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Effects of carnitine on medium-chain fatty acid metabolism in newborn pigs

van Kempen, Theo A. T. G., Ph.D.
University of Illinois at Urbana-Champaign, 1993
EFFECTS OF CARNITINE ON MEDIUM-CHAIN FATTY ACID METABOLISM IN NEWBORN PIGS

BY

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THESIS

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutritional Science in the Graduate College of the University of Illinois at Urbana-Champaign, 1993

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EFFECTS OF CARNITINE ON MEDIUM-CHAIN FATTY ACID METABOLISM IN NEWBORN PIGS

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Metabolism of medium-chain fatty acids (MCFA) is considered to be independent of carnitine because of their passive diffusion into the mitochondrion. However, upon feeding medium-chain triglycerides (MCT) to piglets (n=16) followed by serial blood sampling, we observed significant changes in free and acyl-carnitines in plasma as determined using a newly developed HPLC procedure which allows for quantification of individual carnitine esters. The most noticeably change was observed in propionyl-carnitine, which increased significantly (from 0 to 4μM, p<.05) upon feeding odd-chain MCT, while it was undetectable when even-chain MCT were fed. This suggests a role for carnitine, likely mediated via buffering the acyl-CoA/free CoA ratio in the mitochondria since propionyl-esters under the conditions employed are mainly produced inside the mitochondrion. To further investigate whether carnitine alters MCFA oxidation in vivo, we infused pigs (n=34) continuously with MCFA via umbilical arterial catheters; and after an adaptation period, carnitine was added to the infusate. The addition of carnitine increased MCFA oxidation (p<.01) when MCFA oxidation provided more than 20% of the metabolic energy (increase= 0.16*[fatty acid oxidation rate - 18], r=0.74, p<.01). In addition, in 18 of these pigs, dicarboxylic acid (DCA, analyzed using HPLC) excretion in urine (collected via a bladder catheter inserted via the umbilicus) was measured over time. Carnitine supplementation decreased DCA excretion by 45% (p<0.05) in urine. This suggests that carnitine lowered the intracellular fatty acid concentration in the liver (possibly due to an increased fatty acid oxidation) since DCA production is correlated with the fatty acid concentration. Carnitine supplementation also increased plasma and liver carnitine and acetyl-carnitine concentrations and urinary acetyl-carnitine excretion. However, no increase was observed in muscle acetyl-carnitine. The increase in liver acetyl-carnitine due to carnitine and fatty acid infusion concurs with the hypothesis that carnitine acts as a buffer to the hepatic intramitochondrial acyl-CoA/CoA ratio, aiding in the export of excess acyl groups and thereby, relieving the mitochondrion of a toxic substance which is capable of inhibiting oxidative processes as well as the urea cycle.
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Enrolled in graduate school, I came to know Dr. Jack Odle as an excellent mentor, and also as a friend. The first years of our joint endeavor, for me to get a Ph.D. and for Jack to get tenure, were plagued by misfortune in our housing conditions; but that created an atmosphere of comradery. Jack's door was always open; and as a result, hours were spent, besides cleaning mud out of the lab, discussing topics from $\beta$-oxidation to social situations. I admire Jack for his tremendous knowledge of biochemistry, but even more for his great personality, keeping in mind that we are all human beings with shortcomings and needs. Jack, it was great to have you as a boss, and I like to thank you for all the guidance and support you provided.

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[Signature]
# Table of Contents

**Introduction** .................................................. 1
   Development of a nutritional supplement ......................... 3
   Carnitine .......................................................... 4
   Carnitine in the fetal and the newborn pig ....................... 8
   Research focus: the role of carnitine in MCFA oxidation in vivo ........................................... 9

**Chapter 1. Toxicity of medium-chain fatty acids and triglycerides** ........ 11
   1.1 Introduction .................................................. 11
   1.2 Accumulation of fatty acids .................................... 11
   1.3 MCAD deficiency ................................................ 13
   1.4 Reye syndrome .................................................. 19
   1.5 Dietary intake leading to toxicity ............................. 22
   1.6 Possible mechanisms for the toxic action of fatty acids ....... 23
   1.7 Future research directions ..................................... 28

**Chapter 2. Quantification of carnitine esters by High-Performance Liquid Chromatography** ..... 29
   2.1 Introduction .................................................... 29
   2.2 Material and Methods ........................................... 30
      2.2.1 Chemicals .................................................. 30
      2.2.2 Equipment .................................................. 31
      2.2.3 Isolation of carnitine esters from plasma samples ....... 31
      2.2.4 Derivatization procedure ................................... 32
      2.2.5 Chromatography ............................................ 33
      2.2.6 Elution profile of carnitine esters ....................... 34
   2.3 Results ......................................................... 34
   2.4 Discussion ..................................................... 37

**Chapter 3. The effect of feeding MCT of varying chain-length on the carnitine ester profiles in newborn pigs** .... 39
   3.1 Introduction .................................................... 39
   3.2 Materials and Methods ......................................... 40
   3.3 Results ........................................................ 40
   3.4 Discussion ..................................................... 44
Chapter 4. Medium-chain fatty acid oxidation in colostrum-deprived newborn piglets: stimulatory effect of L-carnitine supplementation ........................................ 45
  4.1 Introduction ............................................................................. 45
  4.2 Materials and methods ............................................................ 46
    4.2.1 Animals ............................................................................ 46
    4.2.2 Infusion protocol .............................................................. 47
    4.2.3 Analysis ........................................................................... 49
  4.3 Results .................................................................................. 50
  4.4 Discussion ............................................................................ 52

Chapter 5. Dicarboxylic acid (DCA) production of newborn pigs infused with medium-chain fatty acids (MCFA): effects of valproate and L-carnitine co-infusion .... 56
  5.1 Introduction ............................................................................ 56
  5.2 Materials & Methods ............................................................. 57
    5.2.1 Animals ............................................................................ 57
    5.2.2 Infusion protocol .............................................................. 59
    5.2.3 Sample analysis ............................................................... 60
    5.2.4 Data analysis .................................................................... 61
  5.3 Results .................................................................................. 62
  5.4 Discussion ............................................................................ 70

Concluding remarks ........................................................................ 74
  Does carnitine have a role in medium-chain fatty acid metabolism? .... 74
  Neonatal survival ........................................................................ 75

Appendix A. Peroxisomes ............................................................... 77

Appendix B. Effect of infusion of medium-chain fatty acids of varying chain-length on dicarboxylic acid (DCA) excretion .................................................. 80
  Introduction ............................................................................... 80
  Materials & methods ............................................................... 80
  Results .................................................................................... 80
  Discussion .............................................................................. 81

Literature cited ............................................................................. 82

Curriculum Vitae ........................................................................ 96
Introduction

Piglet mortality is a major problem for the swine industry. Mortality varies considerably across different production units from as low as 5% on well-managed farms to as high as 25% on others. High mortality is associated with a significant increase in costs: a larger sow herd is required to produce similar numbers of animals. In the last decade, efforts to improve sow herd productivity have focused on increasing the number of pigs born per litter, but little effort has been made towards decreasing mortality. Several reasons (see Fig. 1) exist for the high mortality. The underlying cause is, however, often associated with a lack of adequate nutrition.

Modern housing systems are designed for optimal hygiene, climate, and ease of use without compromising the performance of the animal. The housing of the sow with her newborn piglets, however, presents a dilemma. The sow needs a fairly cool environment (15°C, Noblet et al. 1990), while the newborn pigs require a warm environment (35°C, Curtis et al. 1970). Achieving these two environments within one building is extremely costly, and often not practical. Therefore, the environment for the newborn pig is compromised. Newborn pigs are often housed in an environment which is below thermoneutrality (optimum temperature for the animal).

Before and at birth, the pigs’ metabolic rate (heat production) is equivalent to the metabolic rate of the sow (per unit of body weight, Gentz et al. 1970). After birth, the pig’s heat loss increases due to evaporating birth fluids and due to the lack of a thermoneutral environment (Fig. 2). Therefore, the pig looses heat at a rate higher than it produces heat (which actually drops after birth), resulting in a drop in body temperature. The drop in body temperature initiates a fatalistic cycle of a lack
of competitiveness (an unresponsive, weak pig), lack of milk intake, and ultimately mortality (Verstegen, personal communication), unless the pig is able to compensate for the drop in body temperature.

For the pig to increase his heat production, energy is required. However, pigs are born with limited energy reserves. Only 1 to 2% of their body weight is fat, from which about 50% can be used as an energy source. Glycogen is available in liver and in muscle (approximately 14.7 g for a pig of 1.2 kg, dependent on the nutritional status of the sow), which supplies the piglet with energy to adequately maintain body functions for approximately 17 h (at 27°C). Together, fat and glycogen provide sufficient energy to remain vivid for approximately 28 h. In small pigs, stores are significantly less, and without food, they can remain vivid for only 8 h (Mellor & Cockburn 1986). Often these energy supplies, especially in a smaller pig (see Fig. 3), are not enough to enable the pig to cope with suboptimal circumstances after birth; and immediate milk consumption is necessary for the pig to survive. Colostrum, if consumed, supplies new energy, activates the intestinal tract, and increases metabolic rate (Gentz et al. 1970).

Fig. 2. Heat loss, heat production, and body temperature of a suckling (solid line) and a fasting (dashed line) newborn pig housed at 32°C (fictional data modelled after Noblet & Le Dividich 1981). Immediately after birth, the pigs' heat loss is much higher than his heat production, which results in a drop in body temperature. Only a healthy, suckling pig is able to compensate for this drop in body temperature. The non-suckling pig is unable to control his body temperature and will become severely hypothermic and eventually die.
Fig. 3. Effect of piglet birth weight on mortality within the first 4 weeks of life (total mortality at the farm studies was 8.2%; van der Aar 1985). The hatched bars indicate the weight class of pigs which have good odds surviving in a well-managed environment, but often die in a poorly-managed environment.

If the pig is not able to suckle, then nutritional supplementation can provide the energy needed and can stimulate the metabolism of the pig. The advantage of a nutritional supplement compared to other methods for decreasing mortality is that it can be used in existing buildings without major investments and can be directed specifically to animals that need the treatment. A small disadvantage is the need for extra labor which can be reduced by combining the labor associated with supplying extra nutrition with other chores, like iron supplementation and teeth clipping.

Colostrum provides excellent nutrition for the pig, but it is not suited for use as a nutritional supplement for weaker pigs. The volume required to supply sufficient energy (e.g. 50% of the metabolic need for 1 day) exceeds the capacity of the pig's stomach. Second, colostrum is also a good growth medium for bacteria; therefore, it has to be prepared fresh prior to use from an artificial formula or from freeze-dried colostrum, or obtained manually from the sow, increasing the need for labor.

Development of a nutritional supplement

Research in our lab focuses on developing an energy supplement which provides the pig with a significant energy dose without suppressing the milk/colostrum consumption. Medium-chain triglycerides (MCT, which are fats with 6-12 carbon atoms: C6-C12) have been used successfully in pediatric nutrition for both premature and term babies in hospital settings and also in commercial
infant formulas as an energy supplement because of their unique metabolic characteristics. In humans, MCT have been found to spare protein, which enhances recovery of trauma patients and growth in premature infants (Bach & Babayan 1982, see also Chapter 3). We, therefore, focus on the use of medium-chain triglycerides as an energy supplement for pigs.

Medium-chain triglycerides are derived from coconut and other tropical oils, and are commercially available. MCT have some advantageous characteristics which make them interesting for consideration as a nutritional supplement (Bach & Babayan 1982):

* absorption and digestion is greatly enhanced compared to the long-chain triglycerides naturally found in sow’s milk (absorbed as fast as glucose)
* can be absorbed without being broken down in the intestinal tract; therefore, they can be absorbed by nonfunctioning intestines
* can not be reesterified (stored) to a great extent; consequently, MCT have to be metabolized and will yield energy to the pig upon administration
* have a high energy density (2 * the density of lactose or glucose, 7 * the density of colostrum)
* can be stored at room temperature in a non-sterile environment
* do not suppress colostrum consumption (Benevenga et al. 1989)

Several studies have been published which look at survival rates of pigs after supplementation with MCT, but in general, no significant improvements were observed (Steinman 1986, Lepine et al. 1989). These experiments were executed at farms with already low mortality rates (as low as 5%), and the doses of MCT given were far beyond what the pig can handle physiologically and pharmacologically, which might have caused gastric distress and possibly led to intoxication (see Chapter 1). This makes interpretation of results to determine if MCT should be used at commercial farms for decreasing mortality difficult. It, however, indicates that our current level of knowledge is insufficient to recommend the use of MCT for commercial application in the swine industry.

**Carnitine**

Birth creates major changes in environment and nutrition, and thus, in the functioning of animals and humans. The fetal pig is supplied with nutrients by an intravenous infusion of a near optimum nutrient solution with carbohydrates as the major energy source. The neonatal pig is dependent
on intake and digestion of milk with fat as the main energy source (up to 70%).

Dietary fatty acids as well as fatty acids derived from lipolysis are presented to cells in the form of lipoproteins. Lipoproteins, however, can not enter the cells; fatty acids are released from triglycerides and cholesterol-esters which are imbedded in their surface by the action of lipoprotein lipases. The resulting free fatty acids are either taken up by the cell directly (via passive diffusion) or bind to albumin, which can transport the fatty acids to other tissues (Carey et al. 1983).

Inside the cell, fatty acids can bind to fatty acid-binding proteins, which probably channel the fatty acids to the mitochondria and peroxisomes. In several organelles, fatty acids can be activated to their respective acyl-CoA esters by acyl-CoA synthetases. The product is an activated fatty acid, which can be utilized for oxidation (or chain-elongation). However, activation by CoA changes the properties of the fatty acids, eliminating their capability to passively diffuse through the inner mitochondrial membrane (Bremer 1983).

To get these acyl-CoA esters inside the mitochondrion, the cell uses carnitine (β-hydroxy-γ-trimethylammonium butyrate, Broquist & Borum 1982). Via the action of carnitine acyl-transferase (see Fig. 4), the CoA group on the fatty acid is replaced by carnitine, creating an acyl-carnitine ester for which a specific carrier protein is present in mitochondria: carnitine translocase. Inside the mitochondrion, carnitine is replaced again by CoA recreating the active fatty acid which can be oxidized (Pande et al. 1990).

Fig. 4. Schematic representation of the transport of acyl-groups over the inner mitochondrial membrane (Pande et al. 1990, CPT is carnitine palmitoyl transferase).
Carnitine translocase is a poorly understood carrier mechanism. It promotes a 1-to-1 exchange of acyl-carnitine and carnitine; but it also allows a slow, unidirectional transport of carnitine (Bremer 1983).

Several forms exist for the carnitine acyltransferase enzyme which are present in different organelles, suggesting different roles in each organelle. The different forms of the carnitine acyl-transferase are based on chain-length specificity of the enzyme. In the mitochondria, two forms are present: carnitine acetyl-transferase (monomer) with a chain-length optimum for C2-C3 and carnitine palmitoyl-transferase (tetramer) with a chain-length optimum for C10-C16 (Bremer 1990). The carnitine palmitoyl-transferase is present in a form which can be inhibited by malonyl-CoA (present in the outer mitochondrial membrane) and in a malonyl-CoA insensitive form (present inside the mitochondrial matrix; both are distinctive proteins, Murthy & Pande 1990). The acetyl- and palmitoyl-transferase are involved in the transport of acyl-groups in and out of the mitochondrion. In peroxisomes, in addition to carnitine acetyl-transferase, a third form is recognized, carnitine octanoyl-transferase (monomer, which is often called carnitine palmitoyltransferase due to the similar chain-length specificity) with an optimum activity towards C6-C10. The role of this acyl-transferase is most likely the export of products from peroxisomal β-oxidation (see Appendix A). Recently, a new form has been recognized in endoplasmic reticulum, but data on its function and chain-length specificity are lacking (Bremer 1990).

Less well-studied roles of carnitine involving similar transport mechanisms are the following:

* Carnitine can aid in the transport of acetyl groups out of the mitochondria to make them available for fatty acid synthesis. The mechanism of this transport is a reversal of the mechanism for fatty acid transport into the mitochondria (Pearson & Tubbs 1967).

* Carnitine can protect the cell against accumulation of acyl-CoA compounds of either endogenous or exogenous origin by trapping such acyl-groups as carnitine esters (no experimental data has been presented to proof this point). The carnitine esters formed may then be transported to the liver for catabolism or to the kidney for excretion in the urine (Stumpf et al. 1985). (Carnitine acetyl-transferase acts most likely in this fashion, since it is only present in the mitochondrial matrix.) Acyl-carnitines can also be broken down to free carnitine and a fatty acid by the action of acyl-carnitine hydrolase, an enzyme situated in the endoplasmic reticulum with a chain-length optimum of C10 and an activity range from C6 to C22 fatty
acids. The function of this enzyme is most likely to prevent accumulation of excess long-chain acyl-carnitines and thus acyl-CoA (Bremer 1983).

* Carnitine esters can form a rapidly available energy supply, which has been shown to be true in e.g. spermatozoa, which have extremely high levels of acetyl-carnitine (Bremer 1990).

* Carnitine might play a role in the synthesis of CoA from pantothenic acid. The rate-limiting enzyme pantothenate kinase is strongly inhibited by CoA and acyl-CoA. This inhibition can be reversed by carnitine (not by acyl-carnitines, Thurston & Hauhart 1992).

The need for carnitine in higher animals has to be satisfied by intake of carnitine (precursors) with food (carnitine is actively absorbed from the intestinal tract) or by de novo synthesis. In general, carnitine and its precursors are low in feedstuffs of plant origin and high in feedstuffs of animal origin (Robles-Valdes et al. 1976).

The synthesis of carnitine is dependent upon the availability of trimethyllysine. Trimethyllysine can be derived from hydrolysis of certain (select) proteins containing trimethyllysine in a peptide linkage. This trimethyllysine is formed via methylation of lysine, which is one of the posttranslational modification reactions that protect polypeptides from degradation (Bremer 1983). Fasting increases carnitine levels because it promotes the breakdown of body protein which contains trimethyllysine (Robles-Valdes et al. 1976).

All tissues are able to convert trimethyllysine to γ-butyrobetaine (see Fig. 5), but only the liver and testes (and in some species the kidney and/or brain) are able to convert γ-butyrobetaine to carnitine (Rebouche & Engel 1980). The concentration of carnitine in these tissues is rather low (400-500 μM in infants, Shenai et al. 1984). However, other tissues such as muscle have very high concentrations of carnitine (3600 μM, Sperl et al. 1992). Siliprandi et al. (1990) suggest that in those tissues an active transport mechanism exists (carnitine/γ-butyrobetaine antiport). This "antiport" is a one-to-one exchange port between carnitine and γ-butyrobetaine, thought to be energy- and sodium-dependent.
Mammals are unable to catabolize carnitine; however, micro-organism in the intestines are able to convert carnitine to trimethylamine (main end product), trimethylamine-oxide, butyrobetaine and crotonobetaine. The extent of this pathway is not known but is generally considered minor. A mammalian system for carnitine degradation has been reported which involves the enzyme carnitine decarboxylase. The data on this enzyme are, however, contradictory, and possibly confounded by microbial action on carnitine (Bremer 1983).

Carnitine in the fetal and the newborn pig

Carnitine concentrations in the fetal pig decline in plasma after the 90th day of gestation (from 32 μM to 7 μM). The concentration in muscle (which is the main storage site for carnitine) increases from day 60 to parturition (from 1.4 μmol/g non-collagen protein to 7.5 μmol/g non-collagen...

At birth, the plasma carnitine concentration is low (6-8 µM) and is considered limiting for the oxidation of palmitic acid and thus for the energy supply of the neonatal pig (Garber & Froseth 1984). Other factors which might limit β-oxidation are the low number of mitochondria (Bischoff et al. 1969) and a lower capacity of the mitochondria for β-oxidation (approximately 50%, Bieber et al. 1973). Although carnitine levels are low at birth, the newborn infant excretes carnitine before the onset of feeding, mainly as acetyl-carnitine (Novak et al. 1979).

Colostrum contains large amounts of carnitine (esters), mainly acetyl- and isovaleryl-carnitine (total carnitine content is 440 nmol/ml, acetyl-carnitine and isovaryl-carnitine make up 45% each), providing the piglet with ample carnitine to resupply his tissue stores. Within 2 days after parturition, the level of carnitine in the milk decreases considerably (Kerner et al. 1984). Carnitine levels exceeding the kidney threshold (50-60 µM, Engel et al. 1981) result in the excretion of (preferably acyl-)carnitine by the kidney (Gross & Henderson 1984, Olson & Rebouche 1989). Novak et al. (1979) suggested that the majority of the excreted carnitine is in the form of acetate esters. Only in adults does the urine contain significant amounts of free carnitine.

The concentrations of carnitine in the pig (Kerner et al. 1984) are different from the concentrations in the rat as determined by Robles-Valdes et al. (1976). For example, pig liver was low in carnitine at day 0 (95 µM) increasing to day 2 (296 µM); while in the rat liver, the concentration declined from day 1 to day 3 from 570 to 400 µM, which was still higher than in the piglet. This raises questions such as whether the rat has a higher need for carnitine, of if the β-oxidation is more important in neonatal rats.

**Research focus: the role of carnitine in MCFA oxidation in vivo**

"The critical need for carnitine coupled with reduced stores of carnitine and reduced biosynthesis capability in the neonate compared with the adult have caused nutritionist to consider the possibility that carnitine may be an essential nutrient for the human neonate" (Baltzell et al. 1987).

The apparent carnitine deficit found in newborn pigs may limit their capacity to oxidize fatty acids during the first days of life because the fatty acids can not be transported into the site of
oxidation. Therefore, they may not be able to optimally contribute to the energy supply of the pig. Indeed, experiments have shown that addition of carnitine can enhance oxidation of long-chain fatty acids in newborn pig-liver cells (Wolfe et al. 1978).

Medium-chain triglycerides, after breakdown to medium-chain fatty acids (MCFA), do not require carnitine for their oxidation. In contrast to long-chain fatty acids, MCFA can enter liver mitochondria by passive diffusion where they can be activated (Groot & Hulsmann 1973, see Chapter 4). Although the dogma states that MCFA can be oxidized in the absence of carnitine, two research groups have suggested recently that carnitine still plays a role in the oxidation of MCFA (Rebouche et al. 1990, Rössle et al. 1990). The data presented by these two groups is, however, not conclusive; and it has not been determined whether carnitine supplementation to a carnitine deficient subject can improve the utilization of MCT. This possible involvement of carnitine in medium-chain fatty acid metabolism has become the focus of this thesis.
Chapter 1. Toxicity of medium-chain fatty acids and triglycerides

1.1 Introduction

In addition to the beneficial effects of MCT as mentioned in the introduction, MCT have been found to be toxic. This toxicity has clinical implications since several disease have been identified in which endogenously produced fatty acids accumulate whose concentration are a determining factor in the outcome of the disease. Shaw (1985) determined that when plasma free fatty acid concentrations exceeded .85 mM the chance of surviving became less than 35 %, while when plasma free fatty acid level remained less than .85 mM the chance of surviving was greater than 70 % in children affected by Reye syndrome.

Toxic concentrations of MCFA can be reached in the pig with oral MCT doses which are at a level considered practical as an energy supplement. Several experiments in our lab have illustrated this. For example, in a preliminary trial for the experiments as described in Chapter 4, three (out of four) pigs died; and the fourth pig seemed to be in an unresponsive coma. Since the infusion rates used were higher than the infusion rates in other trials, combined with the symptoms seen, we had to conclude in retrospect that these deaths were most likely caused by MCFA intoxication. The oral feeding studies as performed by T.M. Wieland and Xi Lin confirmed this notion when emulsifying MCT led to intestinal uptake rates resulting in plasma levels which caused lethargy and coma, and in some cases death.

The mechanism for this toxicity has not yet been determined, but it might explain in part why researchers were unable to see beneficial effects of MCT on survival in piglets. In this chapter, several mechanisms through which medium-chain fatty acids may be toxic will be discussed along with the possible role carnitine has in this toxicity.

1.2 Accumulation of fatty acids

Fatty acids (NEFA, FFA) are normally present in the body and are utilized as a fuel and as a structural component, mainly of biological membranes. In a healthy subject, the concentration of fatty acids is determined by dietary uptake, lipogenesis, and lipolysis, increasing fatty acid levels, and by reesterification and oxidation decreasing fatty acid levels. Lipolysis, reesterification, and oxidation are regulated to maintain a non-toxic fatty acid level (Murray et al.1988). Several
diseases, however, can lead to an accumulation of fatty acids resulting in a high, possibly toxic level of fatty acids. Two of these diseases are Reye syndrome and MCAD deficiency (Medium-chain Acyl-CoA Dehydrogenase deficiency).

Infants appear to be especially susceptible to these diseases (Roe & Coates 1989). Several reasons might explain this, but none are accepted in the literature. One possibility is that the carnitine biosynthesis of infants is hampered. Carnitine can be synthesized de novo; but it is questionable whether newborns and infants can synthesize carnitine at a rate sufficient to compensate for growth, urinary losses, and low body stores if intake (via animal products) is low (Sachan, personal communication). The carnitine stores are even lower when ß-oxidation is hampered; toxic acyl compounds can be excreted in urine as carnitine esters, relieving toxicity from the system, but causing carnitine deficiency (Roe & Coates 1989, Hamosh et al. 1991).

A second reason might be the elevated plasma fatty acid levels of infants. This is poorly illustrated in literature; but data from Rogiers (1981, Fig. 1.1), which does not include the age category at risk, suggests that children indeed have higher levels of free fatty acids than adults (she mentioned that infants had even higher levels of free fatty acids). If it is valid to extrapolate this graph to the age group at risk (6 to 24 months), then this data might explain some of the increased suscepti-

**Fig. 1.1.** Free fatty acids concentration in children and young adults (average per age group). Plasma samples were obtained from 600 students of varying age and free fatty acid levels were determined (Rogiers 1981).
bility of infants for fatty acid toxicity. High levels of fatty acids leave a smaller margin between actual levels of fatty acids and toxic levels of fatty acids; in other words, a small derailment of β-oxidation causes fatty acids to accumulate to toxic levels.

A third factor may be the metabolic rate of a small subject versus a tall subject, e.g. a child versus an adult. The metabolic rate of a child will be higher per kg of body weight than the metabolic rate of an adult; therefore, the amount of fuel needed to supply the body with energy will be higher for the child per kg of body weight (van Es et al. 1983). This means that infants, in which the (greater) flux of fuel to energy becomes interrupted/blocked, this fuel can reach to a toxic concentration in a much shorter time than an adult can since toxicity is most likely related to a concentration of fatty acids.

A fourth (speculative) factor might be the distribution of energy in infants versus adults. Due to hormonal profiles, infants are predispositioned to grow/spare protein (gluconeogenic substrate), while adults are much more prone to use protein for their energy supply (Verstegen, personal communication). This means that in a negative energy balance infants will rely more on lipids than adults will (lipids contribute a larger proportion to the total energy supply). Therefore, the flux of lipids in infants is higher, which in the case of a blockage of β-oxidation will lead to a more rapid accumulation.

1.3 MCAD deficiency

Medium-chain acyl-CoA dehydrogenase deficiency is a recently discovered disease (Gregersen et al. 1977, Stanley et al. 1983) and is caused (most commonly) by an autosomal recessive inherited disorder which leads to the inability to produce sufficient active medium-chain acyl-CoA dehydrogenase, one of the enzymes of the mitochondrial β-oxidation cycle. The symptoms observed in MCAD deficiency are similar to those observed in SIDS (Sudden Infant Death Syndrome) which explains the late discovery of MCAD deficiency. MCAD deficiency is highly lethal; the mortality rate is 59% during the first episode (at less than 2 years of age, Rinaldo et al. 1989).

MCAD patients are still frequently miss-diagnosed with SIDS (Rinaldo et al. 1989), which is a major cause of infant mortality (% of infant mortality in Belgium, Rebuffat et al. 1991). Postmortem analysis is usually not performed (often the child dies at home, and when it arrives in the hospital, it is in an advanced stage of rigor mortis; and there is "no need for such an analysis
since it is SIDS"). Several studies, however, have been performed to determine the real cause of death in SIDS cases; and MCAD deficiency is probably the main genetic defect leading to SIDS (Rinaldo et al. 1989, Harpey et al. 1990). Estimates for the total contribution of MCAD deficiency to SIDS mortality range from 5 to 10 percent (Roe & Coates 1989, Harpey et al. 1990). These estimates are based on a subgroup of SIDS patients with fatty infiltration in liver since it is assumed that only these infants suffered from a lipid oxidation disorder. It is questionable whether there is a causal link between MCAD deficiency and lipid infiltration of the liver since non-fasting fatty acid oxidation in minimally affected in MCAD deficiency. If this link is not present, then these figures could underestimate the occurrence of MCAD deficiency.

Methods for diagnosing MCAD deficiency include urinary acyl-carnitine and acylglycine-conjugate profiles. A carnitine loading assay can be performed to stimulate accumulation of medium-chain carnitine esters in urine. The carnitine profile of an MCAD deficient patient will show accumulation of medium-chain carnitine esters, with approximately 60% of the carnitine present as octanoyl-carnitine (Roe et al. 1985, Roe et al. 1986, Rinaldo et al. 1989). Acyl-glycines (suberyl- and hexanoyl-glycine) can be formed inside the mitochondria of a subject affected by MCAD deficiency (involves glycine-N-acylase system, Treem et al. 1986). Other diagnostic methods which impose an unacceptable high risk on the patient are prolonged fasting (Gregersen et al. 1977) and the use of a ketogenic diet (Divry et al. 1983). Both stimulate β-oxidation to induce symptoms of MCAD deficiency (prolonged fasting does not always trigger MCAD deficiency symptoms).

The most explicit diagnostic tool is the determination of MCAD activity in, for example, cultured fibroblasts or from liver cells (more invasive). Table 1.1 (Duran et al. 1986) shows results from a family in which B (an infant) died from MCAD deficiency. Other family members were submitted to a 17 h fast, and a fibroblast sample was taken. A and K (children) and the father appear to have lowered octanoate (C8) oxidation capacity. This table clearly shows that oxidation of medium-chain fatty acids is decreased in affected subjects compared to controls.

Subjective observations which can be made in MCAD deficient patients are a sweaty foot odor or acrid, fatty smell on breath or skin (Harpey et al. 1987).
Table 1.1. In vitro oxidation of octanoate (C8) to CO₂ (nmol CO₂/10⁶ cells/h) in a family in which B died of MCAD deficiency. Fibroblast samples were obtained after a 17 h fast from family members of B and from controls (2-4 years of age, after a 24 h fast). B underwent a postmortem liver biopsy. Succinate and butyryl-CoA (C4-CoA) are used as a non-MCAD dependent control substrate (Duran et al. 1986).

<table>
<thead>
<tr>
<th>Family member</th>
<th>Fibroblasts</th>
<th></th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C8</td>
<td>Succinate</td>
<td>C8-CoA</td>
</tr>
<tr>
<td>B</td>
<td>0.40</td>
<td>2.79</td>
<td>471</td>
</tr>
<tr>
<td>A</td>
<td>0.29</td>
<td>2.52</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>0.44</td>
<td>3.05</td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>1.45</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>1.97</td>
<td>3.57</td>
<td>3190</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A typical case of MCAD deficiency can be described as follows. The infant will refuse to eat, often due to symptoms associated with a viral infection or immunizations (Harpey et al. 1990). After 24-40 h of fasting (vomiting and/or diarrhea will decrease this time period since this empties the gastrointestinal tract, leading to a decreased energy influx from the gut), the infant will fall into a "deep sleep" (unresponsive, low muscle tone) followed by coma, cerebral edema, cardiac failure, and/or respiratory arrest (Duran et al. 1986). A postmortem analysis will often reveal a cerebral edema and lipid infiltration of the liver (Roe et al. 1985). Affected plasma parameters are glucose (hypoglycemia, due to the lack of fuel some tissue will keep using glucose, Bartlett et al. 1991, Treem et al. 1986), ammonia (hyperammonemia, high levels of fatty acids will inhibit the urea cycle, Trauner et al. 1988, Treem et al. 1986), ketone bodies (hypoketonemia, since no excess acetyl-CoA is available, Bartlett et al. 1991) and, of course, increased levels of fatty acids (see table 1.2, Duran et al. 1986, Stanley et al. 1983).
Table 1.2. Free fatty acid and ketone body concentrations (μmol/l) of a family in which B was hospitalized for MCAD deficiency (same family as in table 1.1). Family members were submitted to a 17 h fast. Control children (2-4 years of age) were submitted to a 24 h fast (nd=non-detectable).

<table>
<thead>
<tr>
<th>Family member</th>
<th>C8</th>
<th>C10</th>
<th>C16</th>
<th>β-HBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>B (dying)</td>
<td>208</td>
<td>90</td>
<td>1082</td>
<td>1639</td>
</tr>
<tr>
<td>K (fasting)</td>
<td>139</td>
<td>24</td>
<td>483</td>
<td>1134</td>
</tr>
<tr>
<td>A (fasting)</td>
<td>115</td>
<td>12</td>
<td>304</td>
<td>620</td>
</tr>
<tr>
<td>Father</td>
<td>76</td>
<td>trace</td>
<td>246</td>
<td>139</td>
</tr>
<tr>
<td>Mother</td>
<td>nd</td>
<td>nd</td>
<td>183</td>
<td>150</td>
</tr>
<tr>
<td>Control (2-4 y old)</td>
<td>nd</td>
<td>nd</td>
<td>342</td>
<td>2855</td>
</tr>
</tbody>
</table>

The cause of MCAD deficiency is the lack of sufficient activity of the MCAD enzyme. If fatty acid mobilization is stimulated, then this will lead to accumulation of fatty acids via the following mechanism (hypothetical): fatty acids (long-chain) are released from triglycerides because of the stimulated action of lipases. These are stimulated first by fasting (low insulin/glucagon ratio) and second by the presence of a viral infection (at least present in 60% of the cases, Treem et al. 1986). This will lead to an excess of fuel entering the cell. Inside the cell, a pool of fatty acids is present which can enter two routes of oxidation: peroxisomal (minor route) and mitochondrial oxidation.

The peroxisomal β-oxidation (see Appendix A) is an incomplete and modified version of the β-oxidation in mitochondria. To enter peroxisomes, fatty acids are supposedly activated to their acyl-CoA ester upon entrance into the peroxisomes. These acyl-CoA are chain-shortened, and acetyl-CoA is produced. The affinity of the long chain acyl-CoA dehydrogenase enzyme for the acyl-CoA decreases with decreasing chain length. At a chain length between C6 and C10 (mainly C8), the fatty acid will exit the β-oxidation and will be exported out of the peroxisome, preferably as a carnitine ester (Vamecq & Draye 1989). Acetyl-CoA will either be utilized in the peroxisome for e.g. cholesterol synthesis, the cytosol, or will be transported into the mitochondria (if carnitine
Peroxisomes, and thus peroxisomal β-oxidation, can be stimulated by high levels of fatty acids (Cannon et al. 1982) and also by aspirin (Vamecq & Draye 1989, see also Reye syndrome), which used to be a common treatment for viral infections in infants.

The second route of fatty acid oxidation is via the mitochondrial β-oxidation. Long-chain fatty acids need to be activated (which can happen in the outer mitochondrial membrane, cytosol, and in peroxisomes). These acyl-CoA compounds are then converted to acyl-carnitine in the outer mitochondrial membrane (Pande et al. 1990); and this acyl-carnitine complex can be translocated into the mitochondrial matrix, where the carnitine is replaced with a CoA. The resulting acyl-CoA is then available for β-oxidation (Bremer 1983 and Fig. 1.2).

The β-oxidation system consists of 4 enzymes from which several consists of subclasses with their own chain-length specificity. The following subclasses are recognized for the acyl-CoA dehydrogenases: long-chain acyl-CoA dehydrogenase (LCAD), with an optimum activity towards C14-C20, medium-chain acyl-CoA dehydrogenase (MCAD), with an optimum activity towards C6-C12 and short-chain acyl-CoA dehydrogenase (SCAD), with an optimum activity towards C4 and C6 (Harpey et al. 1990).

If MCAD activity is lacking or low and fatty acid oxidation is stimulated (high influx of fatty acids into the cell), then long-chain fatty acids will undergo β-oxidation, but medium-chain acyl-CoA will start accumulating in the mitochondria. These acyl-compounds are, however, bound to CoA. Since CoA is present inside the mitochondrion in limited quantities (which respond only slowly to changing circumstances such as fasting, in which intramitochondrial CoA concentration doubles), the accumulation of CoA in medium-chain acyl-CoA esters can deplete a large portion of the CoA, which will lead to a deficiency of CoA in the mitochondria. Excess amounts of acyl-CoA compared to free CoA will inhibited mitochondrial function, since free CoA is required for the TCA cycle and β-oxidation and the ratio of free CoA to acyl-CoA greatly affects the urea cycle. The accumulation of acyl-CoA can, therefore, shut down mitochondrial activity. Carnitine might play a role in this toxicity by exporting acyl-groups from the mitochondrion into the cytosol as a carnitine esters. This will free up CoA but at a cost to carnitine.
The activity of the peroxisomal β-oxidation is (hypothetical, no proof to support this) not hampered during this scheme of CoA depletion since peroxisomes have their own CoA pool and the exportation of end product is not carnitine dependent, but preferably carnitine conjugated (Vamecq 1989). This will lead to a further accumulation of acyl-carnitine in the cytosol, as well as to an accumulation of medium-chain fatty acids (which can explain the high levels of octanoate in plasma and octanoyl-carnitine in urine).

Acyl-carnitines are very water soluble and upon accumulation, they can be cleared by the kidney which preferably excretes acyl-carnitines (≥ C2-carnitine). This excretion can lead to a carnitine deficiency as illustrated in table 1.3, further impairing oxidation capacity (since mitochondrial β-oxidation of long-chain fatty acids is carnitine dependent). It also hampers the only mechanism the body has to relieve CoA from (toxic) acyl-CoA compounds (without oxidizing the acyl-compound, Coates et al. 1985). If CoA is limited, then oxidation (β-oxidation as well as the TCA cycle) will be severely hampered since intermediates of both pathways have to be conjugated to CoA (Stumpf et al. 1985, Harpey et al. 1990). Ultimately, the continuing influx of fatty acids and the lack of oxidation of these fatty acids will lead to accumulation of fatty acids and acyl-CoA to levels which might be toxic.
Table 1.3. Plasma carnitine status (μmol/l) in four patients with identified MCAD deficiency and one patient with an expected MCAD deficiency (DCA=dicarboxylic aciduria). The ratio of free to acyl-carnitine, as well as absolute levels, are indicators for the buffering capacity of carnitine for the CoA pool.

<table>
<thead>
<tr>
<th>Patient</th>
<th>free carnitine</th>
<th>acyl-carnitine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: MCAD</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>2: MCAD</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>7: MCAD</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>10: MCAD</td>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td>8: DCA</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>Control</td>
<td>26-60</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

1.4 Reye syndrome

Reye syndrome is, just as MCAD deficiency, often confused with SIDS, suggesting that symptoms (and mechanisms) are similar. Reye syndrome is fairly lethal: 40% of the infants/children affected die (Ansevin 1980). Reye syndrome is characterized by liver damage, swollen mitochondria and fragmented cristae. Differences in symptoms with MCAD deficiency are that no acyl-glycine conjugates are found in urine (since acyl compounds do not accumulate inside but rather outside the mitochondria?) and that the urine will contain significant amounts of acetyl-carnitine (and relatively low amounts of octanoyl-carnitine). Also ketogenesis is less inhibited (Treem et al. 1986).

The difference between MCAD deficiency and Reye syndrome is the underlying cause. In MCAD deficiency an enzyme deficiency causes accumulation of fatty acids; in Reye syndrome, the mechanism is not clear (Pranzatelli & De Vivo 1987).

Two prime factors are almost always identified in Reye syndrome: a viral infection and the use of medication to cure this, like aspirin (USA) and phrotozombin (Europe, treatment of nasopharyngitis, Shaw 1985).
Table 1.4. Effect of viral infections and aspirin on the oxidation rate of palmitate (μmol CO₂/g protein/h). Mice were infected with an influenza virus and/or fed salicylic acid, with or without carnitine (100 mg/kg BW of D,L-carnitine). 36 h post infection/feeding initiation, mitochondrial palmitate oxidation capacity was determined (Trauner et al. 1988).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oxidation rate</th>
<th>Standard deviation</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.908</td>
<td>0.395</td>
<td></td>
</tr>
<tr>
<td>virus</td>
<td>0.374</td>
<td>0.195</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>control+carnitine</td>
<td>1.420</td>
<td>0.980</td>
<td>ns</td>
</tr>
<tr>
<td>virus+carnitine</td>
<td>1.400</td>
<td>1.010</td>
<td>ns</td>
</tr>
<tr>
<td>salicylic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/kg/d</td>
<td>1.030</td>
<td>0.410</td>
<td>ns</td>
</tr>
<tr>
<td>100 mg/kg/d</td>
<td>0.309</td>
<td>0.090</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>500 mg/kg/d</td>
<td>0.378</td>
<td>0.150</td>
<td>p&lt;0.02</td>
</tr>
<tr>
<td>1 mg/kg/d+ivirus</td>
<td>0.521</td>
<td>0.210</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

The mechanism causing toxicity is suggested by Trauner (1988) to be the destructive effect of both aspirin and virus on the structural integrity of the mitochondrial matrix. This is, however, easily refuted from Trauners’ own data (see table 1.4). Trauner infected mice with an influenza virus and/or fed them aspirin. After 36 h, mice were killed; and mitochondria were isolated to measure the oxidation rate of radiolabelled palmitate in vitro. The viral infection and aspirin both had negative effects on the oxidation capacity and on structural integrity of the mitochondria. The addition of carnitine, however, completely prevented any damage to the mitochondria; and the oxidation rate of palmitate in carnitine fed animals was not decreased. No mechanism is known to exist which suggests that carnitine has an effect on the ability of a virus to infect a cell. Although some treatments are missing (e.g. carnitine plus aspirin with and without virus) and not all treatments are consistent (virus dose in the aspirin fed animals was lower than in the animals which received only virus), these data (table 1.4) suggest that the presence of the virus and/or salicylic acid caused a secondary carnitine deficiency. Other researchers were not able to identify a serum factor responsible
for the mitochondrial damage other than fatty acids (Pranzatelli & De Vivo 1987).

Several factors may contribute to Reye syndrome. Aspirin is a peroxisomal proliferator (Vamecq & Draye 1989), increasing peroxisomal β-oxidation capacity (Corkey et al. 1988) showed higher numbers of peroxisomes in affected infants). Second, aspirin has a very high binding affinity for the fatty acid binding sites on albumin (the main transport protein of fatty acids in plasma). A normal dose of aspirin (supposedly 100 mg/kg body weight) is sufficient to "cover" all fatty acid binding sites on albumin in an infant (Shaw 1985). This will increase the active fatty acid concentration which will stimulate peroxisomal proliferation even more (Cannon et al. 1982). The viral infection increases this problem by enhancing lipolysis (Pranzatelli & De Vivo 1987). Ansevin (1980) showed that added BSA (bovine serum albumin) could completely reverse all symptoms of Reye syndrome as observed on the mitochondria. A recent paper from Deschamps (1991) suggests a third role for aspirin. Salicylic acid can be activated in mitochondria to a CoA ester, thereby depleting free CoA as well as carnitine, which is in agreement with the theory that free CoA depletion might be the underlying reason for medium-chain fatty acid toxicity.

I hypothesize that the excess of free fatty acids leads to an overstimulation of peroxisomal β-oxidation while mitochondrial oxidation "lags behind". The mitochondria are not able to handle the large amount of peroxisomal products (possibly due to salicilyl-CoA intoxication or a "sub-clinical β-oxidation disorder") which will lead to an accumulation of acyl-CoA and carnitine intermediates (acetyl- and octanoyl-carnitine, mainly acetyl-carnitine: Corkey et al. 1988) in the cytosol. These carnitine esters are excreted into the plasma and cleared by the kidney, which will lead to a secondary carnitine deficiency (carnitine deficiency is shown by Treem et al. 1986, Stumpf et al. 1985, data similar to data presented in Table 1.3), which in a catabolic state (relying primarily on fatty acids for fuel), can become detrimental very rapidly. Carnitine stores in the body are limited and can be depleted in a short time (Stumpf et al. 1985). Peroxisomal oxidation is probably less affected by the carnitine deficiency, leading to accumulation of significant amounts of acetyl- and medium-chain acyl-intermediates (extrapolated from Rabinowitz et al. 1978).

Both liver and especially brain are low in carnitine (compared with other tissues and plasma) (Borum, personal communications), and both brain and liver are identified as the tissues with most extensive mitochondrial damage. Whether a causal relationship exists has not been investigated.

Mitochondria are damaged by the high levels of fatty acids, maybe since the fuel transport
in the cells is channelled towards the mitochondria which causes a higher "active concentration" of fatty acids around the mitochondria (or maybe due to the different composition of the mitochondrial membrane). Other organelles will be damaged if severity increases, which will lead to cell necrosis (and e.g. destruction of myelin sheets, Pranzatelli & De Vivo 1987).

1.5 Dietary intake leading to toxicity

A second mechanism by which fatty acids can reach toxic levels is by dietary intake. The only group of fatty acids which have been shown to lead to such massive absorption rates from the gastro-intestinal tract are the water-soluble medium-chain fatty acid (see introduction). The uptake of MCT (Medium-Chain Triglycerides) from the GI tract is more efficient than the uptake of LCT (Long-Chain Triglycerides) due to enhanced digestion and absorption of the MCT (with the maximum efficiency for tricaprin, tri-C6). Lipases have the highest affinity for MCT and will digest MCT preferably and also at higher rates. MCFA (medium-chain fatty acids) can be taken up through the gastric mucosa, leading to an increased absorption area (Hamosh et al. 1991).

After uptake, MCFA can be transported directly (as MCFA or MCT) via the portal vein to the liver. This leads to a much greater influx from the gastro-intestinal tract, leading to a much faster accumulation of fatty acids in the cell (Hamosh et al. 1991).

MCFA are not reesterified in the liver and are not stored in adipose tissue to a great extent. This makes them obligatory fuels and when influx exceeds oxidation capacity, this will lead to accumulation.

MCFA oxidation is not regulated in the cell by the activity of carnitine palmitoyl transferase (major regulator of long-chain fatty acid oxidation via binding of malonyl-CoA, Bieber 1988), since MCFA can diffuse freely through the mitochondrial inner membrane. To what extent carnitine is involved in the oxidation of MCFA is a point of discussion. Activation of fatty acids in the muscle occurs in the cytosol, and activated MCFA can not enter the mitochondria without being converted to acyl-carnitines (Aas 1971). In liver, some of the MCFA will be activated before entering the matrix, and these acyl-CoA are dependent upon carnitine for their oxidation (Bieber 1988).

Questionable is if MCFA feeding can lead to carnitine deficiency, which will impair oxidation of long-chain fatty acids. The carnitine deficiency is, however, not essential for the toxicity, but
is a mechanism by which fatty acid oxidation can be impaired, leading to levels which are toxic.

In MCT feeding trials using pigs, levels of up to 15 mM in plasma were measured (which did not represent a steady state! Odie et al., unpublished data), and according to research from Dahl (1968) and Zieve et al. (1974) these levels exceed toxic levels. Indeed, animals died during these feeding trials.

1.6 Possible mechanisms for the toxic action of fatty acids

Weber (1966) determined the effect of high levels of fatty acids (C8, octanoate) on the activity of enzymes of the glycolysis and found that all unidirectional enzymes (not involved in gluconeogenesis) were inhibited up to 100% by high levels of fatty acids leading to a decreased flux of pyruvate into the TCA cycle. The effect of this inhibition in the case of an MCAD deficient patient is a further decrease in energy production and an impairment of the oxidation of acetyl-CoA. Inhibition of glycolysis is not a causal factor in fatty acid toxicity as presented in this paper, since inhibition of the glycolysis would lead to an impairment of the TCA cycle, which would lead to excessive production of ketone bodies.

The inhibition of enzymes shown by Weber might be attributable to acyl-CoA, but his work does not determine the causal factor. Acyl-CoA compounds are accepted as being toxic and can
inhibit several essential enzymes, for example the two enzymes of the urea cycle present in the mitochondria: ornithine transcarbamoylase and carbamoyl synthetase (Trauner et al. 1988).

ω-oxidation is an alternative pathway of fatty acid oxidation which becomes active if fatty acid levels increase. Enzymes involved are cytochrome P<sub>450</sub> (endoplasmic reticulum), alcohol dehydrogenase and aldehyde dehydrogenase (cytosol). The activity of this pathway is directly correlated to the concentration of fatty acids. The function of this pathway is unclear, and hypothesized is that it might either detoxify high levels of fatty acids (which is unlikely since its products are toxic) or that it might be a mean of converting fatty acids to TCA cycle intermediates (succinate, Mortensen 1980, Draye & Vamecq 1989). The second function is more likely, and fits better with existing evidence.

Table 1.5. Urinary dicarboxylic excretion (mmol/mol creatinine) of a MCAD patient (Severine) during experimental fasting and while healthy, compared to ketotic, MCT fed and fasted controls. No details were given on the composition of the MCT diet (nd=non-detectable).

<table>
<thead>
<tr>
<th>Case</th>
<th>Adipic; DC6</th>
<th>Suberic; DC8</th>
<th>Sebasic; DC10</th>
<th>5-OH-DC6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hypoglycemic</td>
<td>950</td>
<td>332</td>
<td>45</td>
<td>700</td>
</tr>
<tr>
<td>normoglycemic</td>
<td>7</td>
<td>17</td>
<td>8</td>
<td>nd</td>
</tr>
<tr>
<td>ketotic control</td>
<td>6-300</td>
<td>5-50</td>
<td>1-18</td>
<td></td>
</tr>
<tr>
<td>MCT fed control</td>
<td>122</td>
<td>149</td>
<td>973</td>
<td></td>
</tr>
<tr>
<td>MCT fed control</td>
<td>23</td>
<td>58</td>
<td>247</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>1-13</td>
<td>1-5</td>
<td>1-3</td>
<td></td>
</tr>
</tbody>
</table>

Dicarboxylic acids can undergo β-oxidation for several cycles. In peroxisomal β-oxidation, medium-chain dicarboxylic acids are formed, which will eventually be excreted into the urine (adipic, suberic and sebacic acid: C6, C8 and C10 dicarboxylic acid, leading to aciduria). Mitochondrial β-oxidation of dicarboxylic acids might lead to formation of succinate (Vamecq
& Draye 1989). Dicarboxylic acids are acidic, and accumulation of these acids will lead to acidosis. Presence of dicarboxylic acids in the urine is an indicator for β-oxidation disorders (see table 1.5, Mortensen 1986).

High levels of fatty acids or acyl-CoA as well as a decrease in the ATP/ADP ratio (Stumpf et al. 1985) will inhibit the urea-cycle (at the level of ornithine transcarbamoylase and carbamoyl synthetase) which will lead to accumulation of ammonia (Trauner et al. 1988). Ammonia is toxic by itself but does not cause the symptoms of fatty acid toxicity; Ansevin (1980) showed that NH₃ toxicity did not mimic the symptoms of Reye syndrome, while octanoate did. Ammonia will, however, interact with fatty acids in causing toxicity (see Fig. 1.4).

![Fig. 1.4. Interaction of ammonia and fatty acids in inducing coma in rats (created dataset). Rats were injected with octanoate and ammonia at different concentrations (I.P.). Upon induction of coma (*), blood was collected and analyzed for fatty acid and ammonia level. No coma state was achieved in animals marked O (Zieve et al. 1974).](image)

Fatty acids are detergents. A simple example of this is the use of SDS-PAGE to determine the size of a protein: the fatty acid-sulfate degenerates the protein, unfolding it to a linear strand and rendering the enzyme inactive. The detergent effect might also act on the cell membrane, leading to disruption and altered fluidity. This will lead to altered transport functions and binding functions of enzymes associated with the cell membrane (Shaw 1985, Schmidt & Lankisch 1978). The biological significance of the detergent effect in fatty acid intoxication is, however, not clear.

The chain length of the involved fatty acid is important in this aspect. Long-chain fatty acids are more effective detergents, but their solubility is lower. Medium-chain fatty acids are less potent detergents, but their solubility is up to 100 times higher (octanoate versus palmitate, Hamosh...
et al. 1991), which might make their overall toxicity more significant. No data is available (to my knowledge) which quantified the detergent effect of fatty acids in vivo.

In vivo detergent degeneration was observed during an infusion trial using C10. Lysis of red blood cells was observed, which seemed to be dependent on the infusion rate of the C10. In the most severe cases, "plasma" became dark red, and urine dark red to black, suggesting a severe lysis of red blood cells leading to hemoglobinuria. These animals died during the infusion. C8 infusions were carried out at levels which were 2 fold higher without visible lysis of red blood cells, suggesting that the chain length of the fatty acid is a major factor in this detergent effect. Since the in vivo toxicity symptoms with C10 were very different than with C8, it appears unlikely that a detergent effect is the cause of mortality when C8 is infused.

Kim et al. (1983) showed that octanoate can produce extensive ultrastructural and functional damage to mitochondria of the choroid plexus. This might suggest that the effects seen are mediated by detergent activity of fatty acids, since Trauner (1988) and others reported similar findings in Reye syndrome (Trauner, however, attributed the ultrastructural changes to the action of a virus). Another possible (hypothetical) mechanism of toxicity is overstimulation of protein kinase C (PKC). PKC levels are especially high in brain tissue (Sekiguchi et al. 1988) where it has a role in membrane conductance (Nishizuka 1989).

The brain does not normally oxidize fatty acids, and it achieves this most likely by active reesterification of fatty acids upon passage through the blood-brain barrier (Pardridge 1991). This reesterification might, however, be the fatal link in MCFA toxicity, since the esters of glycerol and MCFA are watersoluble, and diglycerides of C8 are the most potent stimulators of PKC in vitro (Conn et al. 1985). No experiments were done in which glycerol esters of MCFA were measured in brains (reticular formation) of infants affected by fatty acid toxicity.

Evidence for involvement of PKC is limited. Arguments to justify research in this area might be: especially tissue which is very dependent on ion-gradients are affected by fatty acid toxicity and carrier-mediated transport of some ions seems to be impaired by the fatty acid toxicity (Kim et al. 1983, transport of many factors is dependent on the Na\(^+\) gradient over the cell membrane). PKC overstimulation will lead to an inhibition of the Na\(^+\)/K\(^+\) ATPase activity (decreases ion-gradient, Blacksheard et al. 1988) and also stimulates (decrease of sensitivity to intracellular H\(^+\)) the Na\(^+\)/H\(^+\) exchange protein (increasing intracellular pH, Sekiguchi et al. 1988). Dahl (1968) demonstrated
that there was a high correlation between the concentration of fatty acids causing coma in 50% of the treated rats, and the concentration of fatty acids leading to a 50% inhibition of the Na\(^+\)/K\(^+\) ATPase in vitro, using different chain-length fatty acids as a substrate (Fig. 1.5). (NH\(_3\) interferes with the Na\(^+\)/K\(^+\) pump by substitution for K\(^+\), and will also deplete the TCA cycle from \(\alpha\)-keto-glutarate (Pranzatelli & De Vivo 1987), which will lead to a further impairment of the oxidation capacity.) Sodium-potassium-ATPase (as well as the Na\(^+\)/H\(^+\) exchange protein) is involved in regulating the ion-balance over cell membranes and plays an important role in functioning of neural cells. Dahl (1968) however reported that ouabain, an inhibitor of Na\(^+\)/K\(^+\) ATPase did not cause coma/death, suggesting that the effect of fatty acids on the Na\(^+\)/K\(^+\) ATPase were correlated with the causal factor, but not by itself the cause of the toxic effects.

In support of involvement of PCK is the data from McCandles (1985), who injected octanoate into mice at toxic levels, and looked at energy metabolism of neurons. He concluded that oxidative phosphorylation and mitochondria were not affected, however, the ATP concentration was lowered, and the electrical output of the cells was affected. Miles (1991) used EEGs to measure brain activity after injection of octanoate and concluded that there was a slowing and a reduction of the electric amplitudes. These findings suggest again that membrane potentials are affected by fatty acids through affecting PKC, however, changing membrane integrity might have similar effects on the concentration gradients of certain ions over the cell membrane, and therefore, this data does not prove a role for PKC.

Fig. 1.5. Concentration of fatty acids which induced coma in 50% of the animals compared to the concentration of fatty acids required (in vitro) to inhibit the Na\(^+\)/K\(^+\) ATPase activity to 50% of the control value for different chain-length fatty acids (Dahl 1968).
1.7 Future research directions

Of major importance is to elucidate the mechanism of toxicity in MCAD deficiency and Reye syndrome, as well as in feeding of MCT. MCT are being used more and more in hospital settings for both parenteral and enteral nutrition (imagine the effect of feeding MCT to an MCAD deficient person!). Feeding and infusion trials (Chapter 3 and 4) did not use excessive doses, but levels meeting basal energy requirements of the animals for approximately \( \frac{3}{4} \) to \( 1\frac{3}{4} \) day. MCAD-like symptoms resulting in mortality was the result!

Several other diseases, besides MCAD deficiency and Reye syndrome, in which fatty acid toxicity might be involved, exist, some with even a greater (estimated) incidence than MCAD deficiency. Understanding the underlying mechanism is a step towards finding a cure for a severely comatose patient arriving at intensive care, their last stop if we stay reluctant.
Chapter 2. Quantification of carnitine esters by High-Performance Liquid Chromatography

2.1 Introduction

The functions of carnitine make it an important compound in lipid metabolism, and studying the composition of the carnitine-ester pool can add valuable information to our understanding of metabolism. Indeed, metabolism of carnitine has been studied extensively with regard to its role in infant nutrition (Tibboel et al. 1990), ketogenesis (Bohles & Akcetin 1987, Brass & Beyerinck 1988), aciduria (Yount et al. 1989) and metabolic disorders involving oxidation of fatty acids, such as acyl-CoA dehydrogenase deficiencies (Harpey et al. 1987). The carnitine profile in tissues and plasma can function as an indicator of steps limiting the oxidation of fatty acids. Accumulated acyl-CoA esters are converted to carnitine esters, which can be excreted into the cytosol and can enter the plasma pool. Plasma carnitine esters are incompletely reabsorbed in the kidney, resulting in the excretion of these esters in urine (Olson & Rebourne 1989). Determination of the composition of the urinary or plasma carnitine esters can therefore provide information about the steps in oxidation which may be limiting.

Currently available methods to analyze the different carnitine esters are, however, limited. A commonly used assay is the radio-isotopic exchange assay (Cederblad & Lindstedt 1972). While this method is adequate for crude separations of free, acid-soluble (short- and medium-chain) and acid-insoluble (long-chain) acyl-carnitines, it is incapable of distinguishing specific carnitine esters that are of interest in metabolic studies.

Carnitine esters have been analyzed using radio-isotopic exchange, followed by HPLC separation and quantification with a β-flowmonitor (Schmidt-Sommerfeld et al. 1989). The general principle of this method involves the incubation of the sample with radiolabeled carnitine and carnitine acyltransferase, allowing the radio-active carnitine to exchange with the carnitine from the carnitine esters. The composition of the carnitine esters is determined by HPLC, coupled with an in-line β-flowmonitor which results in a radiochromatogram depicting the composition of the different

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1
esters. If the radiolabeled carnitine exchanged with all esters to a similar extent, this method would yield good results. Unfortunately, carnitine acetyltransferase (E.C. 2.3.1.7) which is commonly used for this assay, is not sufficiently active toward long-chain acyl-carnitines to obtain quantitative yields, nor do medium-chain acyl-carnitines and branched-chain acyl-carnitines yield quantitative information without inclusion of appropriate standards. Another confounding issue is the presence of an acyl-carnitine hydrolase associated with commercial preparations of carnitine-acetyl transferase, which results in the breakdown of acyl-carnitines (Di Lisa et al. 1992). Although this method is extremely sensitive, these complications make it of limited applicability.

Other methods to quantify acyl-carnitines have been reported which use HPLC separation followed by UV detection of derivatized acyl-carnitines (Minkler et al. 1990, Bhuiyan & Bartlett 1988). The derivatization procedure employs phenacyl reagents which react with the carboxyl group of carnitine, creating a UV-absorbing chromophoric group. Although these methods do not have the limitation imposed by the limited chain-length specificity of commercial acyl-carnitine acetyl transferase, the use of complex derivatization reagents, ternary gradients or atypical columns prompted us to develop a simplified method.

The objective of this paper was therefore to describe a method to analyze carnitine esters using commonly available equipment and chemicals.

2.2 Material and Methods

2.2.1 Chemicals

Biosafe II scintillation cocktail was obtained from Research Products International (Mount Prospect, IL). ¹⁴C-carnitine was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Methanol (HPLC grade) was purchased from Baxter Healthcare Corp. (Muskegon, MI). Phenacyl-8 was purchased from Pierce (Rockford, IL). Acetonitrile (HPLC grade) was from Fisher (Fairlane, NJ), and ninhydrin was obtained from Beckman (Palo Alto, CA). All other chemicals and the Kieselgel 60 were supplied by Sigma (St. Louis, MO). Vacutainers were obtained from Becton Dickenson and Company (Rutherford, NJ).
2.2.2 Equipment

A Beckman system Gold HPLC was used (Beckman, Palo Alto, CA). The system consisted of two 110B pumps, a model 406 analog interface and a model 166 variable wavelength UV detector. The system was equipped with a model 507 autoinjector with a 50 µl injection loop. The column used was an Ultrasphere C18-IP column (5 µm particle size, 25*0.46 cm, Beckman) with a cartridge system guard column (Absorbosphere HS C18, 5 µm particle size, Alltech, Deerfield, IL). To accommodate three eluents, pump A was equipped with a three-way switching valve (General Valve, Fairfield, NJ), connected with a 12 volt power supply (Radio Shack, Tandy Corp., Fort Worth, TX), controlled by a relay in the 406 module (using a second 3 volt power supply and a relay). The valve was positioned so as to switch solvents at the inlet line of the pump. From the same pump A, the pulse dampener (6 ml void volume) was removed to reduce the volume between the pump and the injector (final volume was approximately 4 ml).

To verify retention times of phenacyl carnitine-esters and to determine recovery of ¹⁴C-carnitine, a model 265 β-flowmonitor was used (Radiomatics, Tampa, FL). Fractions containing radioactivity were collected using a model 204 fraction collector (Gilson, Middleton, WI) and quantified using a model LS6000IC liquid scintillation counter (Beckman).

2.2.3 Isolation of carnitine esters from plasma samples

Carnitine esters (including free carnitine and γ-butyrobetaine) were isolated from plasma using a methanol extraction procedure modified after Minkler et al. (1990). The isolation and derivatization procedure is summarized in Fig. 2.1. This procedure can be used to isolate carnitine esters from liver as well (unpublished data).

The internal standard (hexanoyl-carnitine or ¹⁴C-carnitine) was added to a known amount of plasma (250 µl). The plasma was extracted with 5 volumes of 100% methanol and centrifuged at 30,000 g in a 2 ml microcentrifuge tube (non-siliconized). The supernatant was transferred into another vial, and 50 µl of ninhydrin was added. This mixture was evaporated to dryness using a model AS160 speedvac (Savant, Farmingdale, NY) allowing the ninhydrin to react with amino acids contained in the methanol extract. The methanol-soluble products were reconstituted in 1 ml 100% methanol, and recentrifuged if cloudy. The methanol fraction (supernatant) was then loaded onto a 1 ml silicagel column (Kieselgel 60) contained in a pasteur pipet, and washed 3...
times with a volume of methanol equal to the column volume. Carnitine esters were subsequently eluted using 1.25 ml of a mixture of methanol:water:acetic acid (MWA, 50:45:5 v/v/v) into a glass vial. The MWA fraction was then evaporated to dryness.

The efficiency of the isolation procedure was assessed by adding a known amount of $^{14}$C-carnitine to the plasma samples before methanol extraction. In subsequent steps of the isolation procedure "waste" was collected, and radio-activity in this waste was determined.

### 2.2.4 Derivatization procedure

Acyl-carnitines were derivatized using commercially available phenacyl-bromide which reacts with the carboxyl group of carnitine forming a phenacyl-ester (Durst et al. 1975). The dried MWA fraction was reconstituted in (in the order specified): 20 µl H$_2$O, 0.1 ml phenacyl-8 (0.1 M phenacyl-bromide and 0.005 M crown ether in acetonitrile) and 0.9 ml acetonitrile. This mixture was either heated at 80°C under vigorous stirring or sonicated at 60°C in an ultrasonic water bath (model 3200, Branson, Danbury, CT). Similar derivatization efficiencies of $^{14}$C-carnitine were obtained from each procedure.

The reaction mixture was evaporated to dryness, and the phenacyl-carnitine esters were reconstituted in 500 µl of a mixture of acetonitrile and water (25/75 v/v). Up to 50 µl of this mixture was injected into the HPLC.

The efficiency of derivatization was determined based on recovery of $^{14}$C-carnitine and measured as the percentage of radioactivity associated with the UV peak corresponding to phenacyl-carnitine, compared to total radio activity per injection. Underivatized carnitine does not co-elute with derivatized carnitine.

To allow quantification of phenacyl-carnitine esters, $^{14}$C-carnitine and $^{14}$C-hexanoyl-carnitine of known specific activity were derivatized and chromatographed. The peak area and the radio-activity associated with the corresponding peaks was determined, and the absorption area per nmol of phenacyl-carnitine ester was calculated (analogous to a molar extinction coefficient).

To verify that the isolation and derivatization procedure yielded similar results for free carnitine and medium-chain acyl-carnitines, the recovery of exogenous $^{14}$C-carnitine and hexanoyl-carnitine from plasma samples was determined.
2.2.5 Chromatography

A constant flow rate of 1.6 ml/min was utilized, which resulted in a head pressure ranging from 1700 to 2500 PSI over the course of the gradient employed. Three eluents were used with the following composition (by volume):

I) ACN/H₂O: 70% acetonitrile and 30% water
II) H₂O: 100% water
III) ACN/TEAP: 80% acetonitrile, 20% water, .8% triethylamine, 0.64% phosphoric acid

All eluents were passed through a 0.2 μm filter (Anodisk 47, Alltech) and degassed using an Alltech filter apparatus. The water used was distilled and deionized (Barnsted 4-module E-pure system, Barnsted, Dubuque, IA).

The gradients are presented graphically in Fig. 2.2. The ACN/H₂O eluent (100% I) was used to equilibrate the column and was maintained until 1 min after an injection was made (dependent on the void volume of the system between switch valve and column; for our system this was approximately 4 ml). One min after injection, the switch valve was activated on pump A, causing a flow of 100% water (eluent II). The acetonitrile remaining in the system was sufficient to elute all excess derivatization agent. In our experience, extending eluent I for longer duration caused free and acetyl-carnitine phenacyl-esters to co-elute in the void volume.

Eluent II was subsequently maintained at 100% for 5 min. A shorter time shifted the free carnitine- and acetyl-carnitine derivatives to a shorter retention time, and resulted in very poor peak shapes. Times of 2 min, however, resulted in good peak shapes for carnitine- and acetyl-carnitine derivatives, but not for the medium-chain ester derivatives.

Six min after injection, a gradient was initiated, delivered through pump B, with the ACN/TEAP eluent (III) initially at 15%. This was increased linearly over a 25 min time period to 100% (replacing eluent II). Thirty-six min after the injection, ACN/TEAP (III via pump B) was switched to 100% ACN/H₂O (eluent I via pump A). The switch valve was typically deactivated 25 min after injection such that heat produced by the switch valve was not a problem. The system was re-equilibrated for 19 min; injections could be made at 55 min intervals.

This method was optimized for short- and medium-chain carnitine esters (C2-C10) and tested up to palmitoyl-carnitine. Preliminary analyses indicated that long-chain carnitine esters were not present at detectable levels in samples used for this and similar studies. Extending the 100%
ACN/TEAP period elutes longer-chain esters.

Phenacyl-carnitine esters have a maximum UV absorption at 245 nm (based on spectral analysis obtained with a scanning Gilford Response II spectrophotometer, Ciba Corning Diagnostics, Oberlin, OH). Therefore, the detector was programmed to monitor absorbance at 245 nm. Acetonitrile and triethylamine absorbed at 245 nm, and this resulted in a gradual rise in baseline with the progression of the gradient. This baseline shift could be reduced by using a higher wavelength, e.g. 254 nm, but this results in a 20% decrease in sensitivity.

2.2.6 Elution profile of carnitine esters

Retention times of different carnitine esters were determined in order to identify peaks in chromatograms obtained from plasma samples. Carnitine esters were either purchased or synthesized according to the following procedure. CoA esters (0.5 mM) were suspended in a 100 mM TRIS (tris(hydroxymethyl)aminomethane) buffer (pH 8.0) containing 1 mM of L-carnitine (plus γ-carnitine). To this mixture was added carnitine acetyl-transferase in order to convert the CoA esters to carnitine esters.

The elution profile of γ-butyrobetaine (deoxycarnitine) and trimethyllysine was also evaluated. Both components are precursors for the de novo synthesis of carnitine and have been detected in plasma (Rebouche et al. 1986). The addition of ninhydrin during the purification of carnitine esters destroys trimethyllysine; therefore, ninhydrin should be omitted for the quantification of total carnitine precursors.

2.3 Results

A graphic summary of the isolation procedure as well as the losses of 14C-carnitine internal standard in the subsequent steps is given in Fig. 2.1. 14C-carnitine was lost in the methanol precipitate (10.9 ± 0.3% of initial 14C present), in the waste-effluents from the silicagel column (2.7 ± 4.5%) and in non-specified losses (1%; transfer pipettes, remnants in vials). The overall percentage of carnitine recovered in the phenacyl-carnitine peak was 79.9 ± 6.7%.

The efficiency of derivatization was 94.2 ± 4.1% (n=6). The recovery of 14C-carnitine and hexanoyl-carnitine (UV) correlated with an r² of 0.95, and the absorption area per nmol of carnitine (7.42) and hexanoyl-carnitine (7.70) were not statistically different (p > 0.1).
Fig. 2.1. Graphic summary of the isolation procedure for acyl-carnitines from plasma samples, and the loss of $^{14}$C-carnitine in the subsequent steps of the isolation procedure (ppt = precipitate, spt = supernatant, MeOH = methanol, MWA = methanol/water/acetic acid (50/45/5 v/v/v)).

The complete elution profile of all tested esters and $\gamma$-butyrobetaine is given in Fig. 2.2. This elution profile is actually a composite of 2 chromatograms since the reaction conditions used to synthesize the acyl-carnitines caused a broad carnitine peak, overlapping $\gamma$-butyrobetaine (see dashed line). To illustrate the separation of carnitine esters, another chromatogram (10-15 min) with carnitine, $\gamma$-butyrobetaine, and acetyl-carnitine is overlaid.
In Fig. 2.3, the retention time observed for the phenacyl esters of acyl-carnitines is regressed as a function of the chain-length of the acyl group. A linear relationship was observed for the straight-chain acyl-carnitines.

Two chromatograms, obtained from plasma-sample analysis (equivalent to approximately 20 μl plasma) for carnitine esters are given in Fig. 2.4. Both chromatograms show the presence of free carnitine, the carnitine precursor γ-butyrobetaine, acetyl-carnitine, and C4-carnitine. In the chromatogram obtained from a pig fed tri-C7 (Fig. 2.4B), propionyl-carnitine and possibly valeryl-carnitine were present.
Fig. 2.4A and B. Samples of carnitine-ester plasma profiles obtained from pigs 4 h after feeding 6 ml of medium-chain triglycerides containing (saturated) fatty acyl-chains of 6 (A) or 7 carbons (B).

2.4 Discussion

The method presented provides a powerful tool to analyze carnitine esters. Because it employs standard reversed-phase column chemistry and commercially available reagents, this method is easy to implement in laboratories using an HPLC. The observation that odd-chain MCT can give rise to propionyl-carnitine in plasma (Fig. 2.1A) and (abnormally) elevated γ-butyrobetaine (Fig. 2.3) in one litter of pigs illustrates the importance of a method which can distinguish between different carnitine esters in plasma samples and, therefore, detect potential abnormalities in the profile. Several metabolic disorders can be characterized via analysis of the carnitine profile as observed in plasma (Harpey et al. 1987, Roe et al. 1985).

The isolation procedure of plasma carnitine esters proved to be effective for both free and medium-chain acyl-carnitines. Stearic hinderance of the carboxyl group of (medium-chain) acyl-carnitines by the fatty-acid chain, which could possibly prevent efficient derivatization, as mentioned
by Minkler (Minkler et al. 1984) was not observed to be a problem using phenacyl-bromide as a derivatization agent.

The linear dependence of the retention time of acyl-carnitine derivatives on chain-length of the acyl group (Fig. 2.3) suggests that the hydrophobicity of the acyl-group is a determining factor in the elution profile (given the use of a linear gradient).
Chapter 3. The effect of feeding MCT of varying chain-length on the carnitine ester profiles in newborn pigs

3.1 Introduction

The role of L-carnitine (β-hydroxy-γ-trimethylaminobutyric acid) in the transport of long-chain fatty acids across the inner mitochondrial membrane has long been acknowledged. Carnitine replaces the CoA group of acyl-CoA by the action of carnitine acyl-transferase. The formed acyl-carnitine is then transported across the inner mitochondrial membrane by carnitine acyl-translocase, and the carnitine is again replaced by a CoA, forming acyl-CoA (Fritz & Schultz 1965).

More recently, carnitine has been shown to have a similar role in the export of the products of β-oxidation from the peroxisomes (Mannaerts & Debeer 1982). Rebouche et al. (1990) proposed that carnitine has a role in releasing mitochondrial CoA from acyl-CoA when the free CoA supply becomes limiting due to activation and subsequent accumulation of metabolites in the mitochondrion. Carnitine might act as a buffer to the mitochondrial acyl-CoA/CoA ratio by translocating excess acyl-groups into the cytosol.

The carnitine profile in tissues and plasma can function as an indicator of steps limiting the oxidation of fatty acids. Accumulated acyl-CoA esters are converted to carnitine esters, which can be excreted into the cytosol and can enter the plasma pool. Plasma carnitine esters are incompletely reabsorbed in the kidney, resulting in the excretion of these esters in urine (Bremer 1983). Determination of the composition of the urinary or plasma carnitine esters can therefore provide information about the steps in oxidation which may be limiting.

Carnitine is considered to be a conditionally essential nutrient, especially for the neonate. This nutritional aspect of carnitine has been widely accepted when long-chain fatty acids (≥ C14) provide the majority of the energy for the neonate (Bremer 1983). Recent evidence, however, has suggested that carnitine supplementation might also be important in neonates fed medium-chain triglycerides (MCT, containing C6 to C12 fatty acids). Rebouche et al. (1990), although not proposing a beneficial effect of supplemental carnitine, reported the accumulation of medium-chain acyl-carnitine intermediates.

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2Data as presented in this chapter has been published, together with Chapter 2, in the Journal of Chromatography, Biomedical Application 584;157-165 (1992) © Elsevier Science Publishers
ates in plasma after feeding infants with MCT, suggesting a role of carnitine in the oxidation of these medium-chain fatty acids.

The objective of this chapter was to determine which acyl-carnitines were affected by feeding MCT to piglets. Since no easy to use, accurate method was available for this analysis, a new HPLC method was developed for this assay. This method is described in Chapter 2.

3.2 Materials and Methods

To determine the effect of feeding MCT on the plasma carnitine profile, plasma samples were obtained from 16 MCT-fed pigs. The pigs were removed from the sow 24 h after birth (4 pigs per litter), and were fasted for an additional 4 h while housed in pens maintained at 32°C. MCT oils consisting of different chain-length (saturated) fatty acids (4, 5, 6 or 7 carbons, indicated as tri-C4, tri-C5, tri-C6 or tri-C7 respectively) were subsequently fed via gastric intubation in doses of 6 ml so that all 4 pigs per litter received MCT oil containing a different chain-length fatty acid. Blood samples were obtained via jugular venipuncture using heparinized vacutainers at 0, 1, 2, 4, and 8 h after oral gavage. Plasma was obtained following centrifugation at 4°C and was stored at -20°C until analyzed. The samples used were obtained in the course of another study (Wieland et al. 1992).

The data were analyzed by the analysis of variance procedure using SAS (1982) according to a split-plot design, blocked by litter (Steel & Torrie 1980).

3.3 Results

The acyl chain-length of the MCT fed to the piglets significantly affected the concentration of free carnitine as well as the concentration of propionyl-carnitine. Propionyl-carnitine was undetectable in animals fed even-chain MCT, as well as at time 0 in all treatments. In animals fed odd-chain MCT (tri-C5 or tri-C7), propionyl-carnitine increased with increasing oxidation rate of the odd-chain fatty acids and declined in a similar fashion as the oxidation rate declined (see Fig. 3.1A).

Plasma free carnitine concentrations varied considerably during the sampling period. A significant chain-length effect (p < .06) as well as a significant time effect was observed (p < .05). Odd-chain MCT invoked a higher plasma free carnitine concentration compared to even-chain MCT. A significant increase in plasma carnitine concentration was observed 1 h after gastric intubation, while
no increase was observed 2 h after gastric intubation (plasma fatty acid concentrations peaked between 1 and 2 h (Wieland et al. 1992).

![Graph A](image1.png)

![Graph B](image2.png)

**Fig. 3.1.** The effect of feeding MCT of varying chain-lengths (tri-C4 through tri-C7) on the concentrations of propionyl-carnitine (A) and free carnitine (B) in plasma of newborn pigs. Propionyl-carnitine: time effect \( p < .01 \) and chain-length effect \( p < .05 \); free carnitine: time effect \( p < .01 \) and treatment effect \( p < .06 \), \( n=3 \) per mean.

No significant effects were observed in \( \gamma \)-butyrobetaine and acetyl-carnitine esters (\( p > 0.1 \)). Acetyl-carnitine varied considerably, and tended to be higher when tri-C6 and tri-C7 were fed; but due to the high variation, no significant effects were observed (data not shown).

The C4-carnitine peak, presumptively identified as butyryl-carnitine, followed a pattern which
mirrored the pattern of free carnitine; however, no significant effects were observed (p > 0.1, see Fig. 3.2).

Fig. 3.2. The effect of feeding MCT of varying chain-length on (A) the concentrations of γ-butyrobetaine and acetyl-carnitine averaged over treatments (n=12 per mean) and (B) C4-carnitine in plasma of newborn pigs (n=3 per mean).

Other peaks occurred in the chromatograms, from which the retention time of one peak corresponded with the retention time of C5-carnitine (from pigs fed tri-C7). Due to sporadic co-elution of a reagent peak, later identified as originating from the use of dated ninhydrin reagent, the C5-carnitine peak could not be quantified consistently (Appendix A, Fig. A4). Other peaks were not
identified since their retention time did not correspond to the retention time of a saturated, straight-chain acyl-carnitine.

With the exception of one litter of pigs, \( \gamma \)-butyrobetaine was low throughout the experiment. These animals were not included in the final analysis of the data. Pigs from this litter exhibited extremely high concentrations of carnitine precursor (\( \gamma \)-butyrobetaine) and relatively low levels of free carnitine. No elevation in free carnitine was observed 1 h after oral gavage, while a rise was observed in \( \gamma \)-butyrobetaine. Fig. 3.3 shows data averaged over treatments from this litter contrasted to data averaged over treatments from the other 3 litters.

Fig. 3.3. The effect of feeding MCT on the carnitine profile over time. A; averaged over 12 pigs (three litters) with normal \( \gamma \)-butyrobetaine (Gbb) levels or B; averaged over 4 pigs (one litter) with high \( \gamma \)-butyrobetaine levels (C2-carn=acetyl-carnitine, C3-carn=propionyl-carnitine, C4-carn=butyryl-carnitine).
3.4 Discussion

The data obtained from pigs fed MCT indicate that MCT feeding does affect the concentration of free carnitine in plasma (Fig. 3.1B). The concentration of free carnitine increased 1 h after feeding, but dropped to the 0-h level at 2 h. The increase in plasma carnitine at 1 h after feeding MCT suggests that the pig has the ability to mobilize carnitine from tissues such as the intestines, as suggested by Li et al. (1992). The biological significance of this possible mobilization is unknown, but raises the question of whether plasma carnitine is a correct parameter for assessing the carnitine status of a subject under certain nutritional and/or disease states.

The drop in plasma carnitine between 1 and 2 h after feeding MCT might be caused by an impaired reabsorption of free carnitine by the kidneys due to the high levels of plasma fatty acids, as suggested by Penn et al. (1992) and Stadler & Rebouche (1992). Further experiments are needed to determine the exact cause.

Odd-chain fatty acids (upon β-oxidation) can yield propionyl-carnitine (as well as valeryl-carnitine). Propionyl-carnitine was present in the plasma of pigs fed tri-C5 or tri-C7, suggesting that the production of propionyl-CoA exceeds the oxidation in certain tissues (Fig. 3.1A). Propionyl-CoA is mainly produced within the mitochondria under the conditions employed. The presence of propionyl-carnitine suggests that carnitine does act as a buffer of the intramitochondrial CoA pool by exporting acyl-groups such as propionate.

The anecdotal observation that one litter of pigs had high concentrations of the carnitine precursor γ-butyrobetaine and relative low levels of free carnitine suggests that these pigs had a genetic deficiency, impairing either the uptake of γ-butyrobetaine by the liver or the synthesis of carnitine from γ-butyrobetaine in the liver (Fig. 3.3). A similar condition was previously observed in an infant by Dr. S.C. Winter (personal communication). Detailed information on this case is, however, lacking as well.
Chapter 4. Medium-chain fatty acid oxidation in colostrum-deprived newborn piglets: stimulatory effect of L-carnitine supplementation

4.1 Introduction

A major problem in the care of premature and/or small-for-gestational-age infants is the provision of sufficient amounts of nutrients, especially energy, to support rapid body weight gain. Underdevelopment of the gastrointestinal tract makes enteral feeding problematic, and consequently, intravenous infusion of nutrients is often employed. A major challenge with intravenous infusion of nutrients is to provide sufficient amounts of energy without negative effects on the health of the subject, such as fluid overload. Thus, lipid emulsions are often used because of their high energy density and low osmolarity (Lima 1989).

Commonly used lipid emulsions are composed of long-chain triglycerides. However, infusion of these lipids is associated with several problems such as hypertriglyceridemia, decreased immunity, hepatomegaly and splenomegaly (Bell et al. 1991, Booth 1992). To avoid these problems, triglycerides composed of medium-chain fatty acids (MCFA, which are fatty acids with 6-12 carbons, often in combination with long-chain fatty acids) are being used increasingly for both parenteral (Goulet et al. 1992) and enteral nutrition (Kelts & Jones 1984, Lima 1989, Bell 1991, Borum 1992).

Carnitine functions as a cofactor involved in the transport of (long-chain) fatty acid groups (after activation to an acyl-CoA) across the inner mitochondrial membrane (Fritz 1968) and has also been suggested to have a role in buffering intramitochondrial free CoA by aiding in the export of acyl-groups (Siliprandi et al. 1990). Neonates are considered carnitine deficient, which could impair their capacity to utilize long-chain fatty acids as a fuel, particularly when fed via total parenteral nutrition (Penn et al. 1981).

Medium-chain fatty acids can passively diffuse through the inner mitochondrial membrane and can be activated intramitochondrially, thereby eliminating the need for carnitine as a cofactor for entrance into the site of oxidation. Although in vitro evidence indicates that carnitine stimulates the oxidation of MCFA in muscle (Aas 1971, Groot & Hulsmann 1973, Otto 1984), MCFA are

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commonly fed to (premature) infants without supplemental carnitine (Lima 1989, Booth 1992). The objective of the research reported herein was to determine whether the assumption that MCFA oxidation is carnitine independent is appropriate in the neonatal pig model (Shenai et al. 1983, Baltzell et al. 1987, Hannon et al. 1990).

4.2 Materials and methods

4.2.1 Animals

Newborn, colostrum-deprived pigs (commercial crossbreds, avg. weight: 1.46±0.34 kg) were obtained from the Swine Research Center of the University of Illinois (trial 1 and 2) and from a commercial swine farm (trial 3 and 4). The animal protocols were approved by the University of Illinois Laboratory Animal Care Advisory Committee and were in accordance with the "Guide for the Care and Use of Laboratory Animals" (NRC 1985).

The animals (littermates within a trial) were removed from the sow immediately after birth, and placed in a heated box (30-35°C). Using a minor surgical procedure, an argyle catheter (3½ french, Sherwood Medical, St. Louis, MO) was inserted into an umbilical artery as described by Benevenga et al. (1992) using general halothane anaesthesia (Halocarbon Laboratories, North Augusta, SC) in combination with local lidocaine anaesthesia (Anthony Products, Arcadia, CA) of the umbilicus. This catheter was inserted 22 cm into the aorta to a position near the heart (as verified by X-ray photography) and was used for infusion of [l-14C]-medium-chain fatty acids.

After recovery from the anaesthesia (1-2h), piglets were randomly placed into one of four respiration chambers consisting of an acrylic tube (15 cm diameter*45 cm long), designed after those described by Benevenga et al. (1992). A heat pad on the outside of the chambers maintained the floor temperature in the chambers at 35°C. The chambers (Fig. 4.1) were mounted onto a metal frame and connected to an air inlet containing a sodasorb canister to remove incoming CO2. The volume of air passing through each chamber (2 L/min) was controlled by flowmeters (Cole Parmer Instrument Co., Niles, IL). Air exiting the chambers was diffused through one of 2 columns containing 75 mL of a 1.8 N sodium-hydroxide solution to trap expired 14CO2. The sodium-hydroxide solution was replaced at twenty min intervals after switching the air flow to the alternate columns. This allowed for continuous collection of all CO2. Preliminary studies verified that under these conditions, in excess of 95% (102.5±3.2%) of the expired CO2 was trapped via this system. Recovery
was assessed using continuous infusion of a sodium bicarbonate (1 mmol/min) solution into a shallow pan mounted within the respiration chamber. The infused bicarbonate was mixed with excess HCl via a mixing T in the chamber resulting in the release of CO$_2$. Infused CO$_2$ (as bicarbonate) was recovered in the sodium-hydroxide-containing columns and analysed as described below.

![Fig. 4.1. Schematic view of the apparatus used to collect expired CO$_2$ and to house the piglets. Using a vacuum pump (A), air (2 L/min) was first drawn through a column of sodasorb (B), subsequently through the chambers in which the piglets were housed (C), after which the air was passed through columns containing sodium-hydroxide (D).]

An infusion line was connected to the arterial catheter of each pig and connected to an eight-channel peristaltic infusion pump (model 07524, Cole Parmer Instrument Co., Niles, IL) which allowed for continuous delivery of infusate from I.V. bags (Baxter Healthcare Corp, Deerfield, IL). Upon infusion of the fatty acid solution, the piglets would generally sleep, but could be aroused easily by moving the chamber.

4.2.2 Infusion protocol

Pigs in trials 1 and 2 were infused for a total of 9 h and pigs in trials 3 and 4 for 12 h. During this time period, a constant infusion of [1-$^{14}$C]-MCFA (appr. 0.05 MBq/h; octanoic acid (C8): American Radiolabeled Chemicals, Inc. St. Louis, MO; heptanoic acid (C7) and decanoic acid (C10): Sigma Chemical Co., St. Louis, MO) diluted with corresponding non-radioactive MCFA (Sigma Chemical Co., St. Louis, MO) was maintained. The infusion rates were varied between
pigs as per Table 4.1. Solutions were made iso-osmotic with NaCl (308 mmol/l), and the pH was adjusted to 7.4. Volume delivery was approximately 25 mL/h throughout the trial.

Table 4.1. Treatments for trials 1-4 in which, for each trial, 4 newborn, colostrum-deprived piglets were used.

<table>
<thead>
<tr>
<th></th>
<th>Trail 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Trial 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCFA infused</td>
<td>C8</td>
<td>C8</td>
<td>C8</td>
<td>C7, C7, C8, C10</td>
</tr>
<tr>
<td>Energy infusion (energy provided(^2)/MR(^3)*100)</td>
<td>150</td>
<td>150</td>
<td>60,84</td>
<td>108,132</td>
</tr>
<tr>
<td>Carnitine dose(^4) ((\mu\text{mol}/(\text{h} \cdot \text{kg}^{0.75})))</td>
<td>0,5, 10, 20</td>
<td>0,5, 10, 20</td>
<td>20</td>
<td>0,20</td>
</tr>
<tr>
<td>Age of piglets (h)</td>
<td>6</td>
<td>6</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Carnitine-free infusion period (h)</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Carnitine infusion period (h)</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^1\); One C7 infused animal served as the no-carnitine control.
\(^2\); The energy content of the different chain-length fatty acids was calculated based on ATP equivalent per mole (C7: 52 ATP, C8: 61 ATP, C10: 78 ATP), and that the synthesis of 1 mole of ATP required 73.3 kJ metabolizable energy.
\(^3\); The energy content of the infusates are expressed relative to the anticipated metabolic rate (MR) of the piglets (200 kJ/(d \cdot \text{kg}^{0.75})).
\(^4\); The carnitine doses were based on estimated normal carnitine intake via milk consumption (approximately 10 \(\mu\text{mol}/(\text{h} \cdot \text{kg}^{0.75})\), Kerner et al. 1984, Den Hartog et al. 1987)).

To ensure maximum sensitivity in detecting possible carnitine effects, each piglet served as its own control. To accomplish this, an initial carnitine-free infusion period was compared to a subsequent period in which carnitine was supplemented. Specifically, the first 5 h (trial 1 and 2) or 7 h (trial 3 and 4) of each infusion was used to determine the rate of fatty acid oxidation.
in the absence of exogenous carnitine (carnitine-free infusion period). Previous research (Odle et al. 1992) showed that 5 h was sufficient to predict the steady-state MCFA oxidation rate. After 5 or 7 h of MCFA infusion, a primed co-infusion of L-carnitine (Lonza AG, CH4002, Basel, Switzerland) was initiated (carnitine infusion period) to determine if carnitine addition could deviate the fatty acid oxidation rate from the trend as determined based on the carnitine-free infusion period. Carnitine infusion rates (see table) were based on the amount of carnitine present in colostrum normally consumed by pigs (approximately 10 μmol/h, Kerner et al. 1984, Den Hartog et al. 1987).

Three pigs did not receive any carnitine in order to validate that the extrapolation/modeling approach accurately predicted the fatty acid oxidation rates in the "carnitine" infusion period. The other pigs received a bolus injection of 12.5, 25 or 50 μmol carnitine (2.5 times the hourly doses) dissolved in 1 mL physiological saline at the start of the carnitine infusion period. During the carnitine infusion period, these pigs then received either 5, 10 or 20 μmol carnitine/(h·kg⁰.⁷⁵) respectively, which was mixed with the MCFA infusate.

After the infusions were completed, pigs were anaesthetized using an i.v. injection of pentobarbital and killed using an intracardial injection of saturated KCl.

4.2.3 Analysis

Sodium-hydroxide samples were analyzed for CO₂ as described by Benevenga et al. (1992). Based on the ratio of the specific radioactivity of the expired CO₂ and the specific radioactivity of the infused MCFA, an instantaneous fatty acid oxidation rate (FA-OR = MCFA-derived CO₂/total CO₂*100) was calculated, assuming that the fate of the [1-¹⁴C] carbon represented the fate of all carbons within the fatty acid molecule. The measured CO₂ radioactivity was not corrected for the lag of expiration of CO₂ due to dilution in the body pool. This results in an underestimation of the actual oxidation rates of the infused [1-¹⁴C]-medium-chain fatty acids. This inaccuracy does not, however, pose a problem for the interpretation of the results which evaluated the relative change in fatty acid oxidation rate elicited by carnitine supplementation.

The data obtained during the carnitine-free infusion period were fitted to a single pool exponential curve which was determined by Benevenga et al. (1992) to accurately model the data:
FA-OR = A*(1-e^{k(t-t_{lag})})

where: FA-OR=fatty acid oxidation rate (%)=(MCFA-derived CO_{2}/total CO_{2}*100)

A=asymptotic maximal FA-OR at t=\text{infinity} (%)

k=fractional rate constant (min^{-1})

\text{t}=\text{time (min)}

\text{t}_{\text{lag}}=\text{lag of start of oxidation of MCFA (min)}

To determine the carnitine response, the curve obtained was extrapolated to the carnitine infusion period, and the estimated values of MCFA oxidation were compared with the actual oxidation rates observed. The differences between observed values and estimated values were determined (carnitine response) and a paired t-test was used to test whether the difference was greater than zero.

4.3 Results

Metabolic rates in this study were calculated assuming that glucose (464 kJ/mol CO_{2}) and MCFA (559 kJ/mol CO_{2}) were the primary fuels, and were found to be 197±26 kJ/(d·kg^{0.75}), which was independent of body size and age (p > 0.1, data not shown). The oxidation rate of fatty acids (as a function of infusion rate) was independent of the age of the animals, and the carnitine response (expressed as a function of the infusion rate) in the 24 h age group showed a significant response which was similar (p > 0.1) to the combined data. Therefore, data were pooled across age groups.

In Fig. 4.2, the oxidation rate of MCFA (asymptotic maximal FA-OR at t=\text{infinity}) is expressed as a function of infusion rate of MCFA, both scaled as a percentage of the metabolic rate of the animal. Based on these data, oxidation of MCFA accounted for 40% of the carbon (p < 0.01), assuming that the fate of the [1-^{14}C] carbon represents the fate of all carbons in the fatty acid molecule.

Two example graphs illustrating the time course of MCFA oxidation to CO_{2}, as well as the CO_{2} production are shown in Fig. 4.3. The top panel represent the data from a pig infused with carnitine in the carnitine infusion period, while the bottom panel represents the data from a control animal which did not receive any carnitine.
Fig. 4.2. The asymptotic maximal fatty acid oxidation rate of MCFA in newborn, colostrum-deprived piglets, as a linear function of the infusion rate, both expressed as energy equivalents as a percentage of the metabolic rate (MR) of the recipient.

Fig. 4.3. Sample graphs of the fatty acid oxidation rate (FA-OR=MCFA-derived CO₂/total CO₂*100) and the CO₂ production as a function of time (μmol/(kg⁻⁰.⁷⁵·min)) of two of the 16 pigs receiving variable amounts of carnitine and MCFA (see table 4.1). The squares represent measured values, while the solid line is the curve as fitted to the fatty acid oxidation rate; the dashed line is the extrapolation of this curve assuming no carnitine effect. The graph in the upper panel is from a piglet infused with MCFA at 111% of its metabolic rate, and carnitine was supplemented at 20 μmol/(h·kg⁻⁰.⁷⁵); the increase in FA-OR was 3.87 (p<0.05). The graph in the lower panel is from a piglet infused with MCFA at 57% of its metabolic rate, but no carnitine was supplemented; the increase in FA-OR was 0.07 (p>0.10). The arrow in the upper panel indicates the start of carnitine infusion.
The response to exogenous carnitine was independent of the level of carnitine infused (data not shown). It may be that the priming doses administered were sufficient to maximize the response. The increase in FA-OR due to exogenous carnitine supplementation was, however, a linear function of the infusion rate of fatty acid (p<0.01, Fig. 4.4). Of the 10 animals infused with carnitine and MCFA at a rate providing more than 75% of their metabolic rate, 8 responded with a significant increase (p<0.1, n=1; p<0.05, n=7) in FA-OR (up to a 20% relative increase in the fatty acid oxidation rate).

![Fig. 4.4. The carnitine response expressed as a linear function of the fatty acid infusion rate. The carnitine response was calculated as the increase in MCFA oxidation rate (FA-OR) during the carnitine infusion period as compared to the curve extrapolated from carnitine-free infusion period. The FA-OR is expressed as the energy derived from fatty acid oxidation as a percentage of the (calculated) metabolic rate (MR) of the piglet. Each point represent the average increase in FA-OR during the carnitine infusion period as calculated based upon data from one pig.](image)

4.4 Discussion

The metabolic rate of newborn, colostrum-deprived pigs is lower than the metabolic rate of 0-24 h fed and colostrum-deprived pigs as reported by others (Noblet & Le Dividich 1981, Curtis 1970). Although the measurements made do not allow for exact determination of metabolic rates, the assumption that all CO₂-carbon is derived from glucose and MCFA allows for fairly accurate estimation of metabolic rates since newborn piglets have a low protein turnover and limited amounts of non-structural endogenous lipids (Mellor & Cockburn 1986). According to our data, the basal metabolic rate of newborn, colostrum-deprived pigs was calculated to be 197 ± 26 kJ/(d·kg⁰.⁷⁵),
which is in agreement with data from Mellor and Cockburn (1986).

Of the MCFA infused, 40% could be recovered as CO₂. This value, which is low assuming that MCFA cannot be readily stored as medium-chain triglycerides (Bach & Babayan 1982), is in agreement with data from Sulkers et al. (1989) and less than the 60% as reported by Odle et al. (1992). The fate of the remaining 60% of the carbon is not clear, but other research has suggested that a substantial portion of the MCFA can either be chain-elongated or converted to long-chain fatty acids via lipogenesis after breakdown to acetyl-CoA (Zurier et al. 1967, Reeve & Franks 1968, Sarda et al. 1987). Zurier et al. (1967) investigated the effect of feeding MCT to rats with a portacaval shunt, and concluded that "striking increases in proportions of MCT in adipose tissue were noted", which was not observed to a similar extent in control (no portacaval shunt but MCT-fed) animals. This suggests that the site of entrance into circulation is a major determinant for the extent of reesterification of the MCFA. In our experiment, MCFA were infused into the aorta, which results in MCFA being transported to all tissues, dependent on blood flow distribution, as was the case in Zuriers' experiment.

Another determinant in the fate (oxidation versus lipogenesis) of MCFA might be the site of activation in liver. According to Aas (1971) and Groot & Hulsmann (1973), liver mitochondria contain an intramitochondrial medium-chain acyl-CoA synthetase. However, in determining extramitochondrial versus intramitochondrial activation, it was observed that a major portion of the MCFA were activated outside of the matrix (Aas: approximately 75%, Groot & Hulsmann: approximately 40%). Low long-chain fatty acid oxidation observed in vitro suggests that uptake of acyl-CoAs into the mitochondria is inhibited, possibly via carnitine palmitoyltransferase I. Long-chain fatty acids are, however, actively reesterified by hepatocytes (Girard et al. 1992). The site of activation might, therefore, determine if fatty acids are oxidized (intramitochondrial activation) or esterified/chain-elongated (extramitochondrial activation).

Consistent with previous research (Odle et al. 1992), MCFA infusion did not increase CO₂ production during the course of infusion (p > 0.1, data not shown). This is somewhat surprising since MCFA are considered obligatory fuels (Bach & Babayan 1982) and have been shown to increase heat production in humans compared to long-chain fatty acids (Seaton et al. 1990). The reason for this discrepancy is not known.

Carnitine clearly increased the contribution of fatty acids to total fuel oxidation if fatty acids
were infused at levels which could supply more than 50% of the energy need of the piglet. The stimulatory effect of carnitine became more pronounced at higher levels of fatty acid infusion.

The effect of carnitine could be due to the lack of sufficiently developed octanoyl-CoA synthetase as reported by Shipp et al. (1982) using guinea-pig liver. The octanoyl-CoA synthetase is the enzyme responsible for the intra-mitochondrial activation of medium-chain fatty acids, and a low activity of this enzyme would make the oxidation of MCFA dependent on their activation by acyl-CoA synthetases on the outer mitochondrial membrane and the endoplasmic reticulum. This would make the MCFA oxidation carnitine dependent since acyl-CoA compounds can not diffuse through the inner mitochondrial membrane.

A second possibility for a role of carnitine might be via its involvement in MCFA oxidation in extrahepatic tissues. Aas (1971) and Groot and Hulsmann (1973) suggested that no specific acyl-CoA synthetase is present in muscle with an optimum activity toward medium-chain fatty acids. This would result in the formation of medium-chain acyl-CoAs which have to be transported into the mitochondrial matrix involving carnitine, as demonstrated by Groot and Hulsmann (1973) using rat muscle mitochondria. The biological importance of this carnitine dependence is generally not considered important, which might be warranted due to the low metabolic rate of muscle (compared to e.g. liver) in combination with the presumed high levels of muscle carnitine, which could prevent metabolic problems when MCFA are oxidized (Negrao et al. 1987). Muscle carnitine levels in term and premature infants are, however, low compared with adult controls (Penn et al. 1985). Further research clearly needs to adress this issue.

A third possibility is that the carnitine effect could be mediated by its "buffering" of the intramitochondrial acyl-CoA/free CoA ratio via export of acyl-groups after conversion to acyl-carnitines (Chalmers et al. 1983) which is supported by data obtained from infants fed MCT from Rössle et al (1990) and Rebouche et al. (1990). The release of CoA by transfer of acyl-groups to carnitine can be regarded as a detoxification, since excess acyl-CoA can inhibit normal mitochondrial function with regard to the urea cycle and normal energy production via both the Krebs cycle and β-oxidation (Stumpf et al. 1985, Harpey et al. 1990). The acyl-carnitines can subsequently be excreted into the urine. This excretion, although beneficial for the short term, can, however, also lead to a secondary carnitine deficiency, obstructing further buffering of the CoA pool (Chalmers et al. 1983). The presence of propionyl-carnitine in plasma of newborn piglets...
fed odd-chain medium-chain fatty acids (Kempen & Odle 1992) confirms that excess acyl-groups can be exported from the mitochondrion, since propionyl-CoA under the conditions employed is mainly produced by β-oxidation of odd-chain MCFA, which occurs inside the mitochondrion. In conclusion, this research suggests that carnitine does play a role in MCFA oxidation in vivo, and opens the question as to whether carnitine should be added to nutrition solutions containing MCT or MCFA.
Chapter 5. Dicarboxylic acid (DCA) production of newborn pigs infused with medium-chain fatty acids (MCFA): effects of valproate and L-carnitine co-infusion

5.1 Introduction

Dicarboxylic acids are produced by hepatocytes (Hare & Wahle 1985) when the supply of fatty acids to the liver exceeds the capacity for β-oxidation (e.g. due to a metabolic disorder) and reesterification (e.g. in MCFA feeding), leading to the accumulation of fatty acids in the cytosol (Sherratt & Veitch 1984, Cerdan et al. 1988, Mortensen 1986, Draye et al. 1988). The accumulated fatty acids can serve as a substrate for ω-oxidation which is initiated by the mixed function oxidase system (including cytochrome P-450) and results in the oxidation of the ω-carbon (methyl-carbon) of the fatty acid in a concentration-dependent fashion (Vamecq & Draye 1989). The metabolic function of ω-oxidation is still unknown but is suggested to be, in conjunction with β-oxidation, the formation of the TCA-cycle intermediate succinate (Mortensen 1992). However, a major (static) portion of DCA produced are excreted in urine (after β-oxidation, Mortensen 1981, Mortensen 1986).

Accumulation of fatty acids resulting in DCA production is more likely to occur in subjects who are fed or infused with MCFA (Mortensen 1981), partly due to the more rapid route of entry (via the portal vein) from the gastrointestinal tract and partly due to the lower rates of reesterification in intestines and liver as compared to long-chain fatty acids (Bach & Babayan 1982). In addition, several researchers (Rössle et al. 1990, Rebouche et al. 1990) have suggested that MCFA feeding might result in the depletion of intramitochondrial free CoA which could impair β-oxidation and because of the resulting increase in fatty acid concentration, in an even greater rise in DCA production. Since the use of medium-chain triglycerides (MCT) as an energy source for both enteral and parenteral nutrition formulations has surged in recent years (Booth 1992, Borum 1992), the concern over the associated production of DCA has also increased (Henderson & Dear 1986, Lima 1989, Borum 1992).

Other conditions exist in which the supply of fatty acids exceeds the capacity of the liver to metabolize these fatty acids, for example, when epileptic patients are treated with the anti-convulsant drug valproic acid (Mortensen 1992, Triggs et al. 1990). The reason for the increased production of DCA in valproate-treated subjects is purportedly related to an intramitochondrial free CoA
depletion caused by the activation of valproate which then renders a poorly catabolyzed CoA ester (Sherratt & Veitch 1984, Zammit et al. 1989, Rozas et al. 1990, Veitch & Hoof 1990).

The putative CoA depletion occurring when MCFA are fed or when valproate is dosed can be counteracted by carnitine. Carnitine can act as a carrier molecule for the export of activated acyl groups (such as valproate and its metabolites) out of mitochondria through carnitine acyl transferase II, which liberates CoA. The resulting acyl-carnitine can be excreted into the cytosol and ultimately into the urine (Becker & Harris 1983). This excretion of carnitine, however, can ultimately lead to a carnitine deficiency (which in itself is known to cause dicarboxylic aciduria, Chalmers et al. 1984).

Previously, we (Kempen & Odle 1993) demonstrated that octanoate oxidation could be stimulated in vivo by L-carnitine supplementation in carnitine deficient subjects, thereby illustrating that carnitine indeed has a role in MCFA oxidation. The purpose of the experiment described herein was to determine the effects of carnitine, MCFA and MCFA+valproate infusion on the dicarboxylic acid excretion in carnitine-deficient newborn pigs (Kerner et al. 1984, Baltzell et al. 1987).

5.2 Materials & Methods

5.2.1 Animals

Newborn, colostrum-deprived piglets (n=21, avg. weight 1.29±0.1 kg) were removed from their dams at birth and deprived of food. Via a minor surgical procedure, two arterial catheters were inserted into the animals via the umbilical arteries, and a bladder catheter was inserted via the urachus. The animal protocol for this research was approved by the University of Illinois Laboratory Animal Care Advisory Committee and was in accordance with the "Guide for the Care and Use of Laboratory Animals".

The surgical procedure used to insert the catheters was as follows. Piglets were sedated using isoflurane (2%, Anaquest, Madison, WI) as a general anesthesia; and in addition, a local, subcutaneous lidocaine injection (3 mg; Anthony Products, Arcadia, CA) was administered. Subsequently, the umbilicus was cut off approximately 1 cm above the body wall (in the region where the tissue is atrophic). This exposes the umbilical arteries and vein (the arteries are smaller in diameter and have a less apparent lumen). The lumen in the artery was opened using curved watchmaker forceps and a catheter introducer (Becton Dickenson, Rutherford, NJ) and a catheter
(argyle umbilical catheter, 3½ French, Sherwood Medical, St. Louis, MO) was inserted. This catheter was threaded (approximately 22 cm for the blood sampling catheter) via the external ileac, cranially up the abdominal artery into the abdominal aorta (verified by X-ray photography). To facilitate insertion of the catheter, the umbilicus was raised slightly (ventrally) and pulled cranially for the insertion of the arterial catheters. This procedure was repeated for the other artery in which the catheter was inserted only 20 cm (infusion catheter). After inserting both arterial catheters, the umbilical arteries were pulled apart exposing the urachus (visible as a small, opaque vessel). The urachus was subsequently dissected free from umbilical tissue until a wider region of the urachus became visible (> 2 mm wide). Using hemostats, the urachus was secured in place, after which a small hole was punctured through the wall using a 22 gauge needle. Using a catheter introducer, the bladder catheter (urethral catheter, 3½ french, Sherwood Medical, modified to have 6 holes over the first 7 cm) was subsequently inserted for approximately 9 cm. After insertion, the catheter was secured in place by suturing it into the urachus; all catheters were sutured to the umbilicus. The incision into the umbilicus was subsequently closed (using sutures), and the exterior parts of the catheters were guided to the back of the animal using a suture and tape.

After recovery from the surgery (1 h), an infusion line was connected to the arterial catheter (inserted 20 cm) of each pig and connected to an eight-channel peristaltic infusion pump (model 07524, Cole Parmer Instrument Co., Niles, IL) which allowed a continuous delivery of infusate from i.v. bags (Baxter Healthcare Corp, Deerfield, IL). The other arterial catheter (inserted 22 cm) was connected to a blood sampling line (flushed with heparinized saline, 5 units/ml). Blood was sampled at 0 (before the start of MCFA infusion), 8½, 12½, 16½ & 20½ h after the start of the fatty acid infusion, and at the end of the experiment (24h). Plasma was obtained after centrifugation of the blood (at 4°C) and was stored at -20°C until analysis.

The bladder catheter was connected to a vacuum manifold (which was adjusted to produce a vacuum of approximately 70 mbar), via a urine collection vial (16 ml Falcon tube placed in ice). Urine was collected quantitatively every hour. To ensure proper collection of the urine, the bladder catheters were flushed frequently to verify complete recovery of the flush solution.

During each of 3 trials, piglets were housed in one of 6 heated (35°C) respiration chambers (Kempen & Odle 1993). The chambers were connected to an air inlet containing a sodasorb canister to remove incoming CO₂, and air was pulled through the chambers using a vacuum pump at a
rate of 2 L/min (controlled by flow meters, Cole Parmer Instrument, Niles, IL). Air exiting the chambers was diffused through a column containing 100 mL of a 1.8 M sodium-hydroxide solution to trap expired $^{14}$C$\text{CO}_2$. The sodium-hydroxide solution was replaced at sixty-minute intervals during which time the air flow was switched to a bypass column for 10 minutes. Preliminary studies (Kempen & Odle 1993) verified that under these conditions, in excess of 95% of the expired CO$_2$ was trapped in the NaOH during the collection period.

5.2.2 Infusion protocol

The treatments to which the animals were allotted are summarized in Table 5.1. Three animals served as the fasted controls (Fasted-ctrl). These animals were submitted to surgery, housed at 35°C, and fed water during the infusion experiment. The other 18 animals were placed in the respiration chambers and were infused for 2 h with physiological saline at a rate of approximately 10 ml/h in order to maintain the animals in a hydrated state.

After this adaptation period, an infusion with [1-$^{14}$C]-octanoate (C8, 20 $\mu$Ci/24 h, American Radiolabeled Chemicals, St. Louis, MO; diluted with non-radioactive C8, Sigma Chemical, St. Louis, MO) was started (time 0 h) and maintained for 24 h at a rate estimated to provide between 35-100% (energy content of C8 calculated to be 4470 kJ/mol) of the metabolic requirements of the animal (estimated to be 200 kJ/(d·kg$^{0.75}$), Kempen & Odle 1993). The octanoate infusion rate was constant for each animal, but varied between animals. No additional treatments were provided to the C8-ctrl animals.

In addition to the octanoate infusion, pigs allotted to the C8-carn treatment were co-infused with L-carnitine (for 8 h) starting 16 h after the start of the octanoate infusion. The carnitine co-infusion consisted of a prime dose of carnitine (50 $\mu$mol/kg$^{0.75}$) and a continuous infusion of 20 $\mu$mol/(h·kg$^{0.75}$), Lonza AG, Ch4002, Basel, Switzerland). The carnitine dose was based on data from Kempen & Odle (1993).

Pigs allotted to the VPA-C8-carn treatment were given, in addition to the octanoate infusion, a pulse dose of valproate equivalent to 180 $\mu$mol/kg$^{0.75}$ (clinically recommended dose per kg body weight per day) two hours before the start of the octanoate infusion. In addition, valproic acid was co-infused with octanoate (from 0-24h) at a rate of 10 $\mu$mol/ kg$^{0.75}$. Carnitine was co-infused to the VPA-C8-carn animals using the same procedure as used for the C8-carn pigs.
All infusion solutions were made iso-osmotic (308 mmol/l) using NaCl and the pH was adjusted to 7.4. The fluid delivery rate was 15-20 ml/(h·kg). Upon infusion of the fatty acid solution, the piglets would generally sleep, but could be aroused easily by moving the chamber.

Table 5.1. Time course of the infusion regimens to which newborn, colostrum-deprived piglets were allotted to determine the effects of octanoate, valproate, and carnitine infusions on dicarboxylic acid production.

<table>
<thead>
<tr>
<th>Infusion of:</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasted-ctrl&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Octanoate (C8)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0-24 h</td>
</tr>
<tr>
<td>Carnitine:</td>
<td></td>
</tr>
<tr>
<td>* prime dose (50 μmol/kg&lt;sup&gt;-75&lt;/sup&gt;)</td>
<td>16 h</td>
</tr>
<tr>
<td>* continuous (20 μmol/(h·kg&lt;sup&gt;-75&lt;/sup&gt;))</td>
<td>16-24 h</td>
</tr>
<tr>
<td>Valproate</td>
<td></td>
</tr>
<tr>
<td>* prime dose (180 μmol/kg&lt;sup&gt;-75&lt;/sup&gt;)</td>
<td>-2 h</td>
</tr>
<tr>
<td>* continuous (10 μmol/(h·kg&lt;sup&gt;-75&lt;/sup&gt;))</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>; Fasted-ctrl animals were only used for tissue and plasma analysis
<sup>2</sup>; The infusion rate of C8 was constant throughout the 24 h infusion for each animal, but varied between animals.

After the infusions were completed, pigs were anesthetized using an i.v. injection of pentobarbital (30 mg/kg body weight) and euthanized via exsanguination. Samples of the liver and semitendinosus muscle were collected, frozen in liquid nitrogen, and stored at -70°C until analysis.

5.2.3 Sample analysis

Urinary excretions were quantified (ml/h) and analyzed for dicarboxylic acids using an HPLC procedure modified after Rebouche et al. (1990). In brief, 250 μl of urine, after the addition of 50 nmol of azelaic acid (DC9) as the internal standard, was acidified using 50 μl of 2 N HCl and then saturated with NaCl. Dicarboxylic acids were subsequently extracted using ethyl-acetate. This extract was evaporated to dryness. Dicarboxylic acids were subsequently derivatized using 2-bromoacetophenone (2 mg) as the chromophoric reagent and diisopropylethylamine as a catalyst.
(7 μl, all reagents were obtained from Sigma, St. Louis, MO, unless noted otherwise) in 400 μl acetonitrile and 100 μl MeOH. The derivatization was performed using an ultrasonic water bath (model 3200, Brandson, Danbury, CT) which was preheated to 60°C.

The HPLC procedure used a C18 Ultrasphere IP column (250×4.6 mm, Beckman Instruments, Palo Alto, CA) and a C18 Absorbosphere HS guard column (Alltech, Deerfield, IL). The column was equilibrated with a mixture of 55% acetonitrile in water, which was maintained until 5 minutes after injection of the sample (20 μl). Over a period of 18 min the acetonitrile concentration was then increased linearly to 83%, which was maintained for 1 min. The column was subsequently re-equilibrated so that injections could be made every 35 minutes. The retention time of DCA was determined using standards (Sigma Chemicals, St. Louis, MO). UV absorbance of bromophenacyl esters of DCA was monitored at 245 nm, and the concentration of DCA in urine was calculated by comparing these peak areas to the peak area of the internal standard, azelaic acid.

Plasma (150 μl), urine (250 μl, from 1, 6, 11, and 16-24 h), liver (10 mg), and muscle (10 mg) were analyzed for carnitine esters using an HPLC procedure after Kempen & Odle (1992) with minor modifications. Protein was precipitated using a mixture of 75% acetonitrile and 25% methanol rather than 100% methanol. Eluent III (triethylamine-phosphoric acid in 80% acetonitrile and 20% water) was started at 4 min after injection and increased linearly over 26 min to 100%.

Sodium-hydroxide samples were analyzed for total CO₂ as described by Benevenga et al. (1992). Radioactivity in CO₂ was determined by mixing 0.5 ml of the sodium hydroxide solution with 1.5 ml NH₄Cl (1 M) and 18 ml scintillation cocktail (Research Products International, Mount Prospect, IL) using a Beckman LS6000IC liquid scintillation counter.

5.2.4 Data analysis

Based on the ratio of the specific radioactivity of the expired CO₂ to the specific radioactivity of the infused MCFA, an instantaneous fatty acid oxidation rate (FA-OR=MCFA-derived CO₂/total CO₂*100) was calculated, assuming that the fate of the [1-¹⁴C] carbon represented the fate of all carbons within the fatty acid molecule. The measured radioactivity in CO₂ was not corrected for the lag of expiration of CO₂ due to dilution in the body pool. This results in an underestimation of the actual oxidation rates of the infused [1-¹⁴C]-octanoate. This inaccuracy does not, however, pose a problem for the interpretation of the results which evaluated the relative change in fatty
acid oxidation rate elicited by carnitine supplementation.

The fatty acid oxidation and DCA excretion data obtained during the carnitine-free infusion period were fitted to a single pool exponential curve (Statgraphics, STSC, MD) which was determined by Benevenga et al. (1992) to accurately model the fatty acid oxidation data:

\[
FA-OR = A^*(1-e^{-kt+bo})
\]

where: FA-OR=fatty acid oxidation rate (%)

\(A=\)asymptotic maximal FA-OR at \(t=\)infinity (%)

\(k=\)fractional rate constant (min\(^{-1}\))

\(t=\)time (min)

\(t_o=\)lag of start of oxidation of MCFA (min)

To determine the carnitine response, the curve obtained was extrapolated to the carnitine infusion period; and the estimated values of MCFA oxidation and DCA excretion were compared with the actual data observed. The differences between observed values and estimated values were determined (carnitine response), and treatment differences were tested using analysis of variance using SAS (Steel & Torrie 1980).

Plasma and urine carnitine and acetyl-carnitine data were analyzed using the general linear models procedure of SAS with time points modelled as repeated measures and with treatment (C8-ctrl, C8-carn and VPA-C8-carn) as the main variable and fatty acid infusion rate as a covariate.

5.3 Results

The rates of fatty acid oxidation and CO\(_2\) production are illustrated for one animal in Fig. 5.1 as an example data set. These data were obtained from a VPA-C8-carn animal. The data from all animals are summarized in Fig. 5.2, in which the increase in fatty acid oxidation rate due to carnitine supplementation is expressed as a function of the fatty acid oxidation rate. The increase in fatty acid oxidation rate (determined using the fatty acid oxidation rate as a covariate) due to carnitine was 2.94 (C8-carn animals at a fatty acid oxidation rate of 41.9%) and 4.18 (VPA-C8-carn animals at a fatty acid oxidation rate of 40.3%); both significantly different (p <0.05) compared to the controls (decrease of 0.57 at fatty acid oxidation rate of 41.9%).
Fig. 5.1. Example graph of the fatty acid oxidation rate (FAOR=octanoate-derived CO₂/total CO₂*100) and the CO₂ production as a function of time for a VPA-C8-carn pig (see table 5.1). The squares represent the measured values of FAOR while the solid line is the curve as fitted to the FAOR of 0-16 h; the dashed line is the extrapolation of this curve. The FAOR deviated (p<0.05) from the extrapolated values, indicating a carnitine response.

The percentage of fatty acid infused which was oxidized to CO₂ under steady state conditions (by extrapolation of the single pool exponential curve) was not affected by valproate coinfection (p=0.11), although it tended to be lower (53.8±1.6% versus 56.6±1.2%) for the VPA-C8-carn pigs compared to the C8-ctrl and C8-carn (data from C8-ctrl and C8-carn pigs was combined
since both groups underwent the same treatment up to 16 h). The oxidation rates as a function of the fatty acids infusion rates are summarized in Fig. 5.3. Oxidation of fatty acids infused, independent of the rate of infusion, accounted for 55.6±1% (r²=0.995) of the fate of carbon for the fatty acids infused.

The data from the suberic acid (DC8) excretion in urine from 2 animals (C8-ctrl versus VPA-C8-carn) and the difference between the observed and estimated (as obtained from the extrapolated curve) values of DC8 excretion during the last 8 h of infusion across treatments for all animals are summarized in Fig. 5.4. The C8-ctrl animals increased DC8 excretion by 68% over that predicted by extrapolation while C8-carn animals decreased DC8 excretion by 8% (p<0.05 compared to controls), and the VPA-C8-carn animals decreased the DC8 excretion by 48% (p<0.05 compared to C8-ctrl & C8-carn).

The relationship between suberic acid and sebacic acid (nmol/h) excretion (adipic acid was virtually absent in urine) was determined by non-linear regression (based on one group of 6 animals, all infused at approximately 100% of their MR; treatments were equally represented) and found to be:

\[
DC10 \text{ excretion (nmol/h)} = 0.117 \times DC8 + 0.000086 \times (DC8)^2, \quad r^2=0.80,
\]

in which DC8 is the suberic acid excretion in nmol/h (data not shown).
Fig. 5.4. Left: example graph of the time course of suberic acid (DC8) excretion in two pigs. Both piglets were infused with octanoate for a period of 24 h. The VPA-C8-carn pig (solid bars) was coin infused with valproate for 24 h and with carnitine during the last 8 h of the experiment. DC8 excretion was determined using HPLC. Right: effect of carnitine on the suberic acid (DC8) excretion. The carnitine response was calculated as the relative increase over estimated (extrapolated data from 0-16 h) and the observed data for 16-24 h.

The relationship between the excretion of DC8 in absence of exogenous carnitine and the infusion rate of fatty acids was analyzed using nonlinear regression. For this analysis, it was assumed that the DC8 excretion from 8-16 hours represented a steady state. The values obtained for the total DC8 excretion per day (3*cumulative value for 8-16 h) was compared with the fatty acid infusion rate (expressed in mmol/d). The relationships between the fatty acid infusion rate (in mmol/d) and DC8 excretion (in nmol/d) were determined to be:

\[
\text{C8-ctrl & C8-carn} : DC8 = 5.52(\pm0.68) \times (\text{C8 infusion})^2 \quad (r^2=0.92)
\]

\[
\text{VPA-C8-carn} : DC8 = 9.74(\pm1.48) \times (\text{C8 infusion})^2 \quad (r^2=0.87),
\]

where C8 infusion is in mmol/d, and DC8 (suberic acid) excretion is in nmol/d.

Both equations were significant (p < 0.05), and the excretion of DC8 in the VPA-C8-carn pigs was significantly higher (p < 0.05) than the DC8 excretion in pigs only infused with octanoate.

The plasma carnitine and acetyl-carnitine concentrations for all animals are summarized in
Fig. 5.5, while the urinary carnitine and acetyl-carnitine excretion are summarized in Fig. 5.6. Prior to octanoate infusion (0 h samples), plasma acetyl-carnitine was 1.7±.8 μM, and free carnitine was 24±6 μM, suggesting a borderline carnitine deficiency (defined as a free carnitine concentration of less than 20 μM, Winter et al. 1987) as expected in newborn piglets (Kerner et al. 1984).

Both plasma carnitine and acetyl-carnitine concentrations increased significantly (7 resp 11 fold) upon carnitine supplementation (p < 0.05), but no significant differences were observed between C8-carn and C8-VPA-carn. In addition, plasma acetyl-carnitine concentrations were significantly correlated with the fatty acid infusion rate (see Fig. 5.8), being higher (p < 0.05) at high infusion rates in carnitine supplemented animals.

Fig. 5.5. Least square means (+ standard error of the mean) of plasma carnitine (top) and acetyl-carnitine (bottom) concentrations. Treatments are as shown in table 5.1. Average values (μM) for plasma free and acetyl-carnitine for 16-24 h were, respectively; C8-ctrl, 21.7 and 2.1; C8-carn, 146 and 23.6; VPA-C8-carn, 166 and 22.6. Carnitine esters were determined using HPLC.
Fig. 5.6. Least square means (+ standard error of the mean; determined within treatments) of urinary carnitine excretion (top) and acetyl-carnitine (bottom) excretion. Treatments are as shown in table 5.1. Average values for urinary free and acetyl-carnitine excretion (nmol/h) for 16-24 h were, respectively; C8-ctrl, 196 and 23; C8-carn, 919 and 1398; VPA-C8-carn, 334 and 420. Carnitine esters were determined using HPLC.

The carnitine esters in urine were mainly free and acetyl-carnitine. Carnitine supplementation increased carnitine and acetyl-carnitine excretion in C8-carn pigs (P<0.05), but not in the VPA-C8-carn pigs as compared with the C8-ctrl pigs. Other carnitine esters detected had retention times corresponding to: propionyl, butyryl, hexanoyl, octanoyl, and valproyl-carnitine (verified to be carnitine esters by radioisotopic carnitine exchange using carnitine acetyl-transferase and 14C-carnitine). The concentration of these esters was, using the sample preparation procedure as described, not sufficient to allow for consistent quantification.
Liver carnitine (Fig. 5.7) and acetyl-carnitine (Fig. 5.8) concentrations (nmol/g wet weight) were not affected by the infusion rates used, but the carnitine-supplemented pigs had significantly elevated carnitine and acetyl-carnitine levels (p < 0.05). No differences were observed (p > 0.10) in carnitine and acetyl-carnitine concentrations (nmol/g) between the C8-ctrl (194 resp. 1.68) and Fasted-ctrl group (119 resp. 0.9), and between the VPA-C8-carn (558 resp. 80) and C8-carn group (520 resp. 105).

Fig. 5.7. Carnitine concentration (nmol/g wet weight, average value for 16-24 h) in plasma, liver, and muscle (semitendinosus) as affected by the fatty acid infusion rate (FAIR=energy infused as a percentage of the metabolic rate of the recipient) and treatment (see table 5.1). The regression line as depicted for muscle carnitine are based on data from C8-carn & VPA-C8-carn animals and is as follows: 188 + 7.2*FAIR (r²=0.49, p<0.05). Carnitine was determined using HPLC.
Muscle carnitine concentrations were affected by both carnitine supplementation and the fatty acid infusion rate (see Fig. 5.7). Carnitine supplementation increased muscle carnitine almost two fold (from 493 to 873 nmol/g).

Muscle acetyl-carnitine levels were affected by the fatty acid infusion rate (see Fig. 5.8, p=0.051), but not by carnitine supplementation (non-supplemented pigs: 109 nmol/g versus supplemented pigs: 165 nmol/g, p > 0.10).

Acetyl-carnitine was determined using HPLC.
5.4 Discussion

Carnitine supplementation caused a similar increase in medium-chain fatty acid oxidation rates (see Fig. 5.2) as determined in a previous experiment (this experiment: 0.16-[fatty acid oxidation rate-18] versus 0.18*[fatty acid ox rate-20], Kempen & Odle 1993). This confirms that carnitine does stimulate octanoate oxidation in vivo when the fatty acid oxidation rate exceeds approximately 20% of the metabolic requirements of the animal, and furthermore, that the stimulatory effect of carnitine increases with increasing rate of fatty acid oxidation.

Fatty acid oxidation accounted for 55.6% of the fate for the fatty acids infused (see Fig. 5.3) under steady state condition. This value is higher than what was found in previous research (40%, Kempen & Odle 1993) but is in agreement with data from Odle et al. (1992). The reason for this difference is not clear. For a discussion regarding the fate of the remaining carbon, see Kempen & Odle (1993) and Odle et al. (1993).

Octanoate competitively inhibits the activation of valproate (Becker & Harris 1983, Draye & Vamecq 1987), since the affinity of the medium-chain acyl-CoA synthetase enzyme, which is required for the activation of valproic acid, is several fold higher for octanoate than for valproate (Draye et al. 1988). To avoid the inhibition of valproate activation, we infused a large (180 μmol/kg0.75) dose of valproate 2 h before the start of the octanoate infusion. Even though valproate was infused before octanoate, the valproate treatment did not significantly affect fatty acid oxidation. The lack of an inhibitory response to valproate in fatty acid oxidation contrasts with in vitro data reported by Bjorge et al. (Bjorge & Baillie 1985) and Ponchaut et al. (Ponchout et al. 1992), who showed decreases in C10 oxidation with valproate treated rat liver homogenates.

The suberic acid excretion increased due to the fatty acid infusion and increased even further (76%) in piglets infused with valproate. This suggests that valproate did have an inhibitory effect on fatty acid oxidation, resulting in a higher intracellular fatty acid concentration, and thus a higher production of DC8. If the action of valproate is limited to the liver, the only tissue with an intramitochondrial medium-chain acyl-CoA synthetase (Groot & Hulsmann 1973, Groot et al. 1976), then the lack of a response of valproate on fatty acid oxidation might simply be caused by a "dilution" effect. If the fatty acid oxidation rate in other tissues was unaltered (or even increased; muscle acetyl-carnitine, which was positively correlated with the fatty acid infusion and thus oxidation rate, was elevated in VPA-C8-carn (p=0.06) but not in C8-carn as compared to C8-ctrl pigs),
inhibition of liver fatty acid oxidation might have gone unnoticed under the conditions employed.

Suberic acid excretion was decreased after carnitine supplementation as compared to C8-ctrls. The decrease was larger in the VPA-C8-carn animals (p < 0.05). Surprisingly, after carnitine infusion, the DC8 production (5.08 versus 5.06 *fatty acid infusion²) as well as the fatty acid oxidation rates (61.1% versus 61.0%) were identical in both treatment groups (VPA-C8-carn versus C8-carn). The decrease in DCA excretion as observed after carnitine supplementation is in agreement with clinical observations that DC8 excretion is lowered after carnitine supplementation (Olson & Nelson 1989, Whyte et al. 1986). The role of carnitine in lowering DC8 excretion might be mediated via increasing the oxidation of octanoate, and therefore decreasing the intracellular concentration of octanoate in liver. (Olson & Nelson (1989) showed a decrease of plasma free fatty acid concentration in infants after carnitine supplementation.) Since the conversion of octanoate to DC8 is concentration dependent, a lower intracellular concentration of octanoate could potentially explain (a portion of) the lower excretion rate of DC8.

The dicarboxylic acid excretion in control animals did not reach a steady state during the 24 h infusion, as indicated by the increase in DC8 excretion during the last 8 h of the experiment versus the modelled data in C8-ctrl animals. This is surprising since the fatty acid oxidation rate did approach a steady state after approximately 8 h of octanoate infusion. A steady state fatty acid oxidation rate in our study implies also a steady state systemic fatty acid concentration since the maximum fatty acid oxidation rate was not imposed by metabolic limits, but rather by the fatty acid infusion rate. It is possible that the observed increase in DC8 excretion represents an adaptation mechanism to the continuous fatty acid load (Hare & Wahle (1985) showed an increased capacity for ω-oxidation in sheep after a fast).

The conversion rate of fatty acids infused to dicarboxylic acids excreted (which is not based on steady state conditions), was approximately 5 fold lower that what has been reported in vitro (Mortensen & Gregersen 1981). However, the average conversion rate as observed in this study (in the non-valproate infused animals) was 0.021±0.0034% of fatty acids infused, which was similar to the values as observed by Mortensen & Gregersen (1981) and Mortensen (1992) in vivo, who reported values of 0.019-0.030% and confirms that ω-oxidation is quantitatively not a major pathway.

The mechanism of action for carnitine on in vivo octanoate oxidation is still open for debate.
Observations in this experiment are that carnitine causes an increase in octanoate oxidation and a decrease in DC8 excretion. The effect of carnitine on octanoate oxidation has been suggested to be via its action on muscle tissue, where octanoate oxidation is carnitine dependent (Groot & Hulsmann 1973). It is, however, unlikely that muscle carnitine was limiting β-oxidation since the levels measured in the non-carnitine supplemented animals (493 nmol/g) were similar to liver carnitine after carnitine supplementation (540 nmol/g, which is the normal range as reported by Shenai et al. 1983) and high as compared to unsupplemented livers (194 nmol/g), while rates of β-oxidation in liver far exceed those of muscle per unit of weight (Ernster & Nordenbrand 1967). Indeed, Watmough et al. (1988) reported that the maximal CO₂ production in isolated muscle mitochondria was achieved in an environment containing 100 μM carnitine.

Tissue analysis revealed that the concentration of acetyl-carnitine in muscle was independent of carnitine supplementation, but rather correlated with the fatty acid infusion rate. It is possible that acetyl-carnitine was formed by peroxisomal β-oxidation (Vamecq & Draye 1987) and functions in the transfer of acetyl units from peroxisomes to mitochondria within the cell. The muscle acetyl-carnitine does not appear to diffuse into plasma, since, as shown in Fig. 5.8, muscle acetyl-carnitine in non-carnitine supplemented pigs was 70% of that in carnitine supplemented animals while plasma acetyl-carnitine did not increase and urinary acetyl-carnitine remained virtually non-detectable in the C8-ctrl pigs.

The increase in plasma acetyl-carnitine did, however, mirror the liver carnitine profile (Fig. 5.8). Liver acetyl-carnitine increased 50 to 60-fold upon carnitine supplementation, suggesting that the liver is the source of plasma and urinary acetyl-carnitine. The effects of carnitine on liver fatty acid oxidation could be mediated via the carnitine-dependent transport of medium-chain fatty acids into the mitochondria, potentially due to insufficient activation of medium-chain fatty acids by intramitochondrial medium-chain acyl-CoA synthetase as suggested by Stanley et al. (1983), which would make (a portion of) octanoate oxidation carnitine dependent. This would decrease the cytosolic fatty acid concentration by enhancing oxidation, and thus, decrease dicarboxylic acid production. Although the data are consistent with this notion, there appears to be a lower limit for fatty acid oxidation below which carnitine does not have any stimulatory effects. This might be explained by some baseline levels of carnitine in tissues but also by the broader chain-length specificity of the intramitochondrial short-chain acyl-CoA synthetases, as the name implies. Since
sufficient active intramitochondrial short-chain acyl-CoA synthetase is present at birth and since this short-chain acyl-CoA synthetase has some affinity for C8, low levels of C8 oxidation could occur in the absence of carnitine (Groot et al. 1976, Aas 1971).

Alternatively, carnitine could have a role in mediating the excess acetyl-CoA formed during uncontrolled β-oxidation of medium-chain fatty acids. Since our model species, the piglet, lacks sufficient ketogenic capacity (Adams & Odle 1993), excess acetyl-CoA formed (or lack of free CoA) is not detoxified (free CoA is not regenerated) via this pathway. Carnitine could potentially
Concluding remarks

Does carnitine have a role in medium-chain fatty acid metabolism?

The primary objective of the research presented in this thesis was to determine whether carnitine has a role in the oxidation of medium-chain fatty acids (MCFA) in our model species, the pig. From the data as presented, I would conclude that carnitine indeed has a role. However, the exact mechanism of this role and whether this role of carnitine is the same in other species and/or older pigs has not been not answered.

The newborn piglet is unique in several ways. First, the newborn pig does not make ketone bodies at rates comparable to other (ketogenic) species (Adams & Odle, 1993). This "metabolic disorder" may have several implications. Due to the absence of ketogenesis, β-oxidation has the potential to occur at a rate which exceeds the liver’s capacity to metabolize the acetyl-CoA formed. This potential increases when MCFA are fed or infused, since their metabolism bypasses the regulatory site of β-oxidation (carnitine palmitoyl transferase I). The newborn pig is, therefore, at risk for depleting intramitochondrial free CoA when MCFA are fed. Carnitine has the potential to counteract this depletion, and thus, improve the metabolic health of the subject (see Chapter 5).

A second, possible idiosyncrasy might be the age of the animals used. Several researchers (Shipp et al. 1982, Stanley et al. 1983) have shown that newborn guinea pigs lack intramitochondrial acyl-CoA synthetase activity. However, no data on medium-chain acyl-CoA synthetase activity and development after birth is available for other species.

The lack of (active) medium-chain acyl-CoA synthetase would render the activation of MCFA dependent on intramitochondrial short-chain acyl-CoA synthetases and extra-mitochondrial (not at the site of β-oxidation) acyl-CoA synthetases. Since the affinity of the intramitochondrial short-chain acyl-CoA synthetase is low for medium-chain fatty acids such as octanoate, a substantial amount of the MCFA would be activated extramitochondrially. These extramitochondrially activated MCFA are oxidized in a carnitine-dependent fashion (Groot & Hulsmann, 1973). The effect of carnitine under these conditions is dependent upon the capacity of the intramitochondrial acyl-CoA synthetase; if it can meet the requirements of the mitochondrion for activated fatty acids, then the carnitine dependent uptake of fatty acids is inhibited, alleviating the role of carnitine. It is more likely, however, that the capacity of the intramitochondrial short-chain acyl-CoA synthetase is not sufficient...
to meet the cell’s requirement for activated fatty acids, suggesting that MCFA oxidation would be carnitine dependent.

If none of these idiosyncrasies caused the effects observed, then carnitine will likely have a role in MCFA oxidation in several species. Currently, we do not know the answer to these questions.

Neonatal survival

As noted in the introduction, the focus of the research in our laboratory is to improve neonatal survival in commercial swine operations. The question addressed in this thesis is beyond the original focus of our lab, although the outcome may have some impact on neonatal survival. I think that it is recommendable to fortify a "neonatal survival kit" containing MCFA, with carnitine.

In addition to the role of carnitine in MCFA metabolism, several other observations are worth mentioning in light of the original focus of our laboratory.

During the first chamber studies, it was discovered that MCFA can be toxic when infused at rates exceeding 400 kJ/(d·kg^0.75). Toxicity of MCFA has been observed by other researchers who used large, extra-nutritional doses; but intoxication is not considered a risk factor when MCFA are fed to, e.g., infants and pigs. Obviously, this risk should be reconsidered, as we learned in infusion trials, and later through feeding trials. (This risk might, however, be limited to non-ketogenic species).

In the same chamber studies, it was also noticed that the metabolic rate of newborn, colostrum-deprived pigs was significantly lower (60%) than what it was estimated to be by Noblet & Le Dividich (1981) and Le Dividich & Noblet (1984). Piglets are born with a metabolic rate only slightly higher than the dam’s metabolic rate, which, due to the large difference in weight, is substantially lower (per kg body weight) than that of an average 1-2 kg mammal (Mellor & Cockburn, 1986).

The metabolic rate of newborn piglets is not increased by MCFA infusion (or feeding), which contradicts the assumption that MCFA are a thermogenic, obligatory fuel. This also means that the low metabolic rate of the piglet is not determined by a lack of fuel, since an excess of MCFA was provided and the oxidation of these MCFA reached up to 80% of the total metabolic requirement of the animal. If the piglet indeed has such a low metabolic rate, then the hypothesis that the metabolic rate of an animal is determined by its surface area can not be correct (Kleiber, 1975). Several
days after birth; these piglets do have a "normal" metabolic rate - twice what it was at birth, but their relative surface area did not change. It seems more plausible that the surface area of an animal would be determined by its adaptation to the environment.

Medium-chain fatty acids are unable to increase the heat production of animals. So how useful are MCFA as a nutritional supplement to improve survival? Since MCFA spare body stores, they can probably extend the life of the piglet. However, in a production environment the piglet will have several litter mates which will start suckling immediately after birth. These animals are growing and will increase their metabolic rate to a normal range within 2 days of life. Although the MCFA-supplemented pig can live longer, it is not better able to maintain his body temperature, and thus, compete with its larger litter mates. So, it is not likely that the fate of the MCFA-fed newborn pig has changed.

To improve piglet survival, the provision of only fat as an energy source is not sufficient. Instead, a substrate which stimulates the intestines to start functioning should be fed such that the piglet increases his heat production. In addition, energy should be provided which enables the piglet to maintain a higher heat production for a sufficient length of time; this will enable the piglet to start suckling. One possible avenue for future research might be to use a mixture of non-emulsified medium-chain triglycerides (containing butyrate and heptanoate as an energy source), proteins (such as casein and immunoglobulins to stimulate the digestive tract), and some caffeine (to raise the animals' heat production). In addition to proper nutrition, it is of major importance to provide the piglet with a (temporary) environment which will protect the piglet from excessive heat loss, or even increase the body temperature of the pig using, for example, microwave energy (Braithwaite et al. 1989).
Appendix A. Peroxisomes

Peroxisomes were discovered in 1954 by Rhodin. They are organelles with a single, easily permeable cell membrane, loaded with enzymes in a crystalline fashion (catalase). Their size varies considerably, from .2 to 5 \( \mu \text{m} \) and they form from budding (incompletely) off of the smooth endoplasmic reticulum with which they will still maintain channel-like contacts. The original role of peroxisomes was thought to be the detoxification of oxygen. The environment used to have a very low oxygen tension. When oxygen started to build up, organisms had to deal with oxygen as a toxic agent. Peroxisomes are capable of capturing oxygen, and making hydrogen peroxide out of it as follows: \( \text{O}_2 \) (oxidase, e.g. flavoproteins) \( \rightarrow \text{H}_2\text{O}_2 \) (catalase + \( \text{O}_2 \)) \( \rightarrow \text{2H}_2\text{O} \). The functions of peroxisomes in an oxygen dependent fashion, however, are not well understood (De Duve 1982).

Several mechanisms have been developed to study the peroxisomes. One of the most common used ones is the study of isolated peroxisomes. Peroxisomes are most often isolated by gradient centrifugation. Due to the very similar densities of mitochondria, lysosomes and peroxisomes, classical centrifugation turned out to be ineffective. The density of lysosomes can be altered by feeding the subject a non-ionic detergent, like Triton X-100 or Triton WR-1339. These compounds are absorbed by the lysosomes, and alter their density. Separation from the mitochondria is less easy. After gradient centrifugation for several hours, sufficient separation can be achieved. However, some mitochondrial contamination will remain present (Vamecq & Draye 1989).

To avoid isolation, specific reactions of the peroxisomes have been studied, like hydrogen peroxide formation (inhibiting catalase). The disadvantage of this system are several, including the disruption of normal processes, the lack of knowledge about the breakdown of hydrogen peroxide, and the damage done by accumulating hydrogen peroxide to normal cell functioning. Most studies employ mitochondrial inhibitors, like malonate, cyanide, rotenone or antimycin, or peroxisomal stimulators, like clofibrate or ciprofibrate (Vamecq & Draye 1989). Questions can be raised on the extrapolation of these results to in vivo because of their dramatic disruption of cell function, and of their side-effects (such as NAD depletion).

In 1976, Lazarow and de Duve discovered a \( \beta \)-oxidation system in the peroxisomes. They reported 3 enzymes, 2 of which similar to the ones in the mitochondria, and a bifunctional enzyme (De Duve 1982). The role of peroxisomal \( \beta \)-oxidation is chain shortening. The current concept
is, that very-long-chain fatty acids (LCFA) and unsaturated fatty acids are taken up by the peroxi-somes. There they will undergo several cycles of β-oxidation. The reaction products are then a medium-chain fatty acid, acetyl groups, NADH (possibly used to form hydrogen peroxide) and hydrogen peroxide. This is summarized in the reaction scheme as depicted in Fig. A.1 from Mannaerts & de Beer (1982).

![Fig. A.1. Peroxisomal β-oxidation (see text for details; Mannaerts & de Beer 1982)](image_url)

The current hypothesis is, that fatty acids undergoing peroxisomal β-oxidation are activated by acyl-CoA synthetase: FA + CoA (+ATP-Mg++) -> Acyl-CoA (the use of ATP makes the peroxisomes ATP dependent, and might increase their action in a way opposite to the mitochondria). This activation can happen after entry of fatty acids into the peroxisomes, or, more likely, upon entry into the peroxisomes (Mannaerts & de Beer 1982).

Three enzymes are involved in peroxisomal β-oxidation: acyl-CoA oxidase, enoyl-CoA hydratase/β-hydroxy acyl-CoA dehydrogenase and β-ketothiolase. The end products of peroxisomal β-oxidation are acetyl-CoA (or acetate or acetyl-carnitine), medium-chain fatty acids (MCFA) and H₂O. The role of this pathway is unknown. It makes LCFA more polar, and maybe a more preferable substrate for the mitochondria (Mannaerts & de Beer 1982).

The β-oxidation in the peroxisomes is not coupled to the synthesis of energy-rich phosphate bonds. The released electrons are captured in molecular oxygen, forming hydrogen peroxide. This hydrogen peroxide is broken down to water and oxygen. The result is a loss of energy but an increase in heat production. NADH which is produced might be transported to the cytosol,
and be involved in synthesis processes or can be converted to $H_2O_2$ and NAD. Detailed information is missing.

After the acyl-CoA undergo some cycles of $\beta$-oxidation, the resulting acetyl groups and the MCFA (decreasing affinity of enzyme for complex) will exit the peroxisome, making them available for the mitochondria, or for lipogenic processes/cholesterol synthesis. Within the peroxisomes, the CoA can be dissociated by an acyl-CoA hydrolase. Carnitine octanoyltransferase (specifity C6, activity towards C4-C16) has a function in the export of these compounds into the cytosol, and maybe in the uptake of these compounds by the mitochondria. Carnitine is supposedly not involved in uptake of the fatty acids into the peroxisomes.

The role of peroxisomes in fatty acid metabolism is not quantified very well. Vamecq & Draye (1989) assume that the majority of the LCFA are shortened in the peroxisomes to MCFA and acetyl-groups. Mannaerts & de Beer (1982) estimates that 2 to 30 percent of the acetyl groups is released in the peroxisomes. The number and the activity of peroxisomes can be stimulated by high fat diets, and certain drugs like clofibrate. After stimulation, the peroxisomes can be responsible for up to 50% of the $\beta$-oxidation (Cannon et al. 1982).

The loss of energy in the case of palmitate due to peroxisomal oxidation is 2-5 ATPs per $\beta$-oxidation cycle. The total loss is dependent on the number of $\beta$-oxidations taking place, and if the endproduct requires activation. Estimates range from 1 to 4 cycles of peroxisomal $\beta$-oxidation per fatty acid molecule, which translates into a loss of 2 to 28 ATP (total ATP yield from palmitate oxidation in mitochondria is 129).
Appendix B. Effect of infusion of medium-chain fatty acids of varying chain-length on dicarboxylic acid (DCA) excretion in newborn, colostrum-deprived piglets

Introduction

One of the problems associated with feeding medium-chain triglycerides is the associated production of dicarboxylic acids. Medium-chain triglyceride formulas are, however, not formulated to minimize the excretion of dicarboxylic acids. The experiment as described herein was designed to determine the effect of the chain length of medium-chain fatty acids (MCFA) on the excretion of dicarboxylic acids in urine.

Materials & methods

The experimental procedure used was similar to that described in Chapter 4 and 5. In brief (including the deviations from the protocols described previously), piglets were infused for a period of 12 h with either C7 (hexanoate, 2 animals), C8 (octanoate, 1 animal) or C10 (decanoate, 1 animal) at a rate to provide approximately 60% of the anticipated metabolic rate of the animals. Urine was collected quantitatively and analyzed for DCA using HPLC as described in Chapter 5.

Results

We experienced unforeseen problems with the infusion of C10. Animals infused with C10 had severe hemolysis leading to hemoglobinuria. Infusing C10 at lower rates (down to 30% of metabolic rate) did not prevent this problem. Due to the severe health problems we decided not to continue this line of investigation. Some of the results obtained are summarized below.

Infusion of C10 resulted in a 6 fold increase in total dicarboxylic acid excretion compared to C8. Octanoate infusion resulted in a 2-fold higher DCA excretion than C7. In addition, the chain-length of the DCA excreted was predominantly equal to the chain-length of the fatty acid infused when C10 or C8 were infused (see Fig. B.1). When, however, C7 was infused, the excretion of DC6 surpassed the excretion of DC7.
Discussion

Both the chain length as well as the infusion rate of the MCFA greatly affected the excretion rate and the excretion pattern of DCA in urine. Our data is in agreement with data from Gregersen (1983) and Mortensen & Gregersen (1981) which suggest that both C8 and C7 results in lower excretion of DCA than C10.

The observation that infusing C7 results in the excretion of DC6 at rates greater than DC7 implies that not the C7 itself but endogenous long-chain fatty acids are the main substrates for omega-oxidation as suggested by Gregerson (Gregersen et al. 1983), which would be in accordance with the chain-length specificity of the enzymes involved (Vamecq & Draye 1989). The absence of DC6 when C8 or C10 is fed suggests that DC6 is utilized by piglet under those circumstances to yield succinate. C7 upon β-oxidation yields propionyl-CoA which can be converted to succinyl-CoA. This excess of TCA cycle intermediates produced during C7 oxidation could potentially lead to the accumulation of DC6 (Rusoff et al. 1960).
Literature cited


Curriculum Vitae

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