Molecular and biochemical aspects of carnitine biosynthesis
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Synthesis of carnitine in full-term newborns from exogenous 6-N-trimethyllysine as determined by a novel method using HPLC-electrospray tandem mass spectrometry

Synthesis of carnitine in full-term newborns from exogenous 6-N-trimethyllysine as determined by a novel method using HPLC-electrospray tandem mass spectrometry

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SUMMARY
In mammals, carnitine is derived from dietary sources and de novo synthesis. Carnitine is synthesised from 6-N-trimethyllysine via 3-hydroxy-6-N-trimethyllysine, 4-N-trimethylaminobutyraldehyde and butyrobetaine. We have developed a fast and easy method to determine the concentrations of the metabolites of the carnitine biosynthesis in urine. After derivatisation with methyl chloroformate, the analytes are separated by reversed-phase ion-pair HPLC using heptafluorobutyric acid as an ion-pairing agent, and detected by electrospray tandem mass spectrometry. All metabolites could be quantified, except 4-N-trimethylaminobutyraldehyde. This newly developed method was first used to determine the concentration of the carnitine biosynthesis metabolites in urines of 40 healthy human subjects. The excretion of 6-N-trimethyllysine and 3-hydroxy-6-N-trimethyllysine relative to that of creatinine varied little between individuals with concentrations of 2.8 ± 0.8 and 0.45 ± 0.15 µmol/mmol creatinine, respectively, whereas butyrobetaine and carnitine levels were more variable with values of 0.27 ± 0.21 and 15 ± 12 µmol/mmol creatinine, respectively.

Secondly, the method was used to analyse the carnitine biosynthesis metabolites in urines of newborns, before and after oral administration of deuterium-labelled 6-N-trimethyllysine. After loading, all urines contained deuterium-labelled 6-N-trimethyllysine, 3-hydroxy-6-N-trimethyllysine, 4-N-butyrobetaine and carnitine. The results presented here show that newborns have the capability to synthesise carnitine from exogenous 6-N-trimethyllysine, albeit at a low rate.

INTRODUCTION
Carnitine (3-hydroxy-4-trimethylaminobutyrate) is a vital compound, which plays an indispensable role in the transport of activated fatty acids across the inner mitochondrial membrane into the matrix, where β-oxidation takes place (1-3). Furthermore, carnitine is involved in the transfer of the products of peroxisomal β-oxidation, including acetyl-CoA, to the mitochondria for oxidation to CO₂ and H₂O in the Krebs cycle (4,5). Apart from the dietary intake of carnitine, most eukaryotes are able to synthesise this compound from 6-N-trimethyllysine (TML) (6-8). This TML is generated within lysosomes by the hydrolysis of proteins containing lysines which are trimethylated at their ε-amino group by a protein-dependent methyltransferase. The synthesis of carnitine from TML is shown in Fig. 1. TML is first hydroxylated at the 3-position by 6-N-trimethyllysine, 2-oxoglutarate dioxygenase
Carnitine biosynthesis metabolites

1. 6-N-Trimethyllysine dioxygenase
2. 3-Hydroxy-6-N-trimethyllysine aldolase
3. 4-N-Trimethylaminobutyaldehyde dehydrogenase
4. 4-N-Trimethylaminobutrate dioxygenase

(TMLD), after which the resulting 3-hydroxy-6-N-trimethyllysine (HTML) is cleaved by 3-hydroxy-6-N-trimethyllysine aldolase (HTMLA) into 4-N-trimethylaminobutyraldehyde (TMABA) and glycine (7,9). TMABA is subsequently oxidised by 4-N-trimethylaminobutyaldehyde dehydrogenase (TMABA-DH) to form 4-N-trimethylaminobutrate (butyrobetaine) (10). In the last step, butyrobetaine is hydroxylated at the 3-position by a second dioxygenase, butyrobetaine, 2-oxoglutarate dioxygenase (BBD), yielding L-carnitine (6,11,8). BBD is differentially expressed since its activity has only been detected in human kidney, liver and brain (12). In our laboratory, we have identified the cDNAs encoding TMABA-DH (13), BBD (14,15), and recently that of TMLH (16).

To further investigate the carnitine biosynthesis pathway and its regulation, determination of the concentration of the metabolites of this pathway in body fluids is of crucial importance. Although several methods have been developed to measure one or more of the carnitine biosynthesis intermediates in urine and/or plasma, these procedures are labour-intensive and the sample preparation differs for each compound (17-24). We have developed a new method which enables fast and easy determination of the concentration of the carnitine biosynthesis metabolites in urine and applied the method to the study of the de novo carnitine biosynthesis in newborns. The method uses derivatisation with methyl chloroformate, separation of the analytes by ion-pair HPLC, and detection by electrospray tandem mass spectrometry (MS).

Previous experiments with full-term infants showed a moderate increase of urinary carnitine excretion after TML loading, which suggested that infants are capable to synthesise carnitine from exogenous TML (25). Using fast atom bombardment MS Melegh et al. could not detect isotope-labelled carnitine in urine of premature infants after a single day of oral 6-N-[Me-\(^2\)H\(_9\)]-TML loading (26). To further investigate carnitine biosynthesis in newborns, we extended the latter study and performed a five-day loading test with deuterium-labelled TML on 7 newborns. We used our newly developed method to analyse the urines, before and after loading, to investigate whether newborns have the capacity to use exogenous TML as precursor for carnitine synthesis.

FIG. 1: Biosynthesis of carnitine from TML.
EXPERIMENTAL PROCEDURES

Chemicals
Analytical-grade methanol and methyl chloroformate were obtained from Merck. Heptafluorobutyric acid was from Pierce chemical company (Rockford, IL). All other reagents were of analytical grade. Deionised water was passed through a MilliQ Labo system (Millipore, Bedford, MA).

The internal standards (IS) used were 6-N-[Me-2H9]-TML, 6-N-[Me-2H6]-TML, 4-N-[Me-2H3]-butyrobetaine and 4-N-[Me-2H]-carnitine. 4-N-[Me-2H3]-carnitine was obtained from Cambridge Isotope Laboratories. Other internal standards were synthesised as described below.

Synthesis of 6-N-[Me-2H9]-TML and 6-N-[Me-2H6]-TML
6-N-[Me-2H9]-TML was synthesised from L-lysine HCl (Sigma, St. Louis, MO) using [2H6]-dimethyl sulphate (Isotec, Miamisburg, OH) as methyl donor by the following method. Two grams of L-lysine were dissolved in 20 ml of distilled water, 2 g alkaline CuCO3 were added and the mixture boiled for 10 min. (For the preparation of alkaline CuCO3 see Melegh et al. (26)). The reaction mixture was cooled to room temperature and filtered over a Whatmann 3 MM paper. The clear filtrate was mixed with 4 ml deuterium-labelled dimethyl sulphate at 20°C, after which 13 ml 10% NaOH solution was added drop wise. The solution containing the deuterium-labelled TML was applied to a 4 ml volume Dowex 50 WX 8 column and after washing with 10 volumes distilled water, the bound 6-N-[Me-2H9]-TML was eluted with 120 ml 2M NaOH. The effluent was concentrated and lyophilised. Chemical purity was determined by TLC and NMR to be greater than 98%. The isotope purity was determined by tandem MS and was greater than 99%.

6-N-[Me-2H6]-TML was synthesised by the same procedure used for the synthesis of 6-N-[Me-2H9]-TML using 6-N-methyl-L-lysine HCl as precursor instead of L-lysine.

Synthesis of 4-N-[Me-2H3]-butyrobetaine
4-N-[Me-2H3]-butyrobetaine was synthesised from 4-N-dimethylaminobutyric acid HCl using [Me-2H3]-methyl-iodide (both from Sigma) as the methyl donor using the following method. One gram (6 mmol) 4-N-dimethylaminobutyric acid was dissolved in 1 ml distilled water, mixed with 3.16 g (10 mmoles) Ba(OH)2 × 8 H2O dissolved in 20 ml distilled water, and 200 ml methanol was added. After addition of 600 µl (9 mmol) [Me-2H3]-methyl-iodide the mixture was stirred overnight at room temperature. The solution was concentrated under mild vacuum to obtain a volume of approximately 20 ml, after which 20 ml distilled water was added. Addition of 3-4 ml 2M H2SO4 precipitated the barium ions as BaSO4, which was removed by centrifugation. The supernatant was alkalised by approximately 7 ml 2M NaOH and the mixture was kept at 65°C for 4 hours. The solvent was evaporated under reduced pressure, the residue was redissolved in 1M HCl, and extracted with 150 ml CCl4. The aqueous phase was concentrated to 1-2 ml in a rotavapor. 4-N-[Me-2H3]-butyrobetaine was purified by Dowex exchange chromatography essentially as described above for deuterium-labelled TML.

6-N-[Me-2H3]-TML loading test
Seven orally fed male infants were studied. The mean gestational age was 38.5 ± 0.87 weeks, range 38-41 weeks; postnatal age at the beginning of the study was 8 (range 5-11) days; body weight 3360 ± 100 grams, (range 3100-4300). The infants were fed daily with 7 × 70 g
of formula (Aptamil, Milupa, Germany). The study period lasted 5 consecutive days. Twenty-four hour urines were collected on the day before administration of 6-N-[Me-2H$_6$]-TML (day 0), and on the last day (day 5) of the study. Blood samples were taken on day 0 and day 5. After the pre-loading control day, the newborns received 0.5 mmol of 6-N-[Me-2H$_6$]-TML daily in their formula during the 5 day study period. The study design was approved by the local Ethics Committee, and informed consent was obtained from the parents. 6-N-[Me-2H$_6$]-TML was used as an IS for TML and 6-N-[Me-2H$_6$]-TML for analysis of the urine samples of the 6-N-[Me-2H$_6$]-TML loading test.

**Carnitine biosynthesis intermediates in control subjects**

The 24-hr urine samples used for the determination of the carnitine biosynthesis intermediates in control subjects were obtained from 40 healthy individuals (23 males, 17 females) with a mean age 21 ± 17 years (range: 2-66).

**Sample preparation**

Urine samples were stored at 4°C if analysed within 1 week, or were stored at -20°C until analysis. Urinary creatinine concentrations were determined by the conventional alkaline-creatinine-picrate method (27). Urine samples were centrifuged at 10,000 × g for 5 min to remove debris and a portion of the cleared urine was diluted to a creatinine value of 1 mM. Urines with creatinine concentrations lower than 1 mM were used undiluted. One hundred microliters of this urine sample was used for the derivatisation with methyl chloroformate. Methyl chloroformate reacts with primary, secondary and tertiary amino groups, but not with quaternary amino groups. TML and HTML both contain a primary amino group, which reacts with methyl chloroformate, and this results in the formation of 2-N-methylformyl-6-N-TML and 2-N-methylformyl-HTML, respectively. Carnitine and butyrobetaine do not react with methyl chloroformate. Interfering compounds (especially amino acids) readily react with methyl chloroformate and can be separated from the compounds of interest by acidic extraction with ethyl acetate. The carnitine biosynthesis intermediates, however, remain in the aqueous phase by virtue of their constitutively positive quaternary amino group. Twenty-five microliters of the IS mixture (25 μM of 6-N-[Me-2H$_6$]-TML or 6-N-[Me-2H$_6$]-TML, 4-N-[Me-2H$_3$]-butyrobetaine and 4-N-[Me-2H$_3$]-carnitine each) and 20 μl of a 1:3 mixture of 1.5 M NH$_4$OH and 0.5 M sodium phosphate buffer, pH 7.2, were added to the urine sample. The derivatisation was started by the addition of 20 μl methyl chloroformate and the mixture was vortexed. After a 5 min incubation at room temperature, the reaction was terminated by the addition of 40 μl 4% (v/v) heptafluorobutyric acid. The reaction mixture was extracted with 1 ml ethyl acetate and 5 μl of the aqueous phase was injected into the HPLC-tandem MS. When stored at 4°C, the reaction mixture remained stable for several weeks.

**HPLC-tandem MS**

The HPLC system consisted of an HP 1100 series binary gradient pump, a vacuum degasser, and a column temperature controller (Hewlett Packard, Palo Alto, CA) and was connected to a Gilson 231 XL autosampler with 402 single dilutor (Gilson, Middleton, WI). The autosampler maintained the temperature of the samples at 4°C. The Phenomenex Aqua analytical column (250 × 2.0 mm; particle size, 5 μm; Phenomenex, Torrance, CA) was protected by a guard column (SecurityGuard C18 ODS; 4 × 2.0 mm; Phenomenex). The column temperature was maintained at 15°C. The mobile phases were as follows: 0.1% (v/v)
heptafluorobutyric acid (eluant A); 90% (v/v) methanol (eluant B). The elution gradient was as follows (flow rate, 0.3 ml/min): 0–3 min, 95% A; 3–6 min, 95% A to 50% A; 6–8 min, 50% A to 30% A; 8–9 min, 30% A to 0% A; 9–10 min, 0% A; 10–16 min, equilibration with 95% A. All gradient steps were linear, and the total analysis time, including equilibration, was 16 min. An LC-packings accurate-splitter (Amsterdam, The Netherlands) was used between the HPLC column and the mass spectrometer (split ratio of 1:20), which introduces the eluant at a flow-rate of 15 μl/min into the mass spectrometer. An electrically operated valve was used so that only the eluant from 5.5 to 16 min was introduced into the mass spectrometer (preventing early-eluting salts from contaminating the mass spectrometer). A Quattro II tandem mass spectrometer (Micromass, Wythenshawe Manchester, United Kingdom) was used in the positive electrospray ionisation mode. Nitrogen was used both as nebulising and drying gas. The collision gas was argon, and the cell pressure was 0.25 Pa. The source temperature was set at 80°C, and the capillary voltage was maintained at 3.5 kV. The detector was used in tandem MS mode using multiple-reaction monitoring to detect a specific transition of precursor ion to fragment for each analyte. The transitions, cone voltages, and collision energies established for each compound are listed in TABLE I.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass Da</th>
<th>Parent ion m/z</th>
<th>Daughter ion m/z</th>
<th>Cone Voltage V</th>
<th>Collision energy eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-N-Methylformyl-TML</td>
<td>247</td>
<td>247</td>
<td>142</td>
<td>35</td>
<td>25</td>
</tr>
<tr>
<td>2-N-Methylformyl-H2-TML</td>
<td>256</td>
<td>256</td>
<td>142</td>
<td>35</td>
<td>25</td>
</tr>
<tr>
<td>2-N-Methylformyl-H3-TML</td>
<td>253</td>
<td>253</td>
<td>142</td>
<td>35</td>
<td>25</td>
</tr>
<tr>
<td>2-N-Methylformyl-HTML</td>
<td>263</td>
<td>263</td>
<td>158</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>2-N-Methylformyl-H3-HTML</td>
<td>272</td>
<td>272</td>
<td>158</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Butyrobetaine</td>
<td>146</td>
<td>146</td>
<td>87</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>3H2-Butyrobetaine</td>
<td>149</td>
<td>149</td>
<td>87</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>3H3-Butyrobetaine</td>
<td>155</td>
<td>155</td>
<td>87</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>Carnitine</td>
<td>162</td>
<td>162</td>
<td>103</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td>3H2-Carnitine</td>
<td>165</td>
<td>165</td>
<td>103</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td>3H3-Carnitine</td>
<td>171</td>
<td>171</td>
<td>103</td>
<td>35</td>
<td>16</td>
</tr>
</tbody>
</table>

**Validation**

The linearity and detection limits for each compound were established by injection of calibration mixtures with different concentrations. The stable-isotope-labelled compound of each analyte was used as IS, except for HTML for which 6-N-[Me-H2]-TML or 6-N-[Me-H3]-TML was used as IS. Analyte concentrations were determined using the slope and intercept of the calibration curve, which were obtained from a linear least-squares regression for the analyte/IS peak-area ratio vs. the concentration of the calibrator. The intra-assay (within-day) variation (CV) of the method was established by measuring 10 times a blank urine, and the same urine enriched with synthetic compounds at low (3-10 μM), medium (30-100 μM), and high (300-1000 μM) concentrations. The synthetic compounds were added before dilution to 1 mM creatinine. The inter-assay (between-day) variation was established by measuring blank urines, and urines enriched with synthetic compounds (30-100 μM) during 5 separate weeks. The synthetic compounds were added before dilution to 1 mM creatinine.
The recovery of the method was established by measuring 13 different urines before and after enrichment with known concentrations of synthetic compounds (10-20 μM, 100-200 μM). The addition of the synthetic compounds was performed after dilution of the urine to 1 mM creatinine. IS mixture was added to compensate for losses in the preparation of samples and to compensate for losses in sensitivity because of quenching of the signal by co-eluting components.

RESULTS

Assay development and validation
To measure the carnitine biosynthesis intermediates in urine, samples were first subjected to derivatization with methyl chloroformate. This enables the separation of the interfering compounds, mainly amino acids, from the compounds of interest. Since all amino acids contain a primary amino group, they react readily with methyl chloroformate. The resulting amino acid derivatives, which are neutral at pH<2, can be extracted with ethyl acetate from the aqueous phase. The derivatives of the carnitine biosynthesis intermediates, however, remain in the aqueous phase by virtue of their constitutively positive quaternary amino group. The removal of the contaminating amino acids, and other ethyl acetate soluble compounds, increased the sensitivity of the analysis dramatically.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Blank†</th>
<th>Low†</th>
<th>Medium†</th>
<th>High†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc.</td>
<td>CV</td>
<td>Conc.</td>
<td>CV</td>
</tr>
<tr>
<td></td>
<td>μmol/mmol creatinine§</td>
<td>%</td>
<td>μmol/mmol creatinine§</td>
<td>%</td>
</tr>
<tr>
<td>TML</td>
<td>4.30 ± 0.02</td>
<td>1</td>
<td>4.69 ± 0.04</td>
<td>1</td>
</tr>
<tr>
<td>HTML</td>
<td>0.59 ± 0.03</td>
<td>4</td>
<td>0.98 ± 0.05</td>
<td>5</td>
</tr>
<tr>
<td>Butyrobetaine</td>
<td>0.23 ± 0.01</td>
<td>5</td>
<td>0.89 ± 0.03</td>
<td>3</td>
</tr>
<tr>
<td>Carnitine</td>
<td>10.7 ± 0.1</td>
<td>1</td>
<td>12.0 ± 0.2</td>
<td>1</td>
</tr>
</tbody>
</table>

†Urine supplemented (before dilution to 1 mM creatinine) with low (3-10 μM), medium (30-100 μM), or high (300-1000 μM) concentration of the four measured compounds. The urine used had a creatinine value of 8.7 after addition of the supplement.

§Mean ± SD; n=10

Although specific transitions were used for each analyte an HPLC step was needed to separate interfering compounds in the urine sample with the same transition from the compounds of interest. This is particularly important for butyrobetaine, which showed two major interfering compounds with the same transition at different elution times (results not shown). Another important benefit of the HPLC step is that salts present in urine elute in the void volume of the column, thereby preventing contamination of the mass spectrometer. The calibration curves for each compound were linear up to at least 250 μM (r²>0.995). Above this concentration, deviation from linearity was observed for most compounds. When the concentration of one or more metabolites were above the linear part of the calibration curve (in practice, only carnitine-treated patients), the samples were diluted within the linear range of the curve. The detection limit was established and defined as the lowest signal with a signal-to-noise ratio of 3. The detection limits were as follows: carnitine, 0.05 μM; butyrobetaine, 0.05 μM; HTML, 0.1 μM; and TML, 0.05 μM.
Chapter 6

The intra-assay (within-day) variation, inter-assay (between-day) variation and accuracy data are summarised in TABLES II, III and IV, respectively.

Carnitine biosynthesis intermediates in control subjects
To obtain an indication of the concentrations of the carnitine biosynthesis intermediates in urine of normal subjects, the concentrations of the metabolites were determined in 24 hr urines from 40 healthy subjects with ages varying between 2-66 years. The urinary concentrations of the four metabolites measured with our method are shown in TABLE V and are in agreement with previously published data. The ratio of TML to HTML was relatively constant, with a mean value of 6.5 ± 1.7 (range 4.1-10.3). The ratio of carnitine to butyrobetaine was more variable with a mean value of 58 ± 24 (range 14-134).

<table>
<thead>
<tr>
<th>TABLE III</th>
<th>Inter-assay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>Urine†</td>
</tr>
<tr>
<td></td>
<td>Conc.</td>
</tr>
<tr>
<td></td>
<td>µmol/mmol creatinine§</td>
</tr>
<tr>
<td>TML</td>
<td>4.33 ± 0.57</td>
</tr>
<tr>
<td>HTML</td>
<td>1.28 ± 0.23</td>
</tr>
<tr>
<td>Butyrobetaine</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>Carnitine</td>
<td>11.7 ± 1.0</td>
</tr>
</tbody>
</table>
†‡The results were obtained by measuring the same urines over 5 different weeks
‡Enriched with 30-100 µM of each compound
§Mean±SD; n=5

<table>
<thead>
<tr>
<th>TABLE IV</th>
<th>Accuracy of measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>Conc. added‡†</td>
</tr>
<tr>
<td></td>
<td>10-20 µM</td>
</tr>
<tr>
<td></td>
<td>Recovery‡</td>
</tr>
<tr>
<td></td>
<td>µmol/mmol creatinine§</td>
</tr>
<tr>
<td>TML</td>
<td>3.1 (1.03-4.89)</td>
</tr>
<tr>
<td>HTML</td>
<td>0.55 (0.13-1.13)</td>
</tr>
<tr>
<td>Butyrobetaine</td>
<td>0.49 (0.08-1.26)</td>
</tr>
<tr>
<td>Carnitine</td>
<td>26 (0.87-73)</td>
</tr>
</tbody>
</table>
‡†Urine supplemented (after dilution to 1 mM creatinine) with low (10-20 µM) or medium (100-200 µM) concentration of the four measured compounds
§Mean±SD; n=13
¶Mean (range) of endogenous concentration of compound in urines used for the enrichment

$2H_5$-TML loading test in newborns
To determine whether full term newborns can utilise exogenous TML for carnitine biosynthesis, a loading test with deuterium-labelled TML was performed in 7 healthy newborns. The urinary concentrations of endogenous and $2H_5$-labeled TML, HTML,
butyrobetaine and carnitine, before and after $^2$H$_9$-TML administration, are shown in Fig. 2. Both before and after loading, levels of endogenous TML and HTML appear to be proportional to creatinine (Fig. 2A/B).

The mean concentration of TML and HTML before $^2$H$_9$-TML administration in the urines was 3.6 ± 0.5 and 2.1 ± 0.4 μmol/mmol creatinine, respectively. After $^2$H$_9$-TML administration, the mean concentration of TML and HTML increased significantly (p<0.01) to 4.6 ± 0.5 and 2.7 ± 0.5 μmol/mmol creatinine, respectively. The ratio of TML to HTML remained constant with mean values of 1.8 ± 0.2 before and 1.7 ± 0.3 after loading. The concentration of endogenous butyrobetaine before $^2$H$_9$-TML administration varied greatly, ranging from 0.7-9.7 μmol/mmol creatinine. The butyrobetaine excretion increased considerably after loading, ranging from 1.5 to 6.7 times the original level (Fig. 2C). Carnitine levels showed a similar behaviour, with concentrations ranging from 2.5-48 μmol/mmol creatinine before and increasing 1.5-12-fold after $^2$H$_9$-TML administration (Fig. 2D). For individual subjects, the ratio of carnitine to butyrobetaine was relatively constant and increased slightly from 3.6 ± 1.4 before, to 5.3 ± 2.4 after loading.

The urine samples obtained after $^2$H$_9$-TML administration all contained $^2$H$_9$-TML, $^2$H$_9$-HTML, $^2$H$_9$-butyrobetaine and $^2$H$_9$-carnitine. $^2$H$_9$-TMABA was below the level of detection in these urines. Contrary to their endogenous counterparts, the excretion pattern of $^2$H$_9$-TML and $^2$H$_9$-HTML was not proportional to that of creatinine (Fig. 2E/F) but resembled that of butyrobetaine and carnitine (Fig. 2G/H). With a value of 5.1 ± 1.6, the mean ratio of $^2$H$_9$-TML to $^2$H$_9$-HTML was significantly higher than for the endogenous compounds (p <0.01), which was also true for the carnitine to butyrobetaine ratio; 15.7 ± 8.7 (p<0.01).

Before $^2$H$_9$-TML administration, the ratio of HTML to butyrobetaine is 0.97 ± 0.83 (range 0.27-1.84). After loading with $^2$H$_9$-TML, however, the ratio of $^2$H$_9$-HTML to $^2$H$_9$-butyrobetaine is significantly higher (p<0.01) with a value of 43 ± 22 (range 22-81), suggesting $^2$H$_9$-TML is metabolised differently than endogenous TML.

When considering all 7 subjects, 23 ± 4% of the total amount of excreted $^2$H$_9$-labeled compounds was a product of one or more enzymatic steps of the carnitine biosynthetic

### TABLE V

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. mean ± SD (range)</th>
<th>n</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TML</td>
<td>2.8 ± 0.8 (1.3-4.7)</td>
<td>40</td>
<td>this study</td>
</tr>
<tr>
<td>3.4</td>
<td>3</td>
<td>(18)</td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>25</td>
<td>(37)</td>
<td></td>
</tr>
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<td>5.3 ± 1.7 (3.8-10.4)</td>
<td>13</td>
<td>(17)</td>
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<td>4.7 ± 0.9 (3.5-6.2)</td>
<td>7</td>
<td>(22)</td>
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<td>6.2 ± 0.3</td>
<td>16</td>
<td>(38)</td>
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<tr>
<td>HTML</td>
<td>0.45 ± 0.15 (0.13-0.91)</td>
<td>40</td>
<td>this study</td>
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<tr>
<td>Butyrobetaine</td>
<td>0.27 ± 0.21 (0.05-0.79)</td>
<td>40</td>
<td>this study</td>
</tr>
<tr>
<td>Carnitine</td>
<td>15 ± 12 (1.6-48)</td>
<td>40</td>
<td>this study</td>
</tr>
<tr>
<td>16 ± 11</td>
<td>28</td>
<td>(39)</td>
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<tr>
<td>18 ± 12</td>
<td>5</td>
<td>(25)</td>
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$^2$H$_9$-TMAB was below the level of detection in these urines. Contrary to their endogenous counterparts, the excretion pattern of $^2$H$_9$-TML and $^2$H$_9$-HTML was not proportional to that of creatinine (Fig. 2E/F) but resembled that of butyrobetaine and carnitine (Fig. 2G/H). With a value of 5.1 ± 1.6, the mean ratio of $^2$H$_9$-TML to $^2$H$_9$-HTML was significantly higher than for the endogenous compounds (p <0.01), which was also true for the carnitine to butyrobetaine ratio; 15.7 ± 8.7 (p<0.01).

Before $^2$H$_9$-TML administration, the ratio of HTML to butyrobetaine is 0.97 ± 0.83 (range 0.27-1.84). After loading with $^2$H$_9$-TML, however, the ratio of $^2$H$_9$-HTML to $^2$H$_9$-butyrobetaine is significantly higher (p<0.01) with a value of 43 ± 22 (range 22-81), suggesting $^2$H$_9$-TML is metabolised differently than endogenous TML.

When considering all 7 subjects, 23 ± 4% of the total amount of excreted $^2$H$_9$-labeled compounds was a product of one or more enzymatic steps of the carnitine biosynthetic process.
Fig. 2: Urinary concentrations of endogenous (A-D) and $^2$H$_9$-labeled (E-H) carnitine biosynthesis metabolites in 7 newborns before and after loading with $^2$H$_9$-TML. Gray and black bars represent the concentrations before and after $^2$H$_9$-TML loading, respectively.

The pathway. Ten plus minus three percent of the free carnitine in urine was $^2$H$_9$-labeled carnitine (range: 5-16%). The endogenous and $^2$H$_9$-carnitine concentrations measured in the urine samples were in agreement with the results obtained with the established procedure for the analysis of acylcarnitines by tandem MS (results not shown) (28). Additionally, the acylcarnitine analysis revealed the presence of the following $^2$H$_9$-acylcarnitines in most of the samples after $^2$H$_9$-TML loading: acetyl-$^2$H$_9$-carnitine, propionyl-$^2$H$_9$-carnitine and butyryl-$^2$H$_9$-carnitine.
Carnitine biosynthesis metabolites

DISCUSSION

We have developed an easy and fast HPLC-tandem MS method to analyse four of the five metabolites of carnitine biosynthesis in urine. Urine samples were first derivatised with methyl chloroformate followed by extraction with ethyl acetate. This extraction removes interfering compounds, whereas the carnitine biosynthesis metabolites remain in the aqueous phase. Subsequently, the analytes were separated on a reversed-phase HPLC column equilibrated with the ion-pairing agent heptfluorobutyric acid, and detected by tandem MS. This is the first method which allows analysis of four of the five metabolites of the carnitine biosynthesis pathway using the same sample preparation procedure and detection method. It is highly sensitive and only requires a maximum of 100 μL of urine.

The presence of different compounds with the same transitions makes separation by HPLC mandatory, and underlines the importance of the use of an internal standard, preferably stable-isotope-labelled analogues. The lack of an internal standard for HTML most likely caused the relatively large inter- and intra-assay variation and lower accuracy of measurement for this compound. To overcome this, we intend to synthesise $^2$H$_9$-HTML from $^2$H$_9$-TML enzymatically with purified rat kidney TMLD to obtain better results for this compound.

The only compound that could not be detected was TMABA. Although we have optimised the detection of this compound with a standard (detection limit: 0.1 μM), TMABA could not be detected in urine. Considering the reactivity of aldehydes, this compound might either be chemically oxidised to butyrobetaine, or enzymatically by the action of the enzyme TMABA-DH, which is highly active in human kidney (29).

The analysis of the carnitine biosynthesis intermediates in 40 control subjects (TABLE V) showed that the excretion of TML is proportional to that of creatinine. This is in agreement with previous results obtained from clearance experiments, which showed that TML is not reabsorbed by the kidney (18). Concentrations of HTML in urine have never been reported. The excretion of HTML shows a similar profile as that of TML and is proportional to creatinine excretion, which would suggest that HTML, like TML, is not reabsorbed by the kidney. The ratio of TML to HTML is relatively constant and could be a useful diagnostic parameter to monitor the action of the enzymes TML-dioxygenase and HTML-aldolase. The ratio of carnitine to butyrobetaine is more variable, probably due to the large concentration-difference between the two metabolites. Therefore, this parameter might be less useful in describing the BBD and TMABA-DH function.

The concentrations of butyrobetaine in urine are low (0.3 μmol/mmol creatinine) compared to the concentrations in plasma (4.8 μM (23), 1.8 μM (30,24)). This could be explained by the high activity of BBD in human kidney, which converts most of the butyrobetaine into carnitine. Additionally, butyrobetaine is efficiently reabsorbed by the renal tubules by action of organic cation transporter 2 (OCTN2), which further lowers urinary excretion of butyrobetaine.

When comparing the data from the adult control subjects with that from the newborns before $^2$H$_9$-TML loading, the endogenous levels of butyrobetaine excreted by newborns was higher than adults. Furthermore, the ratio of TML to HTML in newborns was significantly lower than in adults. These results suggest that the amount of excretion of carnitine biosynthesis intermediates is age-dependent, which requires further study.

Rats and guinea pigs both efficiently use exogenous TML and butyrobetaine as substrates for carnitine biosynthesis, evidenced by a considerable increase of urinary carnitine excretion after loading (31,32). Since TML is not reabsorbed by the human kidney (18) and is poorly taken up by tissues (33), exogenous TML is a considerably less efficient carnitine
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precursor than butyrobetaine in human neonates (25,34,26). In a recent article, Melegh et al. could not detect labelled carnitine in urine of premature infants after a single day of oral $^2$H$_9$-TML loading, using fast atom bombardment MS (26). As an extension of this study, we performed a similar experiment with 7 full-term newborns, who were administered $^2$H$_9$-TML for five days, and used our novel assay for the analysis of the urines. After loading, all the metabolites of the carnitine biosynthesis could be detected in urine in $^2$H$_9$-labeled form, except $^2$H$_9$-TMABA. In addition, $^2$H$_9$-carnitine was also incorporated into acylcarnitines. Melegh et al. reported increased urinary endogenous carnitine excretion after $^2$H$_9$-TML/TML administration to premature infants (34,26). This is in agreement with results of the current study, which showed that endogenous carnitine levels are 1.5-12 times the original concentration. This effect, which was also observed for butyrobetaine, could be caused by the competitive inhibition of OCTN2, which mediates the reabsorption of butyrobetaine and carnitine in the kidney, resulting in increased tubular excretion of both compounds. The results presented here show that human infants are able to convert exogenous TML into downstream carnitine precursors, carnitine and carnitine esters at low rate. Most of the TML (>75%), however, was excreted unchanged in urine, which confirms that human infants do not use exogenous TML efficiently as a precursor for carnitine biosynthesis.

An experiment similar to the $^2$H$_9$-TML loading test was performed by Rebouche and Engel (33), where three adult men were given intravenous injections of 1 mCi of tritium-labelled TML, after which the labelled metabolites were monitored in serum and urine. As in our experiment, HTML and carnitine were the predominant metabolites produced from the labelled TML. Furthermore, they observed that the amount of labelled carnitine in urine was 2 to 8 times greater than labelled butyrobetaine. From these data, and experiments where infants and adults were fed TML or butyrobetaine, Rebouche and co-workers concluded that the rate-limiting step of the carnitine biosynthesis lies prior to the conversion of butyrobetaine to carnitine (33,25). This is in agreement with our findings, which also show higher levels of $^2$H$_9$-carnitine than $^2$H$_9$-butyrobetaine.

Surprisingly, after $^2$H$_9$-TML loading, an increased concentration of endogenous HTML was found. One would expect when such high amounts of $^2$H$_9$-TML are administered, the endogenous pool of TML would be diluted and this would result in a reduction of the endogenous HTML formation and its urinary excretion. Furthermore, the ratio of HTML to butyrobetaine, before $^2$H$_9$-TML (0.97 ± 0.83), compared with the ratio of $^2$H$_9$-HTML to $^2$H$_9$-butyrobetaine (43 ± 22) after loading, is significantly different (p<0.01). This would suggest endogenous HTML is more readily converted into butyrobetaine than $^2$H$_9$-HTML to $^2$H$_9$-butyrobetaine. A possible explanation for both observations could be that the endogenous metabolites and the $^2$H$_9$-metabolites are metabolised in different locations in the body. Normally, TML is released from proteins intracellularly within lysosomes and is believed to be converted into butyrobetaine in the tissue of origin. In both man and rat, circulating TML (including exogenously administered $^2$H$_9$-TML) is primarily utilised by the kidney (35,33,36). Therefore, most $^2$H$_9$-metabolites probably result from metabolism in the kidney and reflect the carnitine biosynthesis in this organ. Because the HTMLA activity in the kidney is low (12), the $^2$H$_9$-HTML produced from $^2$H$_9$-TML would not be readily converted into $^2$H$_9$-butyrobetaine. However, if $^2$H$_9$-butyrobetaine is produced, it is rapidly hydroxylated to carnitine by BBD, which is highly active in kidney.

Our future plans include developing a similar assay for plasma, whole blood and cells/tissues. Although defects in carnitine biosynthesis have not been reported so far, the
ability to measure the intermediates of carnitine biosynthesis will facilitate the identification of patients suffering from such a defect.

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