REVIEW ARTICLE

Carnitine biosynthesis in mammals

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Carnitine is indispensable for energy metabolism, since it enables activated fatty acids to enter the mitochondria, where they are broken down via β-oxidation. Carnitine is probably present in all animal species, and in numerous micro-organisms and plants. In mammals, carnitine homoeostasis is maintained by endogenous synthesis, absorption from dietary sources and efficient tubular reabsorption by the kidney. This review aims to cover the current knowledge of the enzymological, molecular, metabolic and regulatory aspects of mammalian carnitine biosynthesis, with an emphasis on the human and rat.

Key words: butyrobetaine, fatty acid metabolism, hydroxytrimethyl-lysine, trimethyl-lysine.

INTRODUCTION

Carnitine (L-3-hydroxy-4-*N*,*N*,*N*-trimethylaminobutyrate) is an essential metabolite, which has a number of indispensable roles in intermediary metabolism. First, carnitine has an important role in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix, where β-oxidation takes place (Figure 1) [1,2]. Secondly, carnitine is involved in the

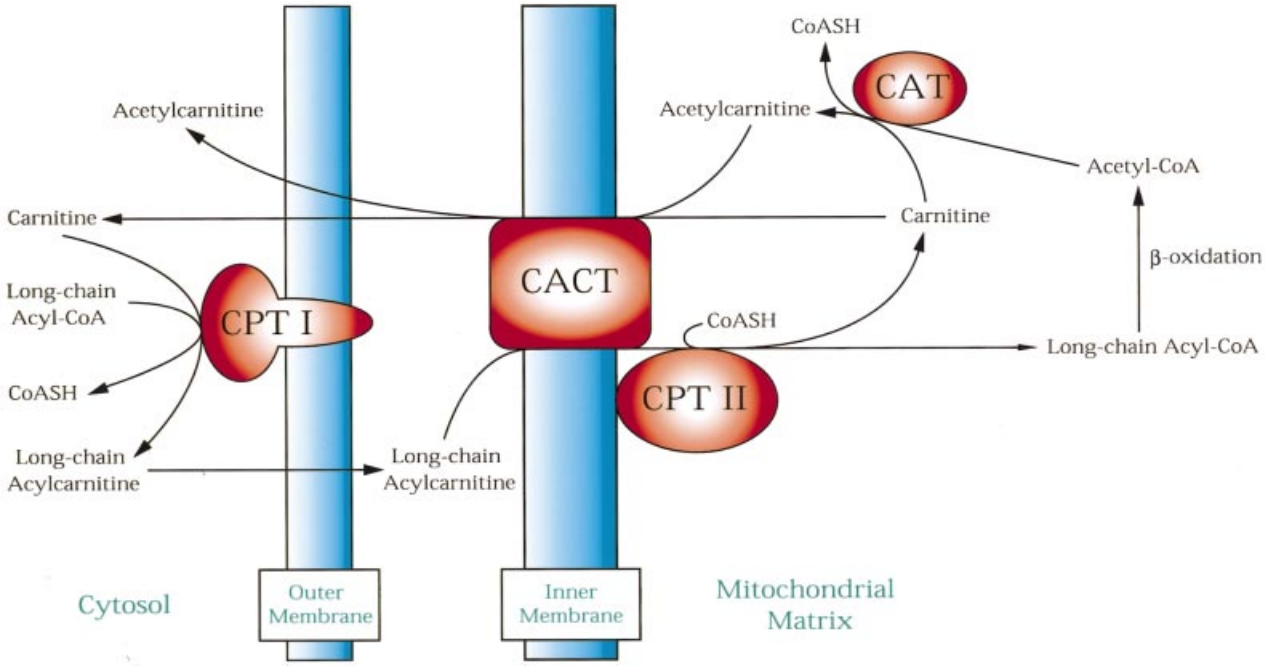

Figure 1 Function of carnitine in the transport of mitochondrial long-chain fatty acid oxidation and regulation of the intramitochondrial acyl-CoA/CoA ratio

Cytosolic long-chain fatty acids, which are present as CoA esters, are trans-esterified to L-carnitine in a reaction catalysed by carnitine palmitoyltransferase I (CPT I) at the mitochondrial outer membrane. In this reaction, the acyl moiety of the long-chain fatty acids is transferred from CoA to the hydroxyl group of carnitine. The resulting long-chain acylcarnitine esters are transported over the inner mitochondrial membrane via a specific carrier, carnitine-acylcarnitine translocase (CACT). At the matrix side of the mitochondrial membrane, the long-chain fatty acids are trans-esterified to intramitochondrial CoA, a reaction catalysed by carnitine palmitoyltransferase II (CPT II). The released carnitine can then leave the mitochondrion via CACT for another round of transport [1]. In the mitochondrial matrix, the enzyme carnitine acetyltransferase (CAT) is able to reconvert short- and medium-chain acyl-CoAs into acylcarnitines using intramitochondrial carnitine. These acylcarnitines can then leave the mitochondria via CACT. Through this mechanism of reversible acylation, carnitine is able to modulate the intracellular concentrations of free CoA and acyl-CoA.

Abbreviations used: ALDH9, aldehyde dehydrogenase 9; BBD, γ-butyrobetaine dioxygenase; CDSP, primary systemic carnitine deficiency; (H)TML, (3-hydroxy-)N^6-trimethyl-lysine; HTMLA, HTML aldolase; JVS, juvenile steatosis; OCTN2, organic cation transporter 2; PPARα, peroxisome-proliferator-activated receptor α; SHMT, serine hydroxymethyltransferase; TMABA, 4-*N*-trimethylaminobutyraldehyde; TMABA-DH, TMABA dehydrogenase; TMLD, TML dioxygenase.

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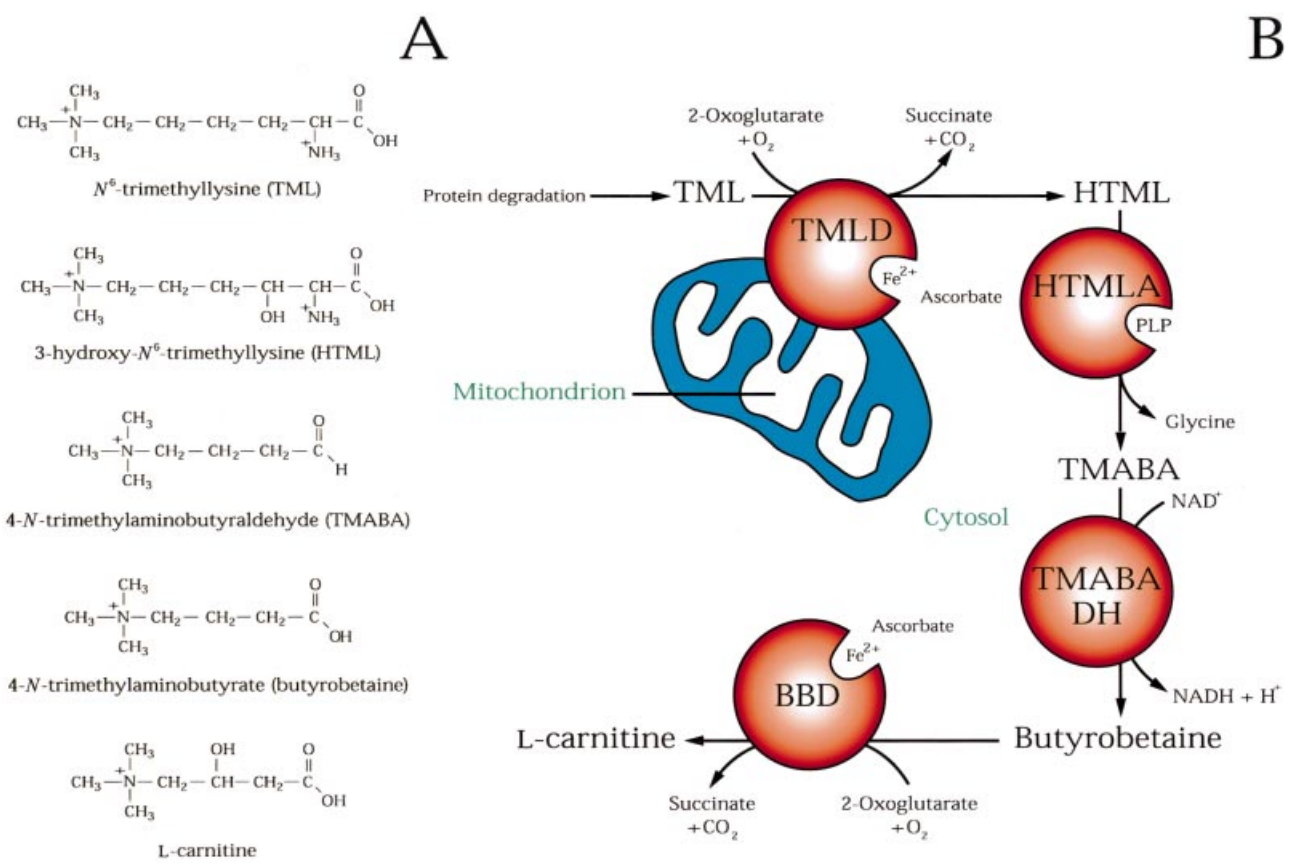

Figure 2 The carnitine biosynthesis pathway

(**A**) The chemical structures of the five carnitine biosynthesis metabolites. (**B**) Carnitine biosynthesis from TML. After release of TML by lysosomal protein degradation, this compound is hydroxylated by TMLD, after which the resulting HTML is cleaved by a specific aldolase, which uses pyridoxal 5′-phosphate (PLP) as a cofactor, into TMABA and glycine. Subsequently, TMABA is oxidized by TMABA-DH to form 4-*N*-trimethylaminobutyrate (butyrobetaine). In the last step, butyrobetaine is hydroxylated by BBD, yielding L-carnitine.

transfer of the products of peroxisomal β-oxidation, including acetyl-CoA, to the mitochondria for oxidation to CO_2 and H_2O in the Krebs cycle [3,4]. Other functions of carnitine include modulation of the acyl-CoA/CoA ratio [1,5], storage of energy as acetylcarnitine [6,5] and the modulation of toxic effects of poorly metabolized acyl groups by excreting them as carnitine esters [7,8]. Carnitine is present in most, if not all, animal species, and in several micro-organisms and plants [9–12]. Animal tissues contain relatively high amounts of carnitine, varying between 0.2 and 6 μmol·g^{-1}, with the highest concentrations in heart and skeletal muscle [6]. Although animals obtain carnitine primarily from the diet, most mammals are capable of synthesizing carnitine endogenously.

Carnitine is synthesized ultimately from the amino acids lysine and methionine. Lysine provides the carbon backbone of carnitine [13,14] and the 4-*N*-methyl groups originate from methionine [15]. In mammals, certain proteins contain N^6-trimethyl-lysine (TML) residues [16]. N-methylation of these lysine residues occurs as a post-translational event in proteins such as calmodulin, myosin, actin, cytochrome *c* and histones [17,18]. This reaction is catalysed by specific methyltransferases, which use *S*-adenosylmethionine as a methyl donor [16]. Lysosomal hydrolysis of these proteins results in the release of TML, which is the first metabolite of carnitine biosynthesis [19,20]. TML is first hydroxylated on the 3-position by TML dioxygenase (TMLD; EC 1.14.11.8) to yield 3-hydroxy-TML (HTML). Aldolytic cleavage of HTML yields 4-trimethylaminobutyraldehyde (TMABA) and glycine, a reaction catalysed by HTML aldolase (HTMLA; EC 4.1.2.'X'). Dehydrogenation of TMABA by TMABA dehydrogenase (TMABA-DH; EC 1.2.1.47) results in the formation of 4-*N*-trimethylaminobutyrate (butyrobetaine). In the last step, butyrobetaine is hydroxylated on the 3-position by γ-butyrobetaine dioxygenase (BBD; EC 1.14.11.1) to yield carnitine. The chemical structure of the intermediates and the enzymes of carnitine biosynthesis are shown in Figures 2(A) and 2(B) respectively.

Because an up-to-date review on carnitine biosynthesis does not exist, while in the past few years the knowledge concerning this pathway has expanded considerably, a review on this topic is required and warranted. The present review aims to describe the current knowledge on carnitine biosynthesis at the enzymological, molecular and metabolic level. First, the individual enzymes of the carnitine-biosynthesis pathway will be discussed, including the recent developments concerning the identification of the genes involved. Secondly, we will discuss the various metabolites of the carnitine-biosynthesis pathway, with an emphasis on their occurrence in biological fluids and on the means employed to determine their concentration. Thirdly, an overview of carnitine biosynthesis will be given for the human and rat. Finally, the transport of carnitine and its precursors will be discussed.

ENZYMES OF CARNITINE BIOSYNTHESIS

Several of the carnitine-biosynthesis enzymes have been isolated and characterized, although identification of the encoding genes has been realized only relatively recently [21–24]. The enzymes involved in carnitine biosynthesis, their cofactors and subcellular localization are depicted in Figure 2(B), and discussed below.

TMLD

Hulse and co-workers [25] were the first to demonstrate that rat liver mitochondria are capable of hydroxylating TML to produce HTML. The enzyme responsible for this conversion was shown to be a non-haem ferrous-iron dioxygenase, which requires 2-oxoglutarate, Fe^{2+} and molecular oxygen as cofactors [25–28]. In this class of enzymes, the hydroxylation of the substrate is linked to the oxidative decarboxylation of 2-oxoglutarate to succinate and CO_2. Molecular oxygen reacts at the active site of the enzyme to form an oxo-ferryl intermediate (Fe^{4+}=O), and this iron-bound oxygen atom is used to hydroxylate the substrate. The other oxygen atom is incorporated into 2-oxoglutarate, resulting in the formation of succinate and the release of CO_2 [29]. TMLD requires the presence of ascorbate (vitamin C) for enzymic activity, presumably to maintain the iron in the ferrous state. Reducing agents other than ascorbate are also effective (dithiothreitol, 3-mercaptoethanol), but ascorbate works best in each of the reactions [25,30].

In most experiments, TMLD activity is measured by using radiolabelled TML and counting the radioactivity of the product HTML after its isolation from the incubation medium by ion-exchange chromatography [25,28,30–33]. An alternative assay was reported by Davis [24], who used unlabelled TML and detected the product (HTML), after ion-exchange chromatography, by reversed-phase HPLC using pre-column derivative formation with *o*-phthalaldehyde. A new method was developed recently to measure the concentration of the carnitine-biosynthesis metabolites in urine using tandem MS, and this was used to measure TMLD activity in tissue homogenates.

In both humans and rats, TMLD activity is present in liver, skeletal muscle, heart and brain, but the highest activity is found in the kidney [28,31]. TMLD was purified previously from bovine kidney by Henderson and co-workers [30,33], who reported that the pure enzyme was very unstable, losing all activity overnight. TMLD has been purified recently from rat kidney, and it was found that the presence of 2 mM ascorbate, 5 mM dithiothreitol and 100 $g \cdot l^{-1}$ glycerol was essential for preserving the enzymic activity during the later purification steps and subsequent storage at -80 °C [24]. TMLD was characterized kinetically, and gel-filtration and blue native PAGE analysis showed that the native enzyme is a homodimer with a mass of approx. 87 kDa [24]. The sequence of two internal peptides of the purified enzyme was determined by quadruple time-of-flight MS. This sequence information, in combination with the data available in the expressed sequence tag database, led to the identification of a rat cDNA of 1218 bp encoding a polypeptide of 405 amino acids with a calculated molecular mass of 47.5 kDa. Using the rat sequence, the authors also identified the homologous cDNAs from human and mouse. Heterologous expression of both the rat and human cDNAs in COS cells confirmed that they encode TMLD [24]. The human TMLD gene is localized at Xq28.

Subcellular localization experiments indicated that the enzyme is associated predominantly with mitochondria [25,27] in contrast with the other three carnitine-biosynthetic enzymes, which are cytosolic. Recently, the mitochondrial localization of TMLD was confirmed by experiments using Nycodenz density-gradient analysis to resolve the different subcellular organelles [24]. The fact that TMLD is localized in mitochondria is remarkable, since the other three enzymes of the carnitine biosynthetic pathway are localized in the cytosol (Figure 2B). The submitochondrial localization of TMLD will have implications for the substrate-flow and regulation of carnitine biosynthesis. Indeed, if TMLD is localized in the mitochondrial matrix, the existence of a transport system to shuttle its substrate (TML) and product (HTML) over the inner mitochondrial membrane would be required. In contrast, if TMLD is present in either the inner membrane space or the outer mitochondrial membrane, no transport system would be needed since the outer mitochondrial membrane is permeable for small molecules. This question needs to be resolved in the future.

HTMLA

Very little is known about HTMLA, which catalyses the aldolytic cleavage of HTML into TMABA and glycine. Rebouche and Engel [31] reported that, in human tissues, HTMLA activity is found almost exclusively ($> 90\,\%$) in the soluble fraction. The highest activity was found in liver, but activity varied greatly, ranging from 8 to 140 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein. HTMLA might be identical to serine hydroxymethyltransferase (SHMT; EC 2.1.2.1), since it has been shown that SHMT purified from rabbit liver acts upon HTML, yielding TMABA and glycine [30,33]. SHMT catalyses the tetrahydrofolate-dependent interconversion of serine and glycine, a reaction that generates one-carbon units for methionine, thymidylate and purine biosynthesis [34]. SHMT also catalyses the aldol cleavage of other β-hydroxyamino acids in the absence of tetrahydrofolate [35], including HTML. Two isoforms of SHMT are present in eukaryotic cells: one localized in the cytoplasm and one localized in mitochondria. In humans, the gene encoding the cytosolic SHMT is located on chromosome 17p11.2, and the gene encoding the mitochondrial isoenzyme is on chromosome 12q13.2 [36]. The human cytosolic SHMT is expressed predominantly in the kidney, liver and skeletal muscle, whereas mitochondrial SHMT is expressed ubiquitously [34]. If HTMLA is identical with one of the two SHMTs, the cytosolic isoenzyme is the most likely candidate, since cytosolic SHMT is expressed predominantly in tissues reported to contain HTMLA activity and HTMLA is localized in the cytoplasm [31]. Like many aldolases, SHMT uses pyridoxal 5′-phosphate, a derivative of pyridoxine (vitamin B_6), as a cofactor. The involvement of a pyridoxal 5′-phosphate-requiring enzyme in carnitine biosynthesis is supported by the observation that synthesis of butyrobetaine and carnitine from protein-bound TML is inhibited by 1-amino-D-proline, an antagonist of vitamin B_6. This compound restricted carnitine biosynthesis by as much as 60–80 %, and leads to the accumulation of HTML [37]. Furthermore, rats maintained on a vitamin B_6-deficient diet showed a significant decrease in carnitine levels in extrahepatic tissues. Moreover, when these rats were fasted for 3 days, liver carnitine levels were significantly lower, as compared with fasted control rats [38]. Repletion of vitamin B_6 resulted in normalization of the carnitine levels in all tissues, supporting further the requirement of this vitamin in carnitine biosynthesis [38]. Whether HTMLA is identical with SHMT, however, remains to be established.

TMABA-DH

TMABA-DH, which catalyses the dehydrogenation of 4-*N*-trimethylaminobutyraldehyde to butyrobetaine, was first isolated by Hulse and Henderson [39] from the cytoplasmic fraction of bovine liver. No activity was detected in either the mitochondrial or microsomal fractions. The same group also reported puri-

fication of this enzyme from rat liver [40]. TMABA-DH has an absolute requirement for NAD^+, and its activity is easily measured spectrophotometrically or fluorimetrically by following the appearance of NADH [39]. In human tissues, the rate of TMABA dehydrogenation is highest in liver, substantial in kidney, but low in brain, heart and muscle [31]. The purification and characterization of TMABA-DH from rat liver cytosol and the identification of the corresponding rat cDNA was reported recently [23]. With this information, the homologous mouse and human cDNAs were also identified. Upon expression of the rat cDNA in *Escherichia coli*, high levels of TMABA-DH activity could be measured in cell lysates, which confirmed its identity as TMABA-DH. The translated coding sequence of rat TMABA-DH cDNA is highly homologous with that of the previously identified human aldehyde dehydrogenase 9 (*ALDH9*, EC 1.2.1.19) enzyme, which is mapped to 1q22–23 (gene name: *ALDH9A1*) [41–43]. This cytosolic ALDH has been reported to act on substrates that resemble TMABA, including 4-aminobutyraldehyde and 2-trimethylaminoethanal (betaine aldehyde). The resulting products of ALDH9, 4-aminobutyrate [the neurotransmitter γ-aminobutyric acid ('GABA')] and betaine, have been implicated in various cellular functions [42–46]. ALDH9 is predominantly expressed in the liver, kidney, heart and muscle [43,46], which are tissues that also contain high levels of TMABA-DH activity [31]. Heterologous expression of ALDH9 in *E. coli* showed that the recombinant protein had the highest activity with TMABA as substrate. In addition, comparison of the kinetic properties for a variety of substrates of rat TMABA-DH with heterologously expressed human ALDH9 showed that these enzymes have highly similar substrate specificities. Therefore, ALDH9 is most probably the human TMABA-DH [23].

BBD

BBD catalyses the stereospecific hydroxylation of butyrobetaine to L-carnitine [47]. Lindstedt and colleagues [48–50] were the first to partially purify a BBD from *Pseudomonas* sp. AK 1, a bacterial strain that can grow on butyrobetaine as the sole source of carbon and nitrogen. They showed that BBD activity was stimulated considerably by 2-oxoglutarate, and that the enzyme requires molecular oxygen, Fe^{2+} and ascorbate for activity; furthermore, this activity was also present in rat liver homogenates. Experiments using an atmosphere of $^{18}O_2$ showed that the enzyme incorporates one atom of molecular oxygen into carnitine and the other into succinate, which demonstrated that BBD, like TMLD, is a dioxygenase [51]. BBD has been purified from various sources, including rat liver [52,53], calf liver [54], human kidney [55] and the bacterium *Pseudomonas* [56]. The complete primary structure of the *Pseudomonas* sp. AK 1 BBD was determined by Edman degradation [57]. Both the *Pseudomonas* sp. AK 1 and bovine enzymes are homodimers of two 43 kDa subunits [56,54]. Lindstedt and Nordin [58] showed by isoelectric focusing and column chromatography that BBD from the human kidney, rat and calf liver are present in three isoforms. However, these results could not be reproduced with purified rat liver BBD, which was eluted as a single peak from a chromatofocusing column used for purification [53]. The significance of the observations of Lindstedt and Nordin remains to be established.

