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Relationship of Carnitine and Carnitine Precursors Lysine, ε-N-Trimethyllysine, and γ-Butyrobetaine in Drug-Induced Carnitine Depletion

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ABSTRACT. Plasma concentrations and rates of urinary excretion of carnitine and some of its precursors were studied in three groups of children receiving drugs known to cause carnitine depletion. Patients in group A received pivampicillin and a molar equivalent of carnitine for 7 d. Patients in group B received pivampicillin with a 5.8-fold molar excess of carnitine for 1 wk. Patients in group C were treated chronically with valproic acid and received a molar equivalent (to valproic acid) of carnitine for 14 d. Patients in group A had markedly increased (16-fold) urinary carnitine ester excretion concomitant with diminished urinary free carnitine and γ-butyrobetaine output and lower plasma free carnitine concentration. Supplementation with one molar equivalent of carnitine (to pivampicillin) was ineffective in preventing the reduction of plasma carnitine concentration observed with pivampicillin treatment alone. For group B patients, administration of excess carnitine resulted in a further increase (35-fold) of urinary carnitine ester output with no decrease of plasma carnitine concentration, urinary γ-butyrobetaine, or free carnitine excretion. For patients in group C, the initially low plasma free and total carnitine concentrations and urinary output of carnitine and carnitine esters markedly increased with carnitine supplementation, but urinary excretion of γ-butyrobetaine remained unchanged. The plasma concentrations and urinary output of L-lysine and ε-N-trimethyllysine remained unchanged within each group before and after treatment. A positive linear correlation was found between urinary ε-N-trimethyllysine and 3-methylhistidine output, indicating that the rate of ε-N-trimethyllysine excretion correlates with the amount of 3-methylhistidine liberated by protein turnover. The data indicate that generation of ε-N-trimethyllysine probably depends on protein breakdown but its use for carnitine synthesis is not necessarily regulated by carnitine status. (*Pediatr Res* 34: 460–464, 1993)

Abbreviations

NMR, nuclear magnetic resonance

Although the carnitine reserves of the humans derive from endogenous synthesis and from alimentary sources (1, 2), carnitine has to be considered as a vitamin-like compound because in healthy humans carnitine depletion can develop under certain conditions, such as with drug therapies (3–5). Pivampicillin (pivaloyloxymethyl ester of ampicillin) generates carnitine depletion because, after enteral absorption of the drug, the liberated pivalate is esterified with carnitine. Sustained renal excretion of this metabolite leads to depletion of the carnitine reserves of the body (4, 6). This decrease of the body stores of carnitine (principally in plasma and muscle) is known to be associated with secondary metabolic changes (hypoketonemia) that can be explained by the function of carnitine in fatty acid oxidation (4, 5). Similarly, by a mechanism not clearly understood, valproic acid therapy has been shown to generate carnitine depletion, characterized by a decrease of plasma and muscle carnitine concentrations and hypoketonemia (3, 6, 7). Rates of endogenous carnitine biosynthesis in patients receiving these therapies are not sufficient to overcome the negative carnitine balance associated with these drugs.

In mammals, carnitine is synthesized from peptide-bound lysine via posttranslational methylation (2, 8). After liberation by proteolytic digestion, ε-N-trimethyllysine is converted to carnitine by four distinct enzymatic reactions (8). Numerous studies have described the characteristics of these reactions *in vitro* (9–12). For humans, the mechanisms underlying regulation of carnitine biosynthesis are not well defined. It is clear that the final enzymatic step in the pathway, catalyzed by γ-butyrobetaine hydroxylase, is not rate limiting (13, 14). In rats, the availability of ε-N-trimethyllysine apparently limits the overall rate of carnitine synthesis (15, 16), but this finding has not been extended unequivocally to humans. This investigation was undertaken to assess the effects of the carnitine-depleting drugs pivampicillin and valproate on carnitine-precursor appearance and metabolism in children acutely (pivampicillin) or chronically (valproic acid) treated with these drugs.

MATERIALS AND METHODS

Patients. Group A: pivampicillin and equimolar carnitine-treated group. Five children (all females; clinical data summarized in Table 1) were selected for the study. All patients were treated previously for urogenital infection with bacteriuria but were not treated with pivampicillin immediately before the start

Table 1. *Characteristics of study participants**

	Age (y)	Weight (kg)	Height (cm)
Group A	9.4 (7–13)	31.9 (24–45)	135 (123–155)
Group B	11.4 (5–16)	35.2 (14–59)	141 (97–162)
Group C	12.0 (7–16)	35.5 (25–51)	144 (124–164)

* Values are means with ranges given in parentheses. $n = 5$ for each group.

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of the study. At the time of the study, they were receiving follow-up treatment to prevent relapse and were healthy with no clinical or laboratory evidence of disease. After a control day (24-h period before start of carnitine and pivampicillin treatment, during which urine and plasma samples were collected; d 0), the patients received 1500 mg (3.24 mmol) of pivampicillin and 525 mg (3.26 mmol) of L-carnitine, divided into three equal doses, daily for 7 d.

Group B: pivampicillin and molar excess carnitine-treated group. Three female and two male children were enrolled in this group (Table 1). These patients were treated for pharyngeal colonization by ampicillin-sensitive streptococci or staphylococci. Their clinical condition at the time of the study was normal, and they were not being treated with pivampicillin immediately before the start of the study. After a control day (d 0), patients in group A were given 1500 mg (3.24 mmol) of pivampicillin per day, divided into three equal doses, for 7 d. L-Carnitine was administered at a higher level, 3 g (18.6 mmol)/d, divided into three equal doses.

Group C: valproic acid and carnitine treated group. Three female and two male epileptic children (asymptomatic and otherwise in good health; age, 12–48 mo; average age, 26 mo) on chronic valproic acid treatment (600 to 1200 mg daily; average, 1050 mg) were enrolled in the study (Table 1). After a control day (d 0), these patients were given a quantity of L-carnitine equal to the valproic acid treatment dose, on a mole-for-mole basis [for each capsule containing 1.07 mmol (150 mg) of valproic acid administered, 172 mg (1.07 mmol) of carnitine was given], for 2 wk.

Procedures. Before the onset (d 0) and on the last day (d 7 or 14) of carnitine treatment, a 24-h urine collection was obtained, and blood was collected after an overnight fast. Plasma and urine were stored at −40°C until analysis. The diet was a standard clinical mixed diet. Informed consent was obtained from the parents of each participant in the study. The study design was approved by the local ethical committee and by the National Pharmacological Institute, Budapest.

Methods. Plasma and urinary acid-soluble carnitine and acylcarnitine esters were quantified as described (17). γ-Butyrobetaine concentrations were determined by conversion of γ-butyrobetaine to carnitine by partially purified γ-butyrobetaine hydroxylase (18), followed by measurement of carnitine formed. ε-N-trimethyllysine in plasma and urine was quantified by fluorescence after HPLC separation of *o*-phthalaldehyde derivatives (19). The presence of pivaloylcarnitine in urine was identified by ^{13}C NMR, using a high resolution GN 500 spectrometer (General Electric, Fremont, CA) at 11.5 T. The acyl groups of carnitine esters in urine were determined by gas-liquid chromatography (4, 6). Concentrations of lysine and 3-methylhistidine were determined by spectrophotometry, after ion-exchange chromatography and postcolumn ninhydrin derivization (20). Urinary total nitrogen output was measured by quantification of ammonia liberated by Kjeldahl digestion, and creatinine was determined spectrophotometrically, as described previously (21).

The *t* test was used for statistical analysis of data within each experimental group. Data for d 0 and d 7 or 14 were paired for each individual to increase the accuracy of comparisons. Between-group comparisons were by analysis of variance, using the SOLO PC program (version 4.0, 1991; BMDP Statistical Software, Los Angeles, CA). Differences were considered significant at $p < 0.05$. The method of least squares was used for linear regression analysis.

RESULTS

Characteristics of patient groups are summarized in Table 1. There were no statistically significant differences in the age, body weight, or height between the three groups. The height and weight of each patient were within the 40th to 60th percentile range using local standards. No statistically significant differences were found in creatinine excretion within groups comparing the data before (d 0) and on the last day (d 7 or 14) of the study (Table 2). Similarly, no differences in creatinine excretion were observed across patient groups.

In the two pivampicillin-treated groups (groups A and B), rates of urinary esterified carnitine excretion were markedly increased after treatment compared with rates before treatment within each group; the difference was much more pronounced in group B subjects than in group A subjects (Table 2). Whereas in group A the output of free carnitine decreased precipitiously after treatment, in group B administration of the higher dose of carnitine resulted in free carnitine output unchanged from the pretreatment rate.

By contrast, subjects in group C excreted significantly less free carnitine on d 0 than their counterparts in groups A and B (who, on d 0, may be regarded as untreated controls for the chronic valproate-treated subjects in group C). After carnitine treatment, free carnitine excretion increased dramatically in group C patients to rates much higher than that in groups A and B. By contrast, water-soluble acylcarnitine ester excretion also increased after carnitine treatment of group C patients but to a much lesser extent than for subjects in groups A and B.

The presence of pivaloylcarnitine as the predominant (or exclusive, >85% of total acylcarnitine esters) carnitine ester in urine was demonstrated in random samples ($n = 7$) selected from patients in groups A and B on d 7 directly by ^{13}C NMR (Fig. 1), or pivaloic acid was determined by gas-liquid chromatography after ester hydrolysis (data not shown).

Acid-soluble acylcarnitine ester concentrations in plasma of subjects in groups A and B were increased with pivampicillin plus carnitine treatment (Table 3). This increase was due perhaps to increased circulating pivaloylcarnitine (6). In group A, a decrease in the free carnitine concentration was observed on the last day of treatment, whereas in group B, a moderate increase was found compared with the pretreatment concentration (on d 0). For patients in group C, the levels of free, acyl, and total carnitine were lower before the start of carnitine supplementation compared with d 0 levels in subjects in groups A and B, and on the last day increases were found in each of these fractions.

The rate of urinary excretion of γ-butyrobetaine was different in the two groups of pivampicillin-treated subjects (Table 4). In group A, a decrease was observed in γ-butyrobetaine output. In three patients, it dropped to the level of detection (0.1 μmol/L) after treatment; by contrast, in group B, the rate of γ-butyrobetaine excretion remained unchanged compared with the pretreatment control day. For all data for patients in groups A and B, urinary excretion of γ-butyrobetaine was positively correlated to excretion of free carnitine ($r = 0.75$; $p < 0.05$; data not shown). The equation describing the regression line was $y = 0.03x + 1.39$. For patients in group C, the rate of γ-butyrobetaine excre-

Table 2. *Urinary excretion of carnitine, acid-soluble acylcarnitine esters, and creatinine**

	Free carnitine (μmol/d)	Acid-soluble acylcarnitine esters (μmol/d)	Total acid-soluble carnitine (μmol/d)	Creatinine (mmol/d)
Group A				
Day 0	115 ± 6.30	109 ± 18.6	224 ± 20.9	3.17 ± 0.66
Day 7	0.06 ± 0.06†	1790 ± 276†	1790 ± 276†	3.44 ± 0.68
Group B				
Day 0	126 ± 48.4	89.4 ± 27.5	216 ± 74.7	4.38 ± 1.07
Day 7	127 ± 49.7	3120 ± 604†	3250 ± 628†	5.05 ± 1.57
Group C				
Day 0	12.1 ± 1.98‡	53.6 ± 9.49	65.7 ± 9.54‡	4.70 ± 0.92
Day 14	910 ± 139†	334 ± 39.8†	1240 ± 12.5†	5.51 ± 1.06

* Values are mean ± SEM; $n = 5$ for each group.
† $p < 0.05$ *vs* d 0 within the same group.
‡ $p < 0.05$ *vs* d 0 in group A or group B.

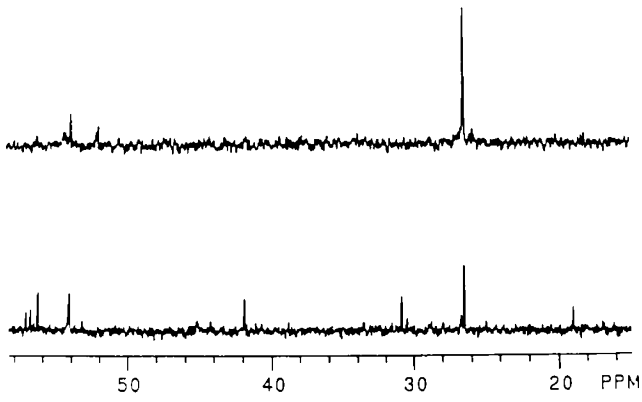

Fig. 1. ^{13}C NMR spectrum of authentic pivaloylcarnitine (*upper spectrum*) and representative ^{13}C NMR spectrum of concentrated urine from a patient in group A (*lower spectrum*). In urine from patients in groups A and B, a characteristic peak was found at about 26 ppm that corresponds to the three methyl groups of pivalate in pivaloylcarnitine. Another peak was found at 54 ppm, corresponding to the three methyl groups of carnitine.

