Rapid Publication

L-Carnitine Therapy in Isovaleric Acidemia

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Abstract. Isovaleric acidemia, resulting from isovaleryl-coenzyme A dehydrogenase deficiency, is associated with marked reduction of free carnitine in both plasma and urine. Fast atom bombardment-mass spectrometry, hydrolysis, and gas chromatography/mass spectrometry have unequivocally identified the existence of isovalerylcarnitine, a new metabolite specific for this disorder. Administration of equimolar amounts of glycine or L-carnitine separately with leucine demonstrated that isovaleryl-coenzyme A is removed by supplemental L-carnitine in the form of isovalerylcarnitine as effectively as it is by glycine, in the form of isovalerylglycine. When L-carnitine is given alone, excretion of isovalerylglycine decreases in preference to enhanced excretion of isovalerylcarnitine and hippurate. Treatment with L-carnitine alone has proven effective in preventing further hospitalizations in a patient with this genetic disorder.

Introduction

Isovaleric acidemia, a defect in leucine metabolism, was first recognized and described by Tanaka and associates (1, 2). Isovalerylglycine (IVG)[1] was identified as a major urinary metabolite (3). Glycine therapy has been demonstrated to be an effective means of removing isovaleryl-coenzyme A (CoA) as IVG (4–6). This disorder characterizes the concept of a relative insufficiency of a normal metabolite, glycine, to meet the requirements for conjugation and excretion of an otherwise toxic intermediate, isovaleryl-CoA, by an existing mitochondrial enzymatic pathway, glycine-*N*-acylation (3).

Recently, examples of the relative insufficiency of another normal metabolite, L-carnitine, have been suggested. In both methylmalonic aciduria and propionic acidemia, the toxic metabolite propