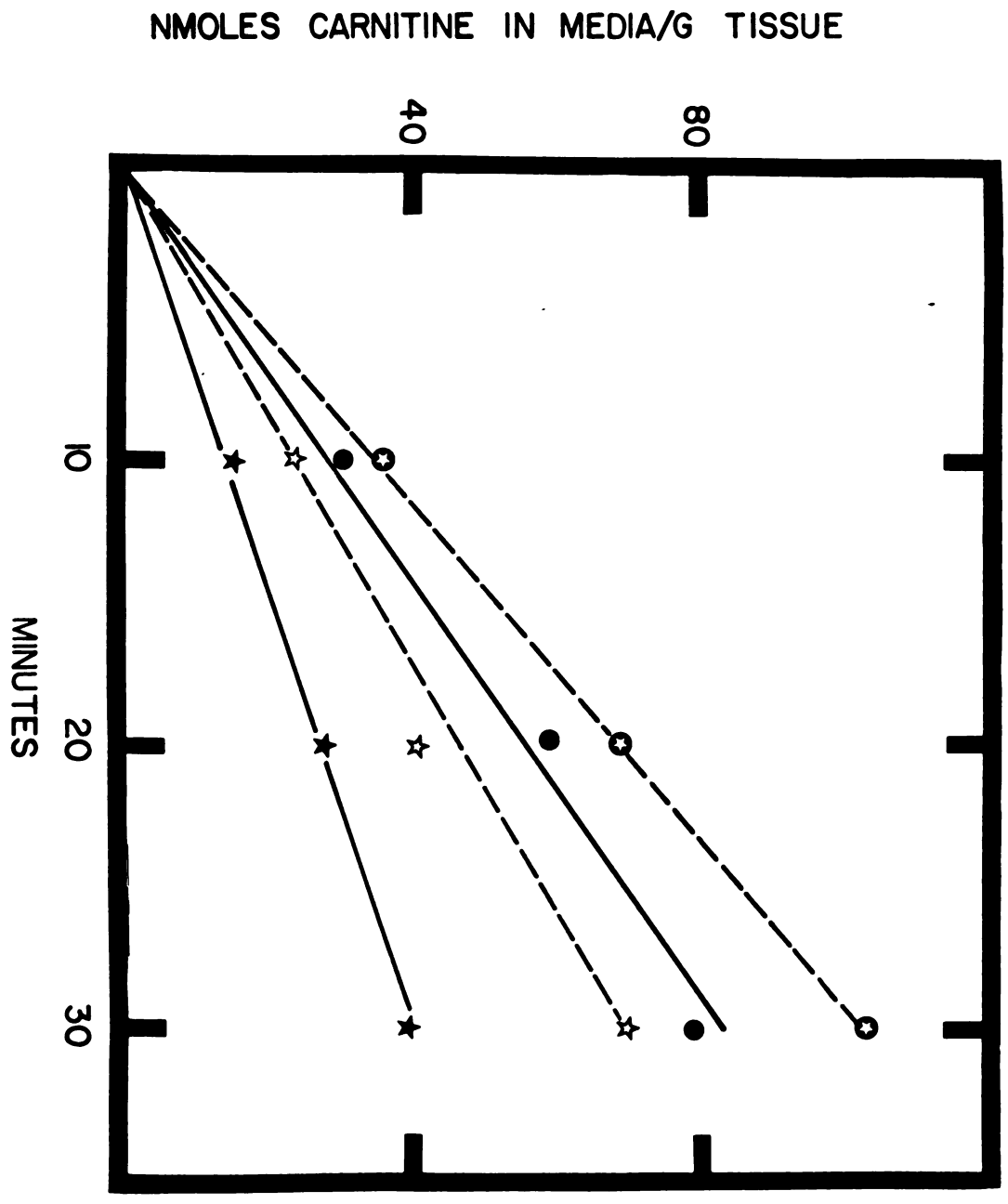
NMOLES CARNITINE IN MEDIA/G TISSUE
40
80
10
20
30
MINUTES

Table I

Effect of Anoxia on Carnitine Levels in Rat Hearts

Experimental Condition	β-Hydroxybutyrate	nmoles/g in tissue: Free Carnitine	nmoles/g in tissue: Short Chain Acylcarnitines	Ratio Free to Short Chain in Heart	nmoles/g released into buffer: Free Carnitine	nmoles/g released into buffer: Short Chain Acylcarnitines	Ratio Free to Short Chain in Buffer
Non-fasted rats							
air-CO_2	+	678 ± 50	291 ± 18	2.33	47.7 ± 3.2	39.2 ± 2.1	1.22
N_2-CO_2	+	701 ± 47	268 ± 22	2.62	*60.3 ± 4.5	44.6 ± 2.1	1.35
48 hour fasted							
air-CO_2	+	561 ± 25	445 ± 29	1.26	38.6 ± 3.2	63.5 ± 4.7	0.61
N_2-CO_2	+	593 ± 31	459 ± 31	1.29	*30.2 ± 3.2	54.8 ± 4.1	0.55
air CO_2	-	568 ± 51	388 ± 29	1.46	34.1 ± 1.1	47.6 ± 1.5	0.72
N_2-CO_2	-	576 ± 47	394 ± 21	1.46	*27.9 ± 1.1	47.3 ± 1.5	0.59

The hearts were split and incubated for 30 minutes at 37° with the appropriate gas mixture by continuous bubbling into the buffer; 5 mM β-hydroxybutyrate was used where indicated. n=6/experiment.
*Two tail t test P value <0.02 by comparing the control (air-CO_2) system to the anoxic (N_2-CO_2) system.

Table II

Short-Chain Acylcarnitines of Normoxic and Anoxic Rat Heart and Incubation Buffer.

Sample Analyzed	Acyl Carnitine	Control nmoles/g	Anoxic nmoles/g
Heart Tissue	acetyl	150.6	183.2
	propionyl	50.7	10.4
	isobutyryl	19.4	80.5
	butyryl	67.7	29.5
	isovaleryl	22.2	18.0
	valeryl	ref.	ref.
	tiglyl	14.8	10.2
	caproyl	29.5	40.0
	sum of individual	354.9	371.8
	Total Short Chain	440	460
Buffer	acetyl	32.6	12.0
	propionyl	11.7	3.6
	isobutyryl	0.3	9.1
	butyryl	10.4	6.0
	isovaleryl	1.2	2.9
	valeryl	ref.	ref.
	tiglyl	1.1	3.0
	caproyl	3.5	8.0
	sum of individual	60.8	44.6
	Total Short Chain	68.0	54.0

The hearts and buffer from 10 experiments described in Table I were analyzed for short-chain acyl carnitines and free carnitine. The short-chain acylcarnitines were isolated and the volatile fatty acids determined by gas chromatography as described in methods (7). Hearts from 10 fasted animals were used and the incubation buffer contained 5 mM β-hydroxybutyrate.

C

Tissue Samp

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Right Vent

Nonperfused
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Std. Dev.

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Table III

Carnitine Levels in Hypoxic and Control Perfused Pig Hearts

Tissue Sample	Nonperfused Hearts nmoles/g Free Carnitine	Nonperfused Hearts nmoles/g Short Chain Acylcarnitines	Perfused Hearts nmoles/g Free Carnitine	Perfused Hearts nmoles/g Short Chain Acylcarnitines
1. Right Ventricle	248	253	213	122
2. Left Ventricle	246	225	239	168
Right Ventricle	247	253	160	105+
3. Left Ventricle*	---	---	185	182
Right Ventricle	248	345	164	135+
4. Left Ventricle	208	274	255	170
Right Ventricle	181	242	159	103+
5. Left Ventricle*	---	---	278	171
Right Ventricle	388	246	207	102+
Nonperfused Mean Value	272	268	Perfused Control	239	173
Std. Dev.	±72	±43		±40	± 6
			Perfused Hypoxic	181	108
				±27	± 9
Percent Difference (Perfused Control: Hypoxic)				-24.4	-37.4
(Nonperfused Control: Perfused Control)				-12.2	-35.6

Nonperfused control hearts were obtained from air ventillated pigs whose blood had been removed for the perfusion studies. For perfused hearts the right ventricle was made hypoxic for 10 minutes by circulating the blood from the right leg and pumping it through a donor lung which contained 0% O_2 (PO_2 150 to PO_2 20). The blood then passed through the pigs lungs to obtain normal PO_2 and then passed through the left ventricle. The pig was sacrificed by stopping the blood flow for 2 minutes and tissue samples were then removed and freeze clamped and the carnitine levels determined. The values in nmol/g tissue for long chain acylcarnitines were: 41.6 ± 4.7 for nonperfused tissue, 61.6 ± 2.1 for the perfused control and 72.5 ± 2.1 for the perfused hypoxic samples; n=4.

*Left ventricle not collected for analyses.

+Wilcoxon's Signed Rank Test ($p < .05$).

Table IV

Effect of Hypoxia on the Short-Chain Acylcarnitine Levels of Perfused Pig Hearts

Sample	Acylcarnitine	nmoles/g
1) Right Ventricle (hypoxic)	acetyl	57.1
	propionyl	13.8
	isobutyryl	11.0
	butyryl	11.4
	isovaleryl	3.3
	valeryl	11.2
	caproyl	10.4
	Sum	118.2
	*Total Short-Chain	108
2) Left Ventricle (normoxic)	acetyl	153.1
	propionyl	3.9
	isobutyryl	1.5
	butyryl	6.8
	isovaleryl	1.8
	valeryl	1.4
	caproyl	1.2
	Sum	169.7
	*Total Short-Chain	173

Equal amounts of tissue from 6 left ventricles of control samples and right ventricles of 6 perfused hearts described in Table III were pooled into separate groups and analyzed for short-chain acylcarnitines. The samples were pooled due to the lack of enough tissue and the inordinate amount of time required to analyze each sample.
*Determined by independent acyl-carnitine analyses.

Affect of Alloxan Diabetes on Carnitine Content in Sheep Livers

This was a joint project with Dr. A.N. Snoswell, from Waite Agricultural Research Institute, Adelaide, Australia. He provided lyophilized perchloric acid extracts from both normal and alloxan diabetic sheep livers. The neutralized perchloric acid extracts were resuspended in water and were then analyzed for the acylcarnitines as described in the methods.

Examples of the gas chromatogram profiles of the acyl residues of acycarnitine from normal (animal no. 2) and diabetic (animal no. 5) sheep livers are shown in figure 3. The various labeled peaks correspond to the peaks obtained during a gas chromatographic run of known fatty acids. Valerylcarnitine was used as the internal standard because priminary studies contained only trace amounts of this acyl residue in both normal and diabetic livers (data not shown).

Carnitine Concentration in Normal and Alloxan Diabetic Sheep Livers

The free-, short-chain acyl-, and total water soluble-carnitine levels in the livers are shown in Table V. There was a significant increase in the carnitine concentration of alloxan diabetic livers, free carnitine increased 2-9 fold while the short-chain acylcarnitine concentration increased 10 to 150 fold when compared to the normals. In a communication with Dr. Snoswell, he described animal no. 6 as being only moderately diabetic due to the lack of fatty material in the liver. But even this animal had increased levels of carnitine in the liver. So the onset of alloxan diabetes leads to increased levels of carnitine in sheep liver.

Quantitation of the Short Chain Acylcarnitines

The amount of each identified acyl residue of the acylcarnitines from both normal and diabetic livers are given in Table VI. In both metabolic states acetylcarnitine was the predominant acyl derivative, 77% in normals and 71% in diabetic, however the absolute amounts of the acetyl carnitine in diabetic livers are increased by at least 40 fold. All of the other acyl residues are also increased many fold in the diabetic livers, although their percent distribution of the total acylcarnitines were similar to the acyl distribution in normal livers.

These data illustrate two major points. One is that the amounts of acylcarnitine can change in response to the metabolic state of the animal, in this case alloxan diabetes. Second is that the tissue contains several different acylcarnitines including propionyl-, isobutyryl-, and isovaleryl-, which could be products of oxidative degradation of branch-chain amino acids. In a personal communication with Dr. A.N. Snoswell he has informed me that the specific carnitine acetyl-, and isobutyryl-transferase have increased activity significantly in the diabetic state, however the medium-chain and palmitoyl transferase activities in both metabolic states are similar. The means of the transferase activitys of acetyl, isobutyryl, octanoyl, and palmitoyl were 3.2, 1.6, 1.4, and 2.0 nmol/min/mg protein in controls and 6.3, 3.4, 2.0, and 1.0 nmol/min/mg protein respectively in alloxan diabetic sheep.

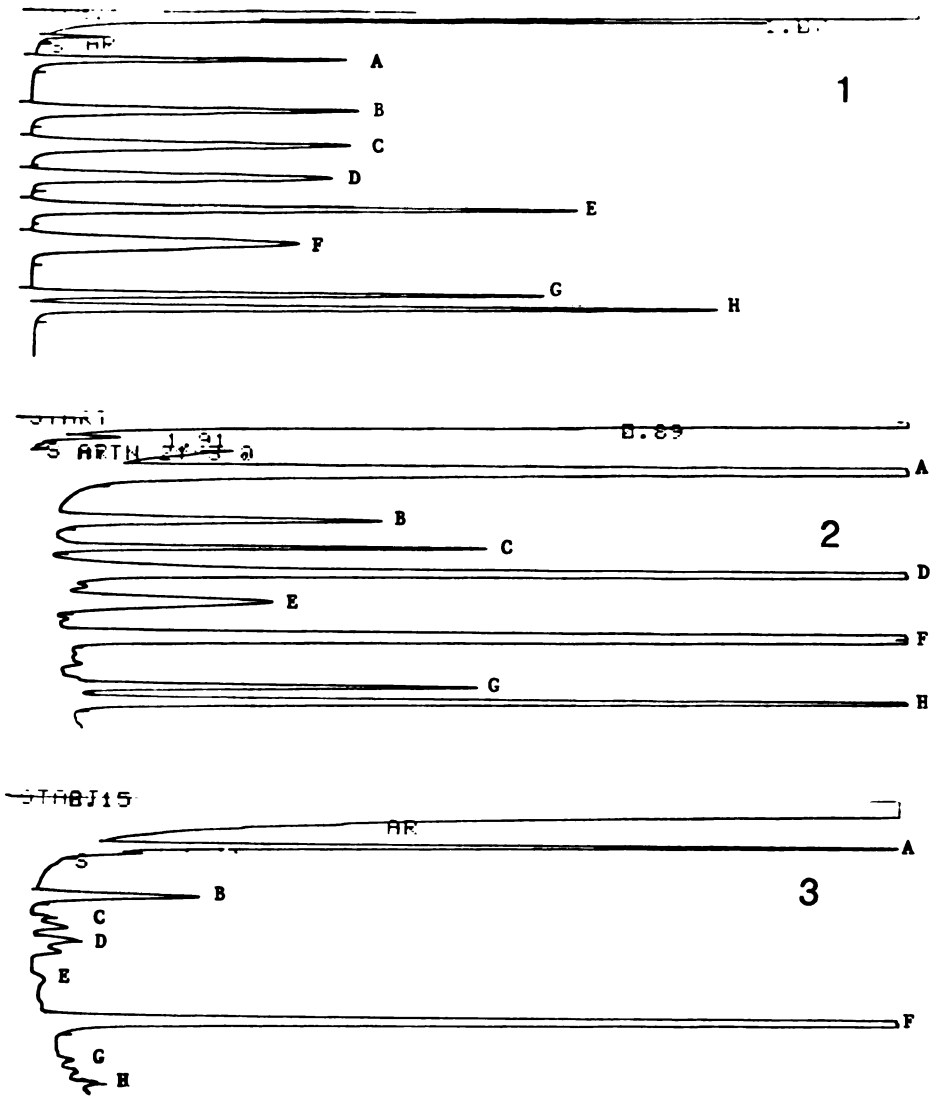


Figure 3. Gas chromatogram profiles of the acyl residues of acylcarnitines from normal and diabetic sheep livers. Acyl residues of the acylcarnitines from alloxan diabetic and normal sheep livers were isolated and injected into a GC as described in the methods. The profile of known fatty acids is presented in the first figure (1) above. The letter after each peak corresponds to these fatty acids; A:acetic; B:propionic; C:isobutyric; D:butyric; E:isovaleric; F:valeric; G:tiglic; H:captoic. Profile (2) is from alloxan diabetic liver number 5 and profile (3) is from control liver number 2.

Table V

Carnitine Levels in Normal and Diabetic Sheep Livers

	free carnitine (nmoles/gram)	short-chain acylcarnitines (nmoles/grams)	total acid soluble carnitines (nmoles/gram)
Normals			
1	155	17	172
2	148	15	163
3	230	10	240
Diabetic			
4	400	2200	2600
5	1614	614	2228
6*	523	116	639

*This animal was not as severely diabetic as the other animals, this was a determination made by the lack of fatty material in the liver.

3 Normal and 3 alloxan diabetic sheep livers were removed and a 5 gm piece of each liver was freeze-clamped and the acid carnitines were extracted with 6% perchloric acid. The extract was neutralized and then lympholized prior to transport to the U.S. The extract was then assayed for carnitine using the methods described previously.

Table VI

Acylcarnitines of Sheep Liver

Acylcarnitine	Normal (nmol/g)					Diabetic (n mol/g)			
	animal #1	#2	#3	mean	%	#4	#5	#6	mean %
Acetyl	10.5	19.5	11.8	13.9	76.5	1004	519	104	71.0
Propionyl	3.06	1.42	1.23	1.9	10.5	383	10.7	26.0	13.6
Isobutyryl	0.20	0.15	0.02	0.12	0.07	94.0	2.9	1.2	2.1
Butyryl	2.36	0.76	0.53	1.22	6.7	216	51.1	5.8	8.1
Isovaleryl	0.23	0.41	0.03	0.22	0.12	68.4	9.8	1.5	2.1
Tiglyl	0.18	0.31	trace	0.25	0.14	17.1	3.9	1.0	0.7
Caproyl	0.91	0.47	0.26	0.55	0.30	62.3	8.7	2.9	2.3

The acylcarnitines were determined using a gas chromatogram as described in the methods. The acid soluble acylcarnitines were obtained from the remaining sample from Table V.

Valerylcarnitine was used as the internal standard.

HUMAN STUDIES

The human may be one of the best systems for demonstrating the various functions of carnitine because of metabolic dysfunctions due to carnitine deficiency or deficiency in carnitine palmitoyltransferase activity as described in the introduction. Urine and serum were analyzed for the total amount of free carnitine and short-chain acylcarnitine content in six normal adults and in two children with metabolic problems.

Sample Collection and Subject Information

Six adult controls (29 ± 9 years old) were fasted overnight for 12 hours prior to collecting 10 mL of whole red blood and total morning urine. The urines were frozen at -80°C and stored for carnitine analysis at a later time as described in methods. The whole red blood samples were centrifuged to obtain serum, which was also stored at -80°C for analysis at a later time. The subjects were fed a high protein breakfast and lunch and both blood and urine were collected 6 hours after the initial morning collection. Patient I had metabolic dysfunctions of unknown origin, with persistent vomiting and hyperactivity resulting in dehydration and acidosis. Dr. P. Maur, at Children's Hospital, Cincinnati, Ohio, sent a sample of lyophilized urine collected during a 24 hour fast because he had observed high amounts of an unusual acid present in the urine. He tested serum

carnitine levels and found that free carnitine was very low, but the short-chain acylcarnitines were much higher than normal (personal communication).

Patient II was a child with Fanconis syndrome (a disease process involving defective absorption of specific ions by the kidneys). Ron Emaus in Dr. Bieber's laboratory at Michigan State University had found low levels of total carnitine in the blood (9 μmolar compared to the normal 40-50 μmolar). A 24 hour urine sample was collected during the time that blood samples were drawn.

Short-Chain Acylcarnitines in the Serum and Urine from Fasted and Fed Humans

Six adults (29 ± 9 years old) were fasted overnight for 12 hours prior to collecting a 10 mL aliquot of whole blood and the total overnight urine. The blood was clotted and was centrifuged to obtain serum. Serum and urine samples were again collected 6 hours later which was also 1 hour after a high protein lunch. Aliquots of the samples were analyzed for total water soluble carnitine content (free carnitine and short-chain acylcarnitines), results shown in Table VII. The data show: 1) Fasting does not affect the ratio of free carnitine to short-chain acylcarnitines in either serum and urine. 2) The concentration of total (free and short-chain acyl) carnitine in urine increases significantly in the fasted samples, while the serum total carnitine concentration remained constant. This may be due to the difference in time allowed for accumulation of carnitine in the urine between the fasted and fed samples. 3) The ratio of free carnitine to short-chain acylcarnitine decreases significantly when compared to the

same ratio in serum. This occurred in both fasted and fed samples indicating the kidneys are preferentially or selectively reabsorbing free carnitine while the short-chain acylcarnitines are retained in the urine.

The composition of the individual short-chain acylcarnitine from urines of both fasted and fed individuals were determined because the acylcarnitine appear to be selectively retained in the urine. These data are shown in Table VIII. The data show that the relative distribution of some of specific acylcarnitines differ between the samples from fasted and fed individuals even though the major short-chain acylcarnitine was acetylcarnitine in all samples. The urine from fed individuals contained a higher portion of propionyl-, isobutyryl-, and isovaleryl-carnitine while the portion of acetylcarnitine decreased. Increases in these acylcarnitines could be derived from branch chain amino acids, reflecting increases in the steady state level of the respective acyl coenzyme A levels and, possibly, increases in the contribution of the metabolic pathways leading to these compounds. The unidentified acylcarnitines were made up of many minor unidentified acyl groups, valerylcarnitine, and 3 peaks containing about 5% of total acylcarnitines. Valerylcarnitine was not be reported because it was used as the internal standard. In 2 separate urine samples in which crotonylcarnitine standard was used, valerylcarnitine represented 3% of the total.

The composition of the individual short-chain acylcarnitines from human serum was also determined to ascertain if the acyl composition of serum is similar to that in urine. Results of the total short-chain acylcarnitine concentration analysis and the acyl composition are given

in Table IX. The major acylcarnitine was acetylcarnitine, but relatively large amounts of propionyl- and butyryl carnitine, both of which might be derived from β-oxidation of long-chain fatty acids, are also present.

The mean values of the proportion of individual acylcarnitines in serum and urine are shown in Table X. The data show that the percent of acetyl-, propionyl-, and butyryl-carnitine in serum is almost double that in urine, while the percent of isobutyryl-, and isovaleryl-carnitine is much higher in urine than in serum. The data indicate that not only is free carnitine reabsorbed into the blood (Table VII) but it appears that acetyl-, propionyl-, and butyryl-carnitine are also selectively reabsorbed, while isovaleryl-, isobutyryl-, and caproyl-carnitine are retained in the urine.

Acylcarnitine Determination of Urine from Patient I

Results of the total amount of free and short-chain acyl carnitine and acylcarnitine composition present in a 24 hour urine collection of the patient suffering from dehydration and acidosis arising from some unknown metabolic problem are given in Table XI. The short-chain acylcarnitine fraction of the patient contained a large proportion of propionylcarnitine. In controls propionylcarnitine is about 5% of the total short-chain acylcarnitines which in the patient propionylcarnitine represents greater than 80% of the total short-chain acylcarnitines. This indicates a possible build-up of propionyl CoA in this individual.

Acylcarnitine Determination of Urine from Patient II

The urine and blood of a person with Fanconi syndrome was collected during a 24 hour fast. The patient had low blood levels of total carnitine but the urine contained large amounts of total carnitine. The short-chain acylcarnitine composition is interesting (Table XII), because of the increased proportion of butyrylcarnitine and decreased proportion of acetylcarnitine compared to control values. The increased proportion of butyrylcarnitine indicates a build-up of butyryl CoA, which should be generated during β-oxidation of long-chain fatty acids. Increased butyryl CoA would be generated by incomplete β-oxidation which may account for the decreased proportion of acetylcarnitine.

Table VII

Carnitine Concentration in Human Serum and Urine
of Fasted and Fed Adults

Sample	Urine (μmolar) Volume (mL)	Total*	Free	Short-Chain Acyl	% Free	Serum (μmolar) Total*	Free	Short Chain Acyl	% Free
#1 Fasted	545	429	249	180	58	41.4	37.4	4.0	90
Fed	660	70	39	31	56	43.2	34.9	8.3	81
#2 Fasted	370	616	353	263	57	39.0	29.9	9.1	77
Fed	200	494	254	240	51	38.4	34.3	4.1	89
#3 Fasted	135	221	32	189	14	35.1	27.1	8.0	77
Fed	270	95	24	71	25	37.2	30.4	6.8	82
#4 Fasted	450	499	278	221	56	39.3	34.6	4.7	88
Fed	300	288	157	131	55	44.0	40.0	4.0	91
#5 Fasted	144	334	102	232	31	38.0	33.8	4.2	89
Fed	185	249	86	163	35	44.5	40.1	4.4	90
#6 Fasted	293	224	110	114	49	48.1	41.3	6.8	86
Fed	552	102	44	58	43	44.4	42.1	2.3	95

The fasted samples were obtained after a 12 hour overnite fast. The serum was obtained from 10 mL of whole blood, and the urine samples were collected at approximately the same time. Serum and urine samples from fed individuals were collected one hour after a high protein meal and 6 hours after the fasted samples. The carnitine concentrations were determined using the enzymatic radiolabeled procedure described in the methods.

*Total represents total water soluble carnitine = free carnitine + short chain acylcarnitines.

Table VIII

Urinary Acylcarnitine Content of Fasted and Fed Adults

Sample	Total μmoles Short-chain Acylcarnitine	% acyl residue of total short-chain acylcarnitine							
		acetyl	propionyl	isobutyryl	butyryl	isovaleryl	tiglyl	caproyl	unidentified
A. Morning Fasted									
#1	98.1	61.6	4.1	10.3	1.2	7.8	0.9	1.4	12.7
2	97.3	48.3	7.2	16.3	4.4	9.8	1.1	2.2	10.7
3	25.5	57.3	4.2	14.7	2.1	4.7	1.3	3.0	12.7
4	99.5	45.6	5.5	18.6	2.1	11.3	0.4	4.8	11.7
5	33.4	60.1	6.1	13.0	3.8	4.3	0.6	2.7	9.4
6	33.5	59.4	4.9	14.6	0.2	8.4	-	2.9	9.6
	Mean	55.4 ± 6.7	5.3 ± 1.2	14.6 ± 2.8	2.3 ± 1.6	7.7 ± 2.8	0.7 ± 0.5	2.8 ± 1.1	11.1 ± 1.5
B. Afternoon Fed									
#1	46.2	54.2	6.7	15.0	2.9	6.3	2.2	3.3	9.4
2	48.0	44.2	8.2	15.0	2.3	10.1	2.3	4.1	13.8
3	19.2	48.7	8.9	15.0	4.5	7.9	0.9	2.4	11.7
4	39.3	31.1	6.6	23.2	2.0	20.6	0.6	6.3	9.6
5	30.2	54.8	7.8	15.1	2.6	9.2	-	1.9	8.6
6	30.1	35.0	13.7	25.1	3.2	9.6	-	2.9	10.5
	Mean	44.7 ± 9.9	8.7 ± 2.6	18.1 ± 4.7	2.9 ± 0.9	10.6 ± 5.0	1.0 ± 1.0	3.5 ± 1.6	10.6 ± 1.9

Total urine excretions were obtained from 6 adults after a 12 hour fast and one hour after a high protein meal (6 hours after fasted sample obtained). The total moles of acylcarnitine were obtained from Table VI. The acylcarnitines were identified and quantified as described in methods, using valerylcarnitine as the internal standard. The percent of acyl represent amount obtained by GC method compared to the total amount of acylcarnitines determined in Table VI. Sample #1 fasted and Sample #1 fed were taken from the same individual. Independent samples showed that valerylcarnitine is about 3.0%.

Table IX

Serum Acylcarnitine Content in Humans

	μmolar		% of total short-chain acylcarntines							
Sample	Total[a] carnitine	Short-chain acylcarnitine	acetyl	propionyl	isobutyryl	butyryl	isovaleryl	valeryl	tiglyl	caproyl
1	38.7	4.4	75.9	10.9	2.4	5.0	0.6	3.8	-	0.7
2	43.8	5.4	75.2	12.9	1.1	6.2	0.0	3.4	-	0.5
3	43.9	4.9	71.0	12.6	2.9	6.1	1.2	4.2	-	1.5
4	44.8	5.6	76.0	10.	2.7	4.5	0.67	5.4	-	1.0
5[b]	39.9	4.1	69.3	13.6	1.9	9.8	1.0	2.8	-	1.0
		MEAN	73.5 ± 3.1	12.0 ± 1.4	2.2 ±0.7	6.3 ± 2.1	0.7 ± 0.4	3.9 ± 1.0	-	0.8 ± 0.4

Whole blood samples were drawn (10 mL) from 6 fed adults and the serum was obtained from the coagulated blood by centrifugation. The serums were then analyzed for carnitine concentration and the acyl components of acylcarnitine as described in the methods. The percent of total represents each acyl residue was obtained by comparing the amount of each acyl determined by the G.C. to the total acylcarnitine determined by the enzymatic labeled method. Crotonyl carnitine was the G.C. internal standard.

[a]Total carnitine represents Free + Short-chain acyl-.
[b]Serum was pooled from 2 individuals; sample provided by Doris Lennon, Univ. Wisconsin, Madison.

Table X

Proportion of Individual Acylcarnitines in Human Serum and Urine

Acycarnitine	In Urine	Serum
acetyl	44.7 ± 9.9	73.5 ± 3.1
propionyl	8.7 ± 2.6	12.0 ± 1.4
isobutyryl	18.1 ± 4.7	2.2 ± 0.7
butyryl	2.9 ± 0.9	6.3 ± 2.1
isovaleryl	10.6 ± 5.0	0.7 ± 0.4
valeryl	3.0 ± 1.1	3.9 ± 1.0
tiglyl	1.0 ± 1.0	0.0 ± 0.0
caproyl	10.6 ± 1.6	0.8 ± 0.4

Tabulation of the mean values of the % of each acylcarnitine in serum and urine were obtained from Tables VIII and IX.

Table XI

Urinary Acylcarnitine Content of Patient I

Component	μmoles/24 hours	% of total short-chain acylcarnitine	Control Normal Adult % of short-chain acyl-carnitine
Free Carnitine	11.0	---	---
S.C. Acyl Carnitine	16.3	100	100
Total (-LC)	27.3	---	---
acetyl-carnitine	.787	4.3	55.4
propionyl-carnitine	13.47	82.6	5.3
isobutyryl-carnitine	.057	0.3	14.6
butyryl-carnitine	.0007	.04	2.3
isovaleryl-carnitine	.041	.3	7.7
tiglyl-carnitine	.002	.01	0.7
caproyl-carnitine	.019	.1	2.8
unidentified	---	12.3	11.1

Urine collected during a 24 hour fast from a patient with metabolic problems (dehydration and acidosis) was analyzed for carnitine concentration and composition of acyl groups of the short-chain acylcarnitines as described in methods. Control values obtained from the urine of six fasted adults (Table VIII).

Table XII

Urinary Acylcarnitine Content of Patient II

Component	μmoles/24 hours	% of total short-chain acylcarnitine	Control Normal Adult % of s.c. acylcarnitine
Free carnitine	113.0		
S.C. Acyl carnitine	78.635		
acetyl-	26.017	33.1	55.4
propionyl-	6.036	7.6	5.3
isobutyryl-	3.909	5.0	14.6
butyryl-	14.591	18.6	2.3
isovaleryl-	4.386	5.6	7.7
tiglyl-	2.140	2.7	0.7
caproyl-	4.980	6.3	2.8
unidentified		21.1	11.1

The total urinary excretion was collected during a 24 fast of a child with Fanconis syndrome. Quantification of the acylcarnitines were performed as described in methods. Control adults values were obtained from the urine of six fasted adults (Table VIII). Data of the amount free and short-chain acylcarnitine was provided by Ron Emaus.

DISCUSSION

Effect of Hypoxia on Heart Acylcarnitines

The data presented herein show that when rat hearts are incubated under anoxic conditions, the tissue preferentially loses short-chain acylcarnitines. This loss of short-chain acylcarnitines and also free carnitine is linear for at least 30 minutes. In our experiments we did not observe a preferential loss of carnitine from cardiac tissue due to anoxia, in contrast to others, but it should be noted that we did not use tissues perfused with blood or media for the rat heart experiments. However, when pig hearts were perfused with hypoxic conditions, a 37% loss of short-chain acylcarnitines occurred compared to the control perfused ventricle and 70% loss occurred when compared to non-perfused left ventricles. These data are in good agreement with the observations of others who have observed a loss of tissue carnitine during hypoxia and ischemia (39,40,50,51).

Our results also show that during hypoxia the long-chain acylcarnitines in tissues of perfused pig heart are increased, presumably due to inhibition of β-oxidation of long-chain fatty acids. In contrast, there was a marked decrease in acetylcarnitine of hypoxic ventricles, again, presumably due to decreased flux of long-chain fatty acids through β-oxidation and, consequently, decreased formation of acetylCoA (the precursor of acetylcarnitine).

Hypoxia caused an increase in the amounts of propionyl-, isobutyryl-, butyryl-, and valeryl-carnitine. Increases in propionyl-, isobutyryl-, and isovaleryl-carnitine should reflect increases in the steady state level of the respective acyl coenzyme A levels and, possibly, increases in the contribution of the metabolic pathways leading to such compounds. These acylcarnitines could be derived from branched chain amino acids (22). Similarily, the increases in butyrylcarnitine and caproylcarnitine could indicate increases in the steady state level of the intermediate products of β-oxidation. The rat heart data are more difficult to interpret since anoxia caused a decrease in the tissue propionylcarnitine but an increase in the isobutyrylcarnitine and lesser changes in the other acylcarnitines. The decrease in the amount of propionylcarnitine in rat heart should reflect a decrease in the steady state level of propionylcoenzyme A. Propionylcoenzyme A is derived from the carbon skeletons of isoleucine, valine and from odd chain fatty acids; hence the decrease could represent decreased isoleucine, valine metabolism and/or decreased oxidation of odd chain fatty acids. This seems reasonable since anoxia should completely inhibit β-oxidation of fatty acids. Alternatively, the data are also consistent with inhibition of the pathways for metabolism of the aliphatic short chain coenzyme A derivatives and their subsequent conversions to acylcarnitines.

Thus, the increased levels of isobutyrylcarnitine observed during limited or no oxygen could reflect a blockage of the enzymatic step for the conversion of isobutyrylcoenzyme A to isobutenylcoenzyme A, a flavin linked dehydrogenase. Carnitine could then be acting as a sink for isobutyryl units at the level of isobutyrylcoenzyme A.

Acylcarnitines of Human Urine and Serum

The data show that the proportion of the total water soluble carnitines represented by free carnitine remains constant in serum and urine when comparing fasted to fed levels, however the percent free carnitine is significantly different between urine and serum for both fasted and fed individuals. This indicates that the kidneys may be retaining short-chain acylcarnitines in the urine while preferentially reabsorbing free carnitine. This may provide the human with a mechanism of removing some acyl residues from the body via the acylcarnitines, which will neutralize the fatty acids and may also allow for the removal of "excess" carnitine, since carnitine is not metabolized in animals and is only removed from the body by urine excretion. Therefore carnitine can act as a carrier which binds and neutralizes the fatty acyl residue for excretion in urine.

Identification of the acylcarnitines of urine showed that acetylcarnitine is the major component, however its proportion was less in urine samples of fed human, while propionyl-, isobutyryl-, and isovaleryl-carnitine were increased slightly. As mentioned earlier, increases in these acylcarnitines could reflect increased levels of branched-chain amino acid metabolites. One would expect increases in branched-chain amino acid oxidation after a high protein meal, which could generate the acylcarnitines found in the urine.

Human serum contains a high proportion of acetylcarnitine, and propionyl-, valeryl-, and butyryl-carnitine represent almost all of the remaining acyl carnitines. It is interesting that the proportion of the acylcarnitines in urine and serum of fed adults give different results: as mentioned above serum contains high proportions of acetyl-,

propionyl-, butyryl-, and valeryl-carnitine, which are almost double the relative proportion of these acyl residues in the urine. However, the urine contains isobutyryl-, and isovaleryl-carnitines, in proportion 10 times greater than these observed in serum. Thus the kidneys not only selectively reabsorb free carnitine and excrete acylcarnitines, but they also preferentially reabsorb acetyl-, propionyl-, butyryl-, and valeryl-carnitine while they excrete isobutyryl-, and isovaleryl-carnitine in the urine. This is related to the data that have shown (101) that the translocase which moves isobutyrylcarnitine into the matrix of mitochondria has a low affinity (high Km) for isobutyrylcarnitine and consequently uptake of carnitine and/or some other acylcarnitines are preferred when all are present. Thus the kidneys may have a lower affinity for reabsorbing isobutyryl-, and isovaleryl-carnitine. Other studies have shown (24) that isobutyrylcarnitine is readily oxidized by beef liver and rat liver mitochondria in the absence of free carnitine but when free carnitine is present the oxidation is inhibited, presumably, due to the competition of free carnitine for isobutyrylcarnitine at the translocase step. The increases in isobutyrylcarnitine during anoxia and hypoxia are consistent if one assumes that isobutyrylcarnitine is in equilibrium with isobutyryl- coenzyme A and that carnitine may be a sink for disposing of excess short-chain acyl residues at level of acylcoenzyme A.

The short chain acylcarnitine composition of the urine from patient I supports the statement that carnitine removes excess acyl residues because the proportion of propionylcarnitine in urine was greater than 80% of the total acylcarnitines while in controls propionylcarnitine is about 5% of the total acylcarnitines. Thus carnitine may be a sink for

excess propionyl CoA which will restore the acyl CoA/CoASH ratio. Apparently the increase of propionylcarnitine indicates a block in the conversion of propionyl CoA to methylmalonyl CoA by the biotin linked carboxylase enzyme, or in the vitamin B_{12} linked mutase that converts L-methylmalonyl CoA to succinyl CoA.

The urine from patient II (Fanconi syndrome) contain large amounts of free carnitine and short-chain acylcarnitines (40% of total water soluble carnitine). The urine contained each of the acyl residues that are present in acylcarnitines from normal human urine. Since the blood had very low levels of total carnitine, it was assummed that the patient had a deficiency in carnitine uptake which resulted in a muscle deficiency. However the presence of acylated carnitines indicates that the carnitine had been intracellular in some tissues prior to excretion in the urine. It seems the kidneys were not reabsorbing carnitine, thus the muscle carnitine deficiency was due to a kidney defect, which would indicate a systemic deficiency instead of a myopathic deficiency.

Diabetic Sheep Livers

The effect of alloxan diabetes on sheep liver acylcarnitines supports the idea that the levels of acylcarnitine reflect the steady state levels of the respective acyl CoA levels. The levels of each acylcarnitine increased many fold in alloxan diabetic liver compared to normal livers, however the relative proportion of the total acylcarnitines represented by each acylcarnitine remains constant between the two metabolic states. For example, acetyl- and propionyl-carnitine were 76.5 and 10.5% of total acylcarnitines in normal livers and these acyls were 71.0 and 13.6% of total acylcarnitine in diabetic livers.

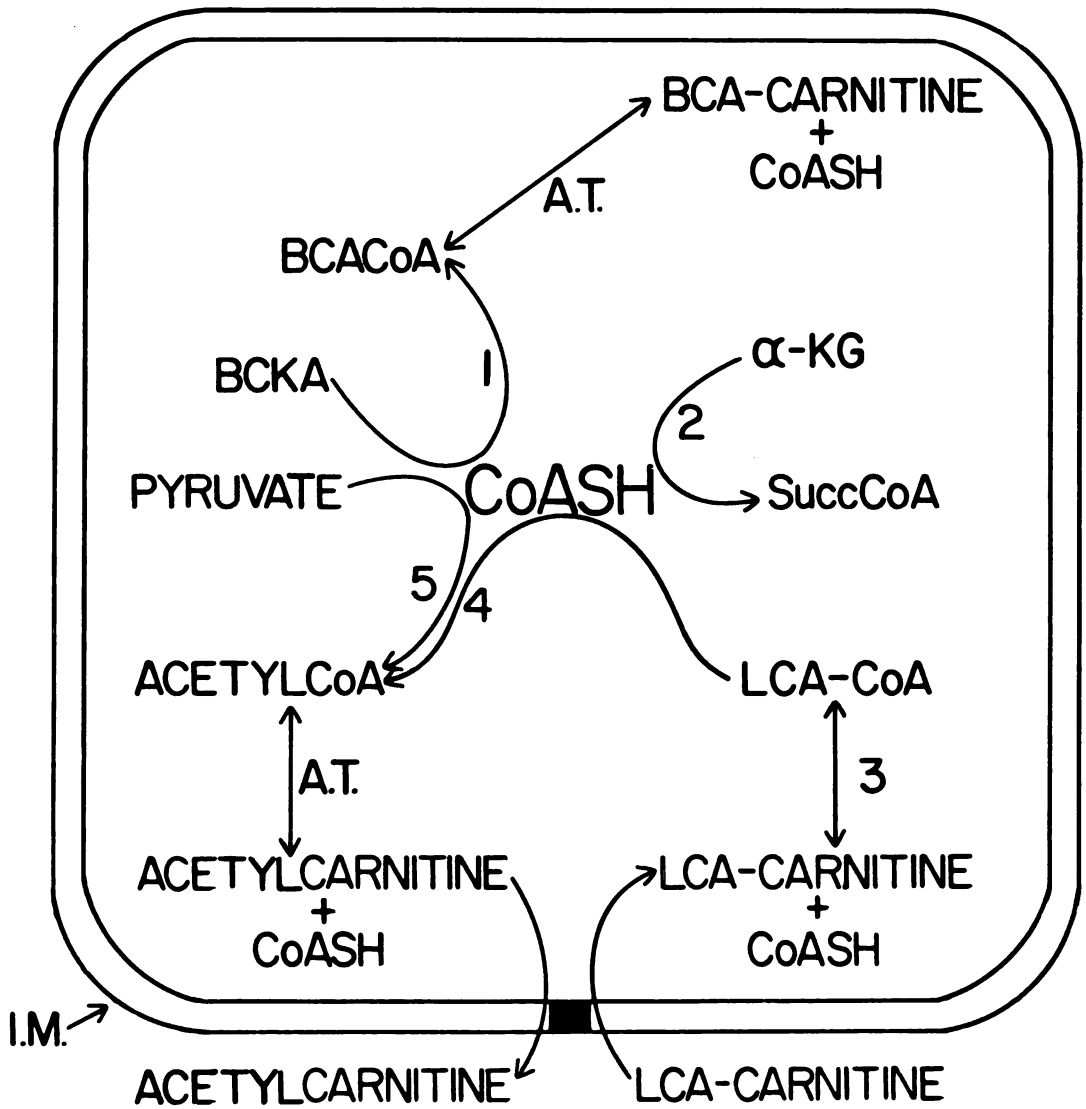
As stated earlier a proposed function for carnitine is to modulate the CoASH/acyl CoA ratio (24,34) by formation of acylcarnitine from the acyl CoA derivatives via the various carnitine acyltransferases that occur in tissues (23). Thus carnitine could buffer the CoASH/acyl CoA ratio, thereby insuring the availability of CoASH for oxidative metabolism in the matrix of mitochondria. CoASH is required at this site for the conversion of: pyruvate to acetyl CoA, long fatty acids to acetyl CoA, α-ketoglutarate to succinyl CoA, and branched-chain α-ketoacids (valine, leucine, and isoleucine) to branched-chain acyl CoAs. Figure 4 depicts the relationship of carnitine to the acyl CoA pool and the regeneration of CoASH in the matrix of mitochondria. Reactions 1 (branched-chain α-ketoacid dehydrogenases), 2 (α-ketoglutarate dehydrogenase), 3 (carnitine acyltransferases), 4 (β-oxidation enzymes), 5 (pyruvate dehydrogenase) all require CoASH, and all of the products except for reaction 2 are in equilibrium with carnitine. Thus generating acylcarnitines via the carnitine acyltransferases liberates CoASH in the matrix of mitochondria, as long as carnitine is available. If carnitine levels are decreased then the "acyl buffer" effect is diminished, which would reduce flux through reactions 1-5 and some reaction (1 and 5) would be modified due to allosteric control of these enzymes by the CoASH/acyl CoA ratio (99,100). Both enzymes are highly regulated and respond to allosteric modulators such as the CoASH/acyl CoA ratio.

During anoxic and hypoxic conditions, cardiac tissue selectively retains free carnitine apparently at the expense of short-chain acylcarnitines which are lost from the tissue. This is consistent with the suggestion that retention of free carnitine would enable the

remaining carnitine to buffer the CoASH to acylcoenzyme A ratio, thereby insuring the availability of CoASH for oxidative metabolism in the matrix of mitochondria. Such a role for carnitine would be consistent with the observed affects of free carnitine on ischemia (24,97) even as it may relate to pyruvate oxidation (43). The reduction in the amounts of short-chain coenzyme A derivatives would also be consistent with the inhibition of adenine translocase by acylcoenzyme A derivatives during ischemia. The reduction of the translocase inhibition by carnitine would be via formation of acylcarnitines.

One could speculate that patient I in Table XI was severely acidotic because of inadequate availability of CoASH and an abnormally high ratio of acylCoA/CoASH due to the large amounts of propionyl CoA.

RELATION OF CARNITINE TO CoASH IN THE MATRIX OF MITOCHONDRIA



SUMMARY

Studies with anoxic and hypoxic hearts

The investigations show that anoxia results in preferential loss of short-chain acylcarnitines from rat hearts. The loss was linear for 30 minutes. Hypoxic perfused pig hearts also preferentially lost short-chain acylcarnitines. Hypoxia caused a 70% decrease in the total carnitine concentration compared to non-perfused hearts. Analysis of the carnitine of hypoxic heart tissue showed increased long-chain acylcarnitines and decreased acetylcarnitine, which indicates a block in β-oxidation of fatty acids. Hypoxia also increased the amount of branched-chain acylcarnitines, which could be derived from amino acid oxidation.

Studies with alloxan diabetic sheep

Alloxan diabetes results in very high levels of free- and short-chain acyl-carnitines in sheep livers when compared to normals. Analysis of the acylcarnitines showed that each of the acyl residues were about equally elevated in the alloxan treated sheep livers. These acylcarnitines should give an indication of the steady state levels of the respective acyl CoAs.

Studies with Humans

Serum samples from fed or fasted adults showed no change in either the total carnitine concentration or in the ratio of free carnitine to short-chain acylcarnitine. The major acyl residue of the acylcarnitines was acetyl with propionyl, butyryl, and valeryl accounting for the remaining residues. When comparing urine samples to serum samples, it was found that the urine samples contained a lower ratio of free carnitine/acylcarnitine ratio and the acylcarnitines contained a lower proportion of acetyl, propionyl, and butyryl residues while isobutyryl and isovaleryl proportions increased. Analysis of the acylcarnitines in urine from patient I showed very high levels of propionyl carnitine suggesting that carnitine could be used as a sink for excess short-chain acyl residues that are generated in the mitochondrial matrix.

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