Table 3. *Concentrations of carnitine and acid-soluble acylcarnitine esters in plasma**

	Free carnitine (μmol/L)	Acid-soluble acylcarnitine esters (μmol/L)	Total acid-soluble carnitine (μmol/L)
Group A			
Day 0	32.3 ± 3.48	11.1 ± 1.94	43.4 ± 3.65
Day 7	9.46 ± 2.69†	20.0 ± 2.91†	29.5 ± 2.69†
Group B			
Day 0	34.0 ± 2.46	9.24 ± 0.99	43.3 ± 1.83
Day 7	44.0 ± 5.70†	16.3 ± 4.14†	60.3 ± 9.12†
Group C			
Day 0	24.6 ± 2.95‡	6.24 ± 1.82‡	30.8 ± 3.73‡
Day 14	46.0 ± 3.19†	10.2 ± 1.17†	56.2 ± 4.11†

* Values are mean ± SEM; $n = 5$ for each group.
† $p < 0.05$ *vs* d 0 within the same group.
‡ $p < 0.05$ *vs* d 0 in group A or group B.

Table 4. *Urinary excretion of L-lysine, ε-N-trimethyllysine, and γ-butyrobetaine**

	L-Lysine (μmol/d)	ε-N-trimethyllysine (μmol/d)	γ-Butyrobetaine (μmol/d)
Group A			
Day 0	32.4 ± 2.28	26.1 ± 4.24	3.60 ± 1.16
Day 7	24.8 ± 5.48	30.4 ± 4.32	0.30 ± 0.30†
Group B			
Day 0	43.1 ± 1.88	21.3 ± 3.67	3.61 ± 0.83
Day 7	42.8 ± 2.22	24.8 ± 7.63	3.14 ± 0.65
Group C			
Day 0	36.8 ± 8.33	27.3 ± 5.84	2.79 ± 0.47
Day 14	41.9 ± 7.83	35.8 ± 6.22	2.91 ± 0.76

* Values are mean ± SEM; $n = 5$ for each group.
† $p < 0.05$ *vs* d 0.

tion was not statistically different from the untreated control subjects (groups A and B, d 0), and remained unchanged on the last day of carnitine administration.

Urinary output of lysine and ε-N-trimethyllysine did not change significantly on the last day of the study period compared with the pretreatment control day (Table 4). Similarly, no changes were found in the plasma concentrations of these amino acids before and after the various treatments (Table 5).

Regression analysis of data for all patients obtained before and on the last day of treatments revealed a positive correlation ($p <$ 0.05) between rates of urinary 3-methylhistidine and ε-N-trimethyllysine excretion (Fig. 2). Although data for each group alone did not provide significant correlations (perhaps due to the small number of observations within each group and clustering of data in group C, and to a lesser extent in group B, within a narrow range of ε-N-trimethyllysine concentrations) the data analyzed in aggregate provide evidence for a significant positive relationship between excretion of ε-N-trimethyllysine and 3-methylhistidine.

Table 5. *Concentrations of L-lysine and ε-N-trimethyllysine in plasma**

	L-Lysine (μmol/L)	ε-N-trimethyllysine (μmol/L)
Group A		
Day 0	178 ± 7.93	0.40 ± 0.03
Day 7	171 ± 16.4	0.46 ± 0.03
Group B		
Day 0	166 ± 18.3	0.42 ± 0.05
Day 7	179 ± 15.6	0.42 ± 0.06
Group C		
Day 0	196 ± 17.3	0.42 ± 0.03
Day 14	176 ± 11.9	0.47 ± 0.04

* Values are mean ± SEM; $n = 5$ for each group.

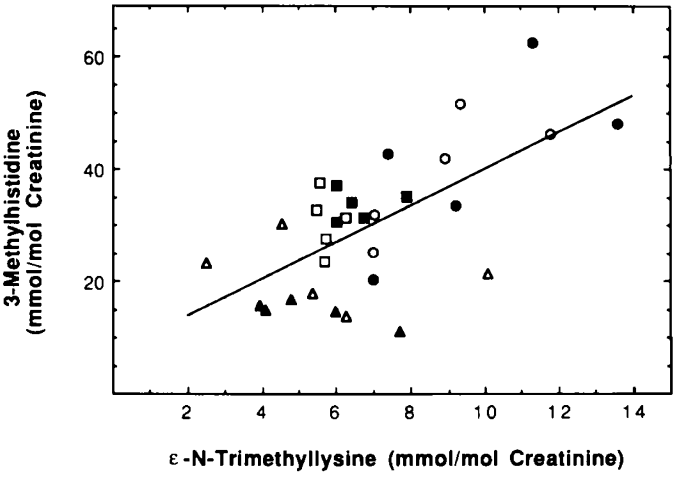

Fig. 2. Relationship between urinary ε-N-trimethyllysine and 3-methylhistidine excretion. For analysis, data from all patients in each of the three groups were used (30 observations from 15 individuals). *Open circles*, group A before treatment; *closed circles*, group A after treatment; *open triangles*, group B before treatment; *closed triangles*, group B after treatment; *open squares*, group C before carnitine supplementation; and *closed squares*, group C after carnitine supplementation. *Solid line* indicates linear regression of the data, described by the equation, $y = 3.26x + 7.37$ ($r = 0.641$).

Urinary total nitrogen and urea excretion were not different within any of the three groups before and after treatment.

DISCUSSION

Pivalic acid is conjugated to several antibacterial drugs to promote their efficient absorption (22, 23). These conjugates, once absorbed, are hydrolyzed to the parent drug and pivalic acid. In humans, pivalic acid is excreted predominantly as an ester of L-carnitine (4, 24). Like other esters of carnitine, fractional excretion of pivaloylcarnitine is greater than that for free carnitine. Administration of pharmacologic amounts of pivalate-conjugated drugs results in carnitine depletion, characterized by reduced concentrations of carnitine in the circulation and tissues, and hypoketonemia. These effects are overcome by pharmacologic administration of carnitine with the drug. Results from this study show that administration of one molar equivalent of

carnitine with pivampicillin is insufficient to prevent the carnitine-depleting effects of the drug. The lack of efficacy of a molar equivalent of orally administered carnitine probably is a consequence of poor absorption of pharmacologic amounts of this compound. Pharmacologic amounts (1 or more g per day) administered orally to humans are less than 20% absorbed (25–27). By contrast, when a 6-fold molar excess of carnitine was given orally with pivampicillin, free carnitine concentration in plasma remained unchanged from the pretreatment level, and the rate of urinary excretion of free carnitine also was not different.

Urinary excretion of γ-butyrobetaine was different in the two groups of pivampicillin-treated subjects (Table 4). In group A, a marked decrease was observed in the γ-butyrobetaine output from d 0 to d 7. By contrast, in group B, the rate of γ-butyrobetaine excretion remained unchanged after carnitine and pivampicillin treatment compared with the rate before treatment. This observation may be explained by changes in one or a combination of metabolic processes, including differences in the tissue and circulating concentrations of γ-butyrobetaine, perhaps affecting its use for carnitine synthesis, and differences in the efficiency of reabsorption of γ-butyrobetaine before and after treatment. The data available are not sufficient to distinguish between these alternative hypotheses.

Numerous studies in experimental animals and humans have shown that chronic valproic acid treatment leads to depletion of carnitine in blood and tissues and reduces the rate of urinary carnitine excretion. The mechanism for this effect is unknown. Unlike pivalic acid, valproic acid is a poor substrate for carnitine ester formation (6, 28). Data from this study are consistent with previous findings: Most of the carnitine supplement administered was not used for ester formation (as was the case with pivampicillin treatment) but was eliminated in the urine as free carnitine.

Valproic acid administration resulted in a lower rate of excretion of carnitine, 1.85 μmol·kg body $wt^{-1}\cdot d^{-1}$ for subjects in group C (d 0), compared with 6.58 μmol·kg body $wt^{-1}\cdot d^{-1}$ for subjects in groups A and B (d 0). A large measure of this decrease was probably due to decreased filtered load of carnitine (plasma total carnitine in valproic acid–treated subjects was 30.8 μmol/L compared with controls, 43.4 μmol/L). Nevertheless, the difference in the rates of excretion of carnitine was larger (by almost 4-fold) than the estimated rate of carnitine synthesis [1.20 μmol·kg body $wt^{-1}\cdot d^{-1}$; estimated from steady state carnitine excretion by strict vegetarians (29)] suggesting that the effect of valproic acid on carnitine homeostasis may be manifest in the efficiency of carnitine absorption, reabsorption, or both.

Circulating concentrations and rates of excretion of ε-N-trimethyllysine were not different before and after treatment in any of the three treatment groups. Thus, ε-N-trimethyllysine production and use does not seem to be altered by substantial changes in carnitine status. ε-N-trimethyllysine excretion correlated with 3-methylhistidine excretion, indicating that the rate of ε-N-trimethyllysine excretion probably is determined by the rate of protein turnover and not necessarily by the rate of carnitine synthesis. The normal rate of ε-N-trimethyllysine excretion (0.71 μmol·kg body $wt^{-1}\cdot d^{-1}$, estimated from d 0 rates by patients in groups A and B) is about 60% of the estimated normal rate of carnitine synthesis. Thus, the rate of carnitine synthesis could be affected significantly if more or less of total ε-N-trimethyllysine were used in this pathway. Principally two factors must regulate the disposition of ε-N-trimethyllysine released by protein digestion into the pathway of carnitine biosynthesis or to excretion by the kidney. The first factor is entry of the amino acid into mitochondria, where ε-N-trimethyllysine hydroxylase is located (10). Proteolysis occurs primarily in extramitochondrial compartments, releasing ε-N-trimethyllysine into the cytosol. To date, no studies have been reported describing the characteristics of entry of this substrate into mitochondria. However, it is well known that ε-N-trimethyllysine is poorly transported across plasma membranes of many tissues (30). Entry of the substrate into mitochondria may present a significant rate-limiting obstacle for use of ε-N-trimethyllysine for carnitine synthesis.

The second factor is the affinity of the hydroxylase for its substrate, relative to the concentration of the substrate. ε-N-trimethyllysine concentration in rat muscle was estimated to be about 24 μmol/L (and somewhat lower in other tissues) (16). The Km for ε-N-trimethyllysine hydroxylase in rat tissues was reported to be about 0.1 mmol/L (31). Thus, the enzyme probably is not saturated under most conditions, and fluctuations in substrate concentration should be reflected in changes in the rate of β-hydroxy-ε-N-trimethyllysine formation. Availability of increased substrate, by oral administration of ε-N-trimethyllysine, significantly increased the rate of carnitine synthesis in both rats (15) and humans (13, 14), suggesting that the capacity both to transport ε-N-trimethyllysine into mitochondria and to hydroxylate this substrate is not fully used under normal conditions. Thus, the appearance of significant amounts of ε-N-trimethyllysine in urine must reflect partitioning of the substrate at the cellular level, between export into the circulation and import into mitochondria and/or hydroxylation within the mitochondria. In humans, ε-N-trimethyllysine is not reabsorbed to any significant extent (32). Coupled with its poor uptake from the circulation by tissues, export from the cell effectively consigns this amino acid to disposal from the body. Because ε-N-trimethyllysine is readily reabsorbed by rats, its efficiency of use for carnitine biosynthesis should be more than in humans. Davis and Hoppel (16) estimated that normally about 80% of ε-N-trimethyllysine is used for carnitine synthesis in rats. Based on relative rates of carnitine synthesis (estimated as described above) and rates of excretion of ε-N-trimethyllysine in this study (d 0 for children in groups A and B), the efficiency of use of ε-N-trimethyllysine for carnitine synthesis in humans is about 63%.

In summary, an excess molar equivalent of carnitine is necessary to overcome the carnitine-depleting effects of pivalate administered as the oral antibiotic ampicillin. Conversely, one molar equivalent of carnitine, administered as an oral supplement, is sufficient to normalize plasma carnitine concentrations and increase the rate of urinary carnitine excretion in patients treated with valproic acid. For all patients taken together, ε-N-trimethyllysine excretion was significantly correlated with 3-methylhistidine excretion, suggesting that the rate of excretion of ε-N-trimethyllysine is determined by the rate of protein turnover and not by the rate of carnitine biosynthesis.

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