In all mammals studied, BBD is localized in the cytosol [50,52,53,55,59], although one group reported the presence of BBD activity in peroxisomes, which could be stimulated by clofibrate, a peroxisome proliferator [60]. Since these results have never been reproduced, additional experiments are needed to resolve whether BBD is also present in peroxisomes.

Simkhovich and co-workers [61] discovered that 3-(2,2,2-trimethylhydrazinium)propionate (mildronate), which has cardioprotective properties during ischaemia, is a competitive inhibitor of BBD. The cardioprotective effect is proposed to be based on a lowering of the carnitine levels in the heart, which results in inhibition of fatty acid oxidation, decreased levels of harmful long-chain acylcarnitines and conservation of ATP [62,63]. It has been shown recently that the reduction of tissue carnitine levels is not based solely on BBD inhibition, since mildronate also inhibits tubular reabsorption of carnitine in the kidney, which results in carnitine loss through urinary excretion [64–66].

BBD activity is usually measured radiochemically using labelled butyrobetaine [67]. The enzyme activity can also be determined by measuring the butyrobetaine-dependent release of $[^{14}C]CO_2$ that is produced from the decarboxylation of 2-oxo-[1-^{14}C]glutarate to succinate. This method, however, requires the measurement of butyrobetaine-independent activity, since the mitochondrial 2-oxoglutarate dehydrogenase complex also produces CO_2 from 2-oxoglutarate. Alternatively, BBD activity can be measured using a two-step procedure in which carnitine produced from unlabelled butyrobetaine is measured in a radioisotopic assay [68,21]. The disadvantage of this assay is that, when tissue homogenates are used, the endogenous carnitine content also needs to be determined.

In mammals, BBD is expressed differentially, and its activity has been found in liver, kidney, brain and possibly in testis and epididymis, but not in other tissues. Butyrobetaine is hydroxylated readily to carnitine in kidney extracts from human, cat, cow, hamster, rabbit and Rhesus monkey sources, and exceeds or equals the BBD activity in the corresponding liver extracts. In contrast, BBD activity is not present, or only at very low levels, in the kidneys of Cebus monkeys, sheep, dogs, guinea pigs, mice and rats [55,59,69,70], in which BBD activity is predominant in the liver. The reason for this species-dependent difference in kidney/liver expression of BBD is not clear. There does not appear to be any evolutionary pattern, since even very closely related species, like the Rhesus and Cebus monkeys, already exhibit a different pattern of expression. Erfle [59] reported BBD activity in sheep muscle. However, this could not be reproduced by Cederblad and co-workers [71]. In contrast with other mammals, the human brain has been shown to contain some BBD activity [31]. The human BBD cDNA was recently identified and shown to contain an open reading frame of 1161 bp, which encodes a protein of 44.7 kDa (the corresponding *BBOX1* gene is localized on 11q14–15). Using the BBD cDNA, it was demonstrated by Northern blot analysis that BBD is expressed in kidney (at a high level), liver (at a moderate level) and also in brain (to a very low level) [21].

There is some evidence to suggest that BBD activity is also present in rat testis and epididymis [72–76]. This was supported by data from Galland and co-workers [22], who identified the rat BBD cDNA and showed that the BBD mRNA is present in liver, testis and epididymis. The size of the mRNA in testis and epididymis, however, is significantly larger than in liver (1.9 kb) and differs from testis (3.5 kb) and epididymis (4.5 kb). These either represent alternatively spliced BBD mRNAs or non-specific cross-hybridizations. Other reports, in which radioactive butyrobetaine or carnitine was administered to rats, showed that the cauda epididymis has a high capacity to take up carnitine, but not to synthesize it from butyrobetaine [75,77]. The capacity of (rat) testis/epididymis to synthesize carnitine thus remains controversial.

Galland and co-workers [22] also investigated the expression of BBD in the liver during development. The BBD mRNA

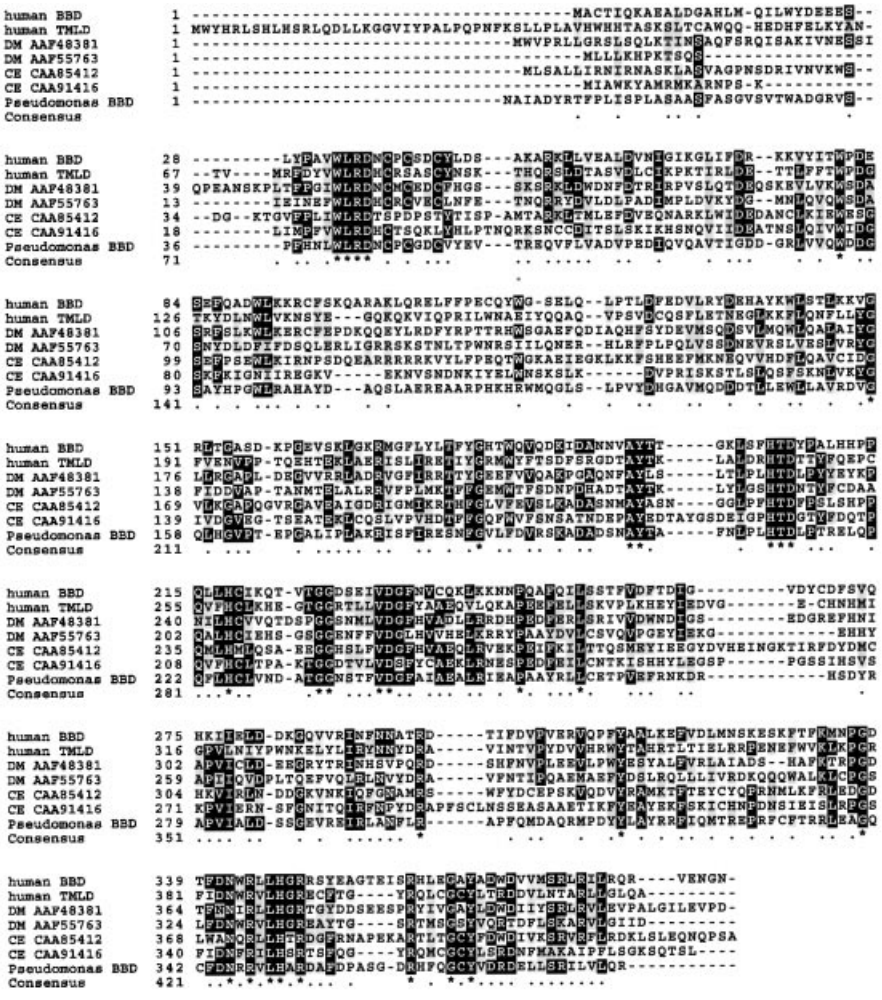

Figure 3 Sequence comparison of human TMLD and BBD with homologues from other organisms

For sequences without a confirmed function, the GenBank® accession number, preceded by an abbreviation of the organism of origin, is used as sequence name. DM, *D. melanogaster*; CE, *C. elegans*. The *Pseudomonas* sp. BBD is derived from strain AK 1 and has GenBank® accession number P80193. Highly conserved residues (black boxes) and residues conserved in more than 50% of the sequences (dark grey boxes) are highlighted.

appeared after weaning, and reached maximal values at the adult stage. These data are in agreement with those of Hahn [78], who showed that BBD activity in liver homogenates increased from low values in the fetus to adult values on the eighth day after birth. In human liver, BBD activity is also low at birth, and increases to adult values during puberty. However, kidney BBD activity is already present at birth [79].

Homologues in other organisms

Similarity searches in the increasing numbers of complete genome sequences available have shown that homologues of carnitine biosynthesis enzymes also exist in organisms other than humans, rats and mice. A comparison of the sequences of human TMLD and human BBD with each other shows that these enzymes share considerable similarity and, since no other homologous proteins are present in the current assembly of the human genome, they appear to form a separate family of 2-oxoglutarate-dependent, non-haem ferrous iron dioxygenases. This is supported by the fact that, when either of the two sequences is used as query to search the non-redundant database using the BLASTp-algorithm, only two to four homologues were found per organism, which include *Caenorhabditis elegans* and *Drosophila melanogaster*. An alignment of a selection of BBD/TMLD homologues found in different organisms is shown in Figure 3.

A BLASTp search in the *C. elegans* Wormpep database using either human BBD or human TMLD as the query yields two homologues, CAA85412 and CAA91416 (both corresponding genes are localized on chromosome II). The protein CAA91416 has higher homology with TMLD, whereas CAA85412 is more homologous with BBD.

When the same search was performed in the *D. melanogaster* genome, four homologues were found. Two of these proteins, AAF48381 and AAF45580, both contain a putative mitochondrial targeting sequence (MitoprotII; [80]) and their corresponding genes are both present on the X-chromosome. The genes of the two other *D. melanogaster* homologues, AAF58383 and AAF55763, are localized on chromosomes 2R and chromosome 3R respectively, and the deduced proteins do not have a putative mitochondrial targeting sequence. If, in *D. melanogaster*, TMLD also is a mitochondrial protein, the two former proteins are the most likely candidates to code for TMLD, whereas the

latter two proteins could represent BBD. The presence of TMLD/BBD homologues in *D. melanogaster* and *C. elegans* suggest that both organisms are capable of synthesizing carnitine. It remains to be established, however, whether these genes indeed code for enzymes of carnitine biosynthesis, and what the function of carnitine is in these organisms. Since these organisms are relatively easy to manipulate genetically, disruption of these homologous genes is possible, and is likely to provide further insight into their functions.

METABOLITES OF CARNITINE BIOSYNTHESIS

Several methods have been described to measure the concentration of the carnitine biosynthesis metabolites in biological fluids and tissues. These methods, and the concentration of the metabolites in plasma and urine, are described below.

TML

Kakimoto and Akazawa [81] were the first to identify TML in human urine. They isolated the basic amino acid fraction by ion-exchange chromatography, and analysed this by standard amino acid analysis. In general, all methods to assay TML in either plasma, urine or tissues samples use the same sample work-up. After protein removal from the sample, TML is purified by ion-exchange chromatography followed by (ion-pair) HPLC analysis with pre- or post-column derivative formation and fluorimetric detection [82–86]. Agents used for the derivative formation of TML include *o*-phthalaldehyde [83–85], phenylisothiocyanate [86] and 1-fluoro-2,4-dinitrobenzene [82]. More recently, a method to measure TML in plasma by tandem MS has been described [87]. This method uses two subsequent derivative-formation steps, propylation and acetylation, to circumvent the interference of homoarginine, followed by tandem MS analysis. In our laboratory, we recently developed a fast and easy method to determine the concentrations of the metabolites of the carnitine biosynthesis in urine. Without prior purification, the urine sample is derivatized with methyl chloroformate, followed by separation of the analytes by reversed-phase ion-pair HPLC using heptafluorobutyric acid as an ion-pairing agent, and detection by electrospray tandem MS. With this method, TML, HTML, butyrobetaine and carnitine can be quantified in a single analysis (unpublished results). This new method is highly reproducible, and has a detection limit of 0.25 pmol for each compound. This method will be adapted to measure the carnitine-biosynthesis metabolites in plasma and cells/tissues.

The concentration of TML in plasma is relatively constant in both human [85,87,88] and rat [83,89], ranging from 0.2 to 1.3 μM. Plasma levels of TML have been shown to correlate with body mass [88]. In man, urinary excretion of TML is proportional to that of creatinine, and TML is not reabsorbed by the kidney [90,91]. In contrast, the rat is capable of tubular reabsorption of TML [89,92]. Urinary TML concentrations in man have been reported to range from 2 to 8 μmol·mmol of creatinine^{-1} [81,85,90,93,94].

HTML

The presence of HTML in plasma has never been reported, and its urinary excretion has only very recently been investigated in our laboratory. With a urinary excretion of 0.45 ± 0.15 μmol·mmol of creatinine^{-1}, HTML shows a profile similar to that of TML, which is proportional to creatinine excretion, and this suggests that HTML, like TML, is not reabsorbed by the human kidney (F. M. Vaz, B. Melegh, J. Bene, D. Cuebas, D. A. Gage, A. Bootsma, P. Vreken, A. H. van Gennip, L. L. Bieber and R. J. A. Wanders, unpublished work).

Butyrobetaine

Previously, the concentration of butyrobetaine in plasma and tissues was determined by isolating butyrobetaine via HPLC or ion-exchange chromatography, and using BBD to convert it into carnitine, which could be quantified readily by established procedures [95,96]. Others have reported methods where butyrobetaine is derivatized with 4′-bromophenacyl trifluoromethanesulphonate, followed by HPLC analysis with UV detection [97,98]. These methods, however, are rather labour-intensive and require considerable amounts of sample. More recently, Sawada and colleagues [87,99] have described an assay based on tandem MS to measure butyrobetaine in plasma. The use of this technique makes prior purification of butyrobetaine unnecessary. In addition, this method requires a small amount of sample (20 μl of plasma; [87,99]), and is considerably more sensitive than previous methods. No assay has been described to measure the tissue content of butyrobetaine by tandem MS. In humans, the level of butyrobetaine in urine is low (≈ 0.3 μmol·mmol creatinine^{-1}; F. M. Vaz, B. Melegh, J. Bene, D. Cuebas, D. A. Gage, A. Bootsma, P. Vreken, A. H. van Gennip, L. L. Bieber and R. J. A. Wanders, unpublished work) compared with the concentrations in plasma (4.8 μM [96]; 1.8 μM [87,99]). This can be explained by the high activity of BBD in human kidney, which converts most of the butyrobetaine into carnitine. Furthermore, butyrobetaine is reabsorbed efficiently by the renal tubules, which lowers further the urinary excretion of butyrobetaine.

Carnitine

Numerous methods have been developed to determine the carnitine concentration in biological fluids and tissues. Since the first assay for carnitine using *Tenebrio molitor* larvae, several (more convenient) assays have been published using enzymic and radiochemical methods [100]. A method which has been used extensively is based on the conversion of carnitine into [^{14}C]acetylcarnitine by carnitine acetyltransferase ('CAT'), using [^{14}C]acetyl-CoA as substrate [71]. At present, the most common method to determine (acyl)carnitine concentrations in biological fluids employs tandem MS [101,102]. This procedure is fast, sensitive and requires a small amount of sample (< 100 μl).

The concentration of carnitine in plasma from both humans and rats is age- and sex-dependent. In humans, the plasma carnitine concentration increases during the first year of life (from ≈ 15 to ≈ 40 μM), and remains the same for both sexes until puberty [103–107]. From puberty to adulthood, plasma carnitine concentrations in males increase and stabilize at a level that is significantly higher than those in females (50 compared with 40 μM) [104,108,109]. This suggests that sex hormones have a role in the regulation of carnitine plasma concentrations [104,108]. The difference in the rat is even more pronounced, in which the adult male has a plasma carnitine concentration that is more than twofold higher as compared with females (50 versus 20 μM).

Like butyrobetaine, carnitine is reabsorbed efficiently by the kidney. However, urinary carnitine excretion is largely dependent on the diet, and the kidney has been shown to adapt to a higher carnitine intake by reducing the efficiency of carnitine reabsorption [92,110]. This results in a variable urinary carnitine excretion, with values of 15 ± 12 μmol·mmol of creatinine^{-1} [79,111]. Like the plasma carnitine concentration, urinary ex-

cretion in the rat has also been shown to be sex- and age-dependent. Male rats excrete less carnitine than female rats, which also could account for the different plasma carnitine concentrations between sexes [103].

CARNITINE BIOSYNTHESIS IN THE RAT

Sites of carnitine biosynthesis

Most of the research on carnitine biosynthesis has been performed in the rat. The primary site of carnitine biosynthesis in this animal is the liver, since this is the only tissue which contains BBD activity. Although testis has been reported to have a limited capability to convert butyrobetaine into carnitine, it remains unclear whether BBD activity is present in the testis [22,72–76]. Even if testis is capable of carnitine synthesis, the contribution to total carnitine synthesis will be small, which is supported by the fact that when the liver is excluded from the circulation, no conversion of labelled butyrobetaine into carnitine is observed [112].

Experiments by Tanphaichitr and Broquist [74] led to the assumption that all rat tissues produce butyrobetaine from TML, after which butyrobetaine is transported to the liver for conversion into carnitine. Subsequently, Carter and Frenkel [113] showed that, in normal rats, [*methyl*-^{3}H]TML administered intravenously rapidly (15–60 min) accumulated in the kidney, and was converted into butyrobetaine and HTML. After longer time periods (60–240 min), labelled carnitine appeared in the liver, while the hepatic levels of radiolabelled TML levels remained low. Bilateral nephrectomy resulted in a marked decrease in the incorporation of label into the liver, showing that initial conversion of TML into butyrobetaine occurs predominantly in the kidney and that, after transport to the liver, butyrobetaine is converted into carnitine [113]. These experiments also suggested that the liver has a low capacity to take up TML from the circulation, in contrast with the kidney, which appears to act as a scavenger of TML. The results obtained by Carter and Frenkel were confirmed by subsequent vascular perfusion experiments with the liver, kidney and small intestine [114]. Both the small intestine and kidney were capable of absorbing TML and HTML, and converted both compounds into butyrobetaine, but not into carnitine. TML and HTML were not taken up readily by the liver. In contrast, TMABA and butyrobetaine were absorbed rapidly by the liver and converted into carnitine [114]. After synthesis, carnitine is released into the circulation by the liver, primarily as acetylcarnitine [115,116], and imported into tissues.

In all the experiments described above, exogenous TML was used, which was introduced via the circulation. Circulatory TML is metabolized primarily by the kidney [113,114,117]. Normally, TML is released from proteins intracellularly within lysosomes and converted into butyrobetaine in the tissue of origin. From experiments in which N^6-[*methyl*-^{14}C]TML-labelled asialofetuin (a glycoprotein that is rapidly taken up into the liver cells and degraded in lysosomes) was injected intravenously into rats, it was shown that the labelled TML residues of this protein were indeed efficiently ($> 56\,\%$) converted into carnitine [19,20]. However, with another labelled protein, agalacto-orosomucoid, only 18 % of the radioactivity was converted into carnitine and 70 % of the radioactivity was released into the medium as TML [20]. Therefore, Rebouche [118] suggested that part of the intracellularly generated TML is converted into butyrobetaine in the tissue of origin, and the rest is released into the circulation. The kidney would then act as a scavenger of circulating TML, since this organ (at least in the rat) actively reabsorbs TML and has a high capacity to convert it into butyrobetaine.

Regulation of carnitine biosynthesis

Administration of butyrobetaine or TML to rats resulted in markedly increased urinary carnitine excretion (65- and 100-fold respectively), as well as increased levels of tissue carnitine [92,119]. This suggests that hydroxylation of either butyrobetaine or TML is not rate-limiting for carnitine biosynthesis. This observation led Rebouche and co-workers [92] and Davis and Hoppel [89] to propose that the availability of TML, which is determined by the extent of peptide-linked lysine methylation and the rate of protein turnover, limits the rate of carnitine biosynthesis. Liver and muscle together produce approx. 2 μmol of TML in 24 h from protein breakdown [118]. The carnitine produced by an adult rat per day has been estimated to be approx. 3 μmol [108]. Since liver and muscle together account for about one-seventh of whole-body protein turnover, total protein turnover provides sufficient substrate for carnitine biosynthesis ([118], and references therein).

Experiments in which carnitine and its precursors were administered to rats suggested that the metabolites of carnitine biosynthesis regulate the activity of the biosynthetic enzymes to some extent. Hepatic BBD activity in rats fed on a 1 % carnitine-supplemented diet was reduced significantly (37 %) when compared with the activity in livers of rats fed on a non-supplemented diet. In contrast, in rats fed 1 % (but not 0.1 %) butyrobetaine, the specific activity of BBD was increased by 57 %. Renal TMLD specific activity was unaffected by both carnitine and butyrobetaine [119]. In the normal diet, the carnitine and butyrobetaine content is much lower, and it is therefore probable that, under physiological conditions, feed-back inhibition by carnitine and/or stimulation of BBD activity by butyrobetaine is not an important regulatory mechanism of carnitine biosynthesis. The high levels of carnitine synthesis from exogenous carnitine precursors suggest that the enzymic capacity to synthesize carnitine from TML and butyrobetaine is much greater than is usually utilized. This is in agreement with the view that only the availability of TML is rate-limiting for carnitine biosynthesis.

By an unknown mechanism, long-term starvation of rats causes a considerable increase in liver carnitine levels, which parallels the ketogenic capacity of the liver [1,120,121]. During fasting, urinary levels of TML fall to 2–6 % of the fed values [89,122]. Urinary excretion of carnitine and butyrobetaine is also decreased upon fasting to 13 % and 33 % of the levels in fed animals respectively [122]. The conservation of carnitine precursors could lead to enhanced carnitine biosynthesis, which would explain the higher levels of carnitine in liver. However, this increase might also result from redistribution of carnitine from tissues to the liver. Further studies are needed to understand this phenomenon.

Paul and co-workers [123] showed that clofibrate, a peroxisome proliferator and ligand for the nuclear receptor peroxisome-proliferator-activated receptor α (PPARα), greatly increased liver carnitine and acylcarnitine concentrations (by 6- and 5-fold respectively). Carnitine and acylcarnitine levels in skeletal muscle, heart, kidney and plasma did not change significantly. The authors clearly showed that these increases were a result of enhanced hepatic carnitine biosynthesis, and not of redistribution of carnitine among tissues or of a decrease in urinary excretion [123]. Clofibrate treatment did increase urinary TML levels and, since clofibrate has been shown to increase protein turnover [124], it was suggested that the increased carnitine synthesis is due to an increased availability of TML [123]. Recent studies have shown that PPARα has a major role in orchestrating the events during fasting by regulating the expression of genes

involved in mitochondrial and peroxisomal fatty acid oxidation, including carnitine palmitoyltransferase I ('CPTI') and peroxisomal acyl-CoA oxidase [125]. Since carnitine is required for efficient fatty acid oxidation, it would appear to be beneficial physiologically to increase carnitine biosynthesis during fasting. However, it remains to be established whether PPARα is involved in the regulation of carnitine biosynthesis.

During late gestation, liver carnitine levels increase considerably ($\approx$ 6-fold), most probably to provide a source of carnitine to the newborn in order to allow it to obtain energy from fatty acids of milk fat [126]. This high level of carnitine is maintained until 3 days *post partum*, but then falls abruptly and returns to normal values at day 9 [120]. Injection of labelled butyrobetaine into the mother after delivery has shown that butyrobetaine is completely converted into carnitine by the mother's liver and then reaches the pup via the milk [120]. The mechanism behind this rapid rise and subsequent normalization of liver carnitine levels remains unclear. Further study is necessary to determine how carnitine biosynthesis and/or transport is regulated in this situation.

Thyroxine, a thyroid hormone, has been reported to increase liver carnitine levels too. In liver, both the carnitine concentration and BBD activity were increased 2-fold in thyroxine-treated rats [127]. Serum carnitine concentrations were increased moderately, whereas levels in the heart, skeletal muscle and urine were not affected. Effects of sex hormones [75], pituitary hormones [128], insulin and glucagon [120,121,129] on carnitine levels have been documented; their direct influence on carnitine biosynthesis, however, has not been investigated.

CARNITINE BIOSYNTHESIS IN MAN

Major sources of carnitine in the human diet are meat, fish and dairy products. Omnivorous humans generally ingest 2–12 μmol of carnitine per day per kg of body weight [10]. This is more than the carnitine produced endogenously, which has been estimated to be 1.2 μmol per day per kg of body weight [8,10]. In omnivorous humans, approx. 75% of body carnitine sources come from the diet and 25% comes from *de novo* biosynthesis [130]. Since carnitine is present primarily in foods of animal origin, strict vegetarians obtain very little carnitine from their diet (< 0.1 μmol per day per kg of body weight). Therefore, strict vegetarians obtain more than 90% of their carnitine through biosynthesis [10]. Plasma carnitine levels of strict vegetarians and lacto-ovo-vegetarians have been shown to be significantly lower than in normal omnivorous adults [131,132]. This difference, however, is probably not of any clinical significance.

Tissue distribution of carnitine-biosynthetic enzymes

The tissue distribution of carnitine-biosynthetic enzymes in humans has been investigated by Rebouche and Engel [31]. TMLD activity is highest in the kidney, but also present in the liver, heart, muscle and brain. HTMLA activity is found predominantly in the liver. In the other investigated tissues, the HTMLA activity is low. The rate of TMABA oxidation is greatest in the liver, with substantial activity also found in the kidney, but is low in brain, heart and muscle. These results show that all the investigated tissues contain the enzymes necessary to convert TML into butyrobetaine. However, only the kidney, liver and brain are capable of converting butyrobetaine into carnitine [31]. A schematic representation of carnitine homoeostasis in humans is shown in Figure 4. BBD activity is 3–16-fold higher in the kidney than in liver [70,31]. Activity in the brain only has been reported by Rebouche and Engel, and is 50% of the activity measured in the liver [31]. As in the rat [78], liver BBD activity is regulated developmentally in humans [31,79]. In contrast, kidney BBD activity is not age-dependent, since BBD activity is already present at adult levels in newborns [79]. No evidence was found that the activity of the other three enzymes in liver is age-dependent [31].

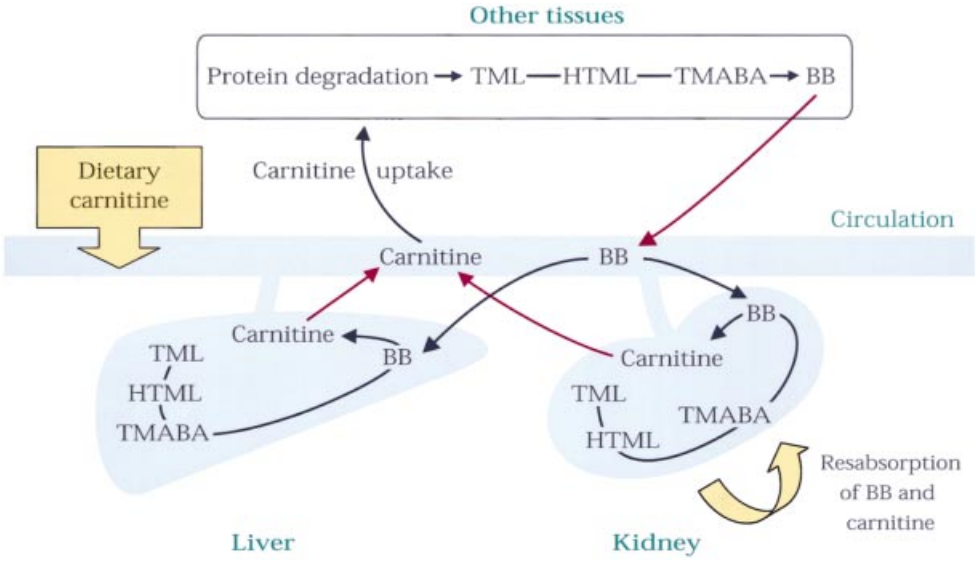

Figure 4 Schematic representation of carnitine homoeostasis in man

Carnitine is synthesized in the kidney, liver and brain (not shown). Other tissues depend on active uptake of carnitine from the circulation (uptake is indicated by black arrows; excretion by red arrows). Protein degradation yields TML, which can be converted into butyrobetaine (BB) in every tissue. However, only the liver, kidney and brain are able to convert BB into carnitine because BBD is expressed only in these tissues. BB is excreted from tissues which lack BBD, and transported via the circulation to liver and kidney, where it is converted into carnitine. The kidney efficiently reabsorbs carnitine and butyrobetaine, thereby minimizing urinary loss of both compounds.

TML and butyrobetaine loading studies

Carnitine biosynthesis was investigated by supplementing adults, which where fed on a low-carnitine diet, excess amounts of the carnitine precursors lysine plus methionine, TML or butyrobetaine [94]. Lysine plus methionine supplementation for 20 days led to an increased carnitine production. However, the effect was small and the underlying mechanism was not determined. A rise in plasma TML was not observed, in contrast with another study in which oral administration of lysine resulted in a 5-fold increase in the plasma TML concentration [133]. Although TML significantly increased carnitine synthesis, the increase was small when compared with that resulting from TML loading in rats. Similarly, the excretion of carnitine only doubled in infants who were fed on TML for 14 days [79]. TML is taken up poorly by both rat [114] and human tissues [117]. The rat kidney is capable of tubular reabsorption of TML, whereas human kidney does not reabsorb this compound. The less efficient use of TML as a carnitine precursor in humans could therefore be ascribed to the low capacity of tissues to take up TML, and the inability of the human kidney to reabsorb this compound. Moreover, TMLD is a mitochondrial enzyme, and its localization also may limit the utilization of TML for carnitine synthesis, since this depends on whether the exact submitochondrial localization of TMLD requires transmembrane transport of TML.

More recently, Melegh and co-workers [134,135] performed a single-day loading study in premature infants using orally administered deuterium-labelled TML. They could not detect incorporation of the deuterium label into urinary carnitine using fast-atom-bombardment MS. As an extension of this study, Vaz and co-workers performed a similar experiment with seven full-term newborns, who received deuterium-labelled TML for 5 days, and used our novel assay for the analysis of the carnitine biosynthesis metabolites in urine. After loading, all the metabolites of carnitine biosynthesis could be detected in urine in deuterium-labelled form, except for TMABA. In addition, deuterium-labelled carnitine was also incorporated into acylcarnitines (F. M. Vaz, B. Melegh, J. Bene, D. Cuebas, D. A. Gage, A. Bootsma, P. Vreken, A. H. van Gennip, L. L. Bieber and R. J. A. Wanders, unpublished work). Most of the TML ($> 75\,\%$), however, was excreted unchanged in urine, which is in agreement with previous findings that humans do not use exogenous TML efficiently as a precursor for carnitine biosynthesis [117,134,135]. These results show that newborns have the capability to synthesize carnitine from exogenous TML, albeit at a low rate.

As in the rat, dietary butyrobetaine dramatically increased urinary carnitine excretion and doubled plasma carnitine concentrations in humans [79]. Muscle carnitine concentrations remained constant, suggesting that the higher carnitine levels were the result of actual biosynthesis and did not originate from release of tissue stores. The same group obtained similar results in human infants, in whom the rate of carnitine excretion increased 30-fold when the infants were fed butyrobetaine [79]. The authors concluded that BBD activity is not rate-limiting for biosynthesis of carnitine in adults, as well as in infants.

Although TML loading studies have made an important contribution to our understanding of carnitine biosynthesis, it should be noted that, in these experiments, the intracellular metabolism of TML is bypassed. Tissues like the heart and muscle normally synthesize TML, but do not readily absorb it from the circulation. It is believed that TML produced intracellularly is converted into butyrobetaine in the tissue of origin, after which butyrobetaine is excreted into the circulation and converted into carnitine in tissues that contain BBD (Figure 4). Unlike TML, butyrobetaine is absorbed readily by the liver and converted into carnitine. These processes could be significant for carnitine biosynthesis.

Another important observation made by Melegh and co-workers [134,135] is that administration of deuterium-labelled TML considerably increased unlabelled carnitine and butyrobetaine excretion. In previous studies in which unlabelled precursors were used [79,94], the carnitine excretion was used to calculate the rate of carnitine biosynthesis, assuming that this carnitine was a result of actual biosynthesis. Especially in the case of TML, these results should be re-evaluated, and additional experiments performed using stable-isotope-labelled carnitine precursors.

Transport of carnitine-biosynthesis metabolites

Although the exact interplay of tissues and metabolites involved in carnitine biosynthesis is not entirely clear, it is evident that transport of metabolites is required for complete synthesis of carnitine. Very little is known about the transport of TML and HTML, and their intracellular/tissue concentrations. The only transport studies performed with these compounds are described above and show that they are absorbed by the intestine [114]. The fact that rat kidney is able to efficiently reabsorb TML implies that (at least in rat kidney) a transporter system exists, which acts on this compound.

Considerably more is known about the transport of carnitine. Since tissues such as the heart, muscle, liver and kidney are highly dependent on the energy generated by β-oxidation, it is essential that these tissues have sufficient amounts of carnitine. Because the carnitine concentration in tissues is generally 20–50-fold higher than in plasma [6,136], and since, in humans, only kidney, liver and brain have the complete set of enzymes to synthesize carnitine, most tissues depend on carnitine uptake from the blood via active transport. Kinetic studies of the plasmalemmal carnitine transporter have demonstrated similar K_m values of 2–60 μM for carnitine transport in muscle [137–139], heart [140,141], placenta [142], and fibroblasts [130,139,143,144], suggesting that they share a common transporter. This high-affinity carnitine transport system is also involved in the tubular reabsorption of carnitine in the kidney [117] and is dependent on sodium ions [130].

In 1998, the cDNA sequence and genomic organization of a new member of the organic cation transporter family (reviewed in [145]), organic cation transporter 2 (OCTN2), was reported by Wu and co-workers [146]. Subsequently, another group showed that the *OCTN2* gene (*SLC22A5*) codes for a high affinity ($K_m \approx 4.3\ \mu$M), sodium ion-dependent carnitine transporter [147]. Carnitine transport is strongly inhibited by acetylcarnitine and butyrobetaine, suggesting that OCTN2 also transports these compounds [147]. Northern blot analysis and *in situ* hybridization studies in rat and human tissues showed that OCTN2 is expressed in the proximal and distal tubules and in the glomeruli of the kidney, in the myocardium, valves and arterioles of the heart, in the labyrinthine layer of the placenta, and in the cortex, hippocampus and cerebellum of the brain [147,148]. Further studies showed that OCTN2 is localized on the apical membrane of renal tubular epithelial cells, demonstrating that OCTN2 is important in the concentrative reabsorption of carnitine after glomerular filtration in the kidney [149].

Much higher K_m values for carnitine transport have been reported for human liver (500 μM) and brain ($> 1000\ \mu$M), and the existence of a low-affinity carnitine transporter therefore has been suggested [150]. Recently, two additional proteins, OCTN1

and $ATB^{0,+}$, have been identified, which also are able to transport carnitine. OCTN1, a homologue of OCTN2, has been shown to be expressed predominantly in the liver, kidney and small intestine [151,152]. The unrelated amino acid transporter $ATB^{0,+}$ has been identified in mouse colon and has a high K_m for carnitine (0.83 mM) [153]. $ATB^{0,+}$ could, therefore, like OCTN1, represent the low-affinity carnitine transporter in liver and brain.

Recent studies have shown that butyrobetaine is transported actively across the basolateral plasma membrane of hepatocytes ($K_m \approx 5\ \mu M$) and that this transport is, like OCTN2, also driven by sodium ions [154]. Butyrobetaine transport is inhibited significantly by propionylcarnitine, but not by TML, D- or L-carnitine, or other acylcarnitines [154]. These results suggest that, in the liver, which does not express OCTN2, another transport system is present that specifically transports butyrobetaine, which is destined for carnitine synthesis. Although carnitine transport into the cell has been relatively well documented, it remains unclear how carnitine synthesized *de novo* is exported from the site of biosynthesis (liver and kidney) into the circulation. Since $ATB^{0,+}$, OCTN1 and OCTN2 all transport carnitine into the cell, the export of carnitine and its metabolites is probably mediated by another transport system, or possibly by passive diffusion. Further research is needed to resolve this issue.

The dependence on carnitine uptake is evident from patients who suffer from primary systemic carnitine deficiency [CDSP; OMIM (Online Mendelian Inheritance in Man): 212140]. These patients show excessive renal and intestinal wastage of carnitine, resulting in very low plasma and tissue carnitine concentrations. Clinically, CDSP patients usually show symptoms of cardiomyopathy, hepatomegaly, myopathy, recurrent episodes of hypoketotic hypoglycaemia, hyperammonaemia and failure to thrive. Studies of cells of CDSP patients have indicated that this disorder is caused by a defect in the active cellular uptake of carnitine into the cell [143,155–158]. The disorder is autosomal recessive, and has been mapped to human chromosome 5q [159]. Shortly after the identification of the high-affinity carnitine transporter OCTN2, which is located on chromosome 5q33.1, it was demonstrated that mutations in this gene cause CDSP [160–164]. In addition, the murine orthologue of OCTN2 has been shown to be mutated in the juvenile steatosis (JVS) mouse, which shows symptoms similar to those of CDSP patients, and which is considered to be the murine equivalent of human CDSP [165–169].

The observation that butyrobetaine excretion in the JVS mouse is 4 times that of control mice supports the concept that OCTN2 also mediates the reabsorption of butyrobetaine [167]. Interestingly, the activity of BBD in liver was twice that of control mice. However, the butyrobetaine content was lower in JVS mice, presumably due to the disturbed reabsorption of this compound in the kidney. The urinary loss of the carnitine precursor butyrobetaine therefore aggravates the carnitine deficiency in the JVS mouse, and probably also the OCTN2 deficiency in man [167].

At present, the importance of carnitine biosynthesis for energy homoeostasis remains unclear, and no patients have been identified in which one of the enzymes of carnitine biosynthesis is deficient. Furthermore, no mutant mice or other organisms with a defect in carnitine biosynthesis have been described. Since omnivorous humans ingest sufficient carnitine from the diet, a defect in carnitine biosynthesis would most probably not manifest itself as systemic carnitine deficiency, except perhaps only when the dietary intake is limited (vegetarians and vegans) or interrupted by illness for a prolonged period [8].

CONCLUSIONS

Despite considerable progress in our understanding of carnitine biosynthesis and metabolism, many questions remain concerning the regulation of carnitine metabolism and the role of carnitine biosynthesis in homoeostasis. The recent identification of three of the four genes of this pathway and the development of an easy method to measure the concentration of the carnitine biosynthesis metabolites allows both the creation and characterization of a mouse model in which one of the carnitine biosynthesis genes has been disrupted. Such a mouse model is expected to provide more insight into the role of this pathway in carnitine and fatty acid metabolism.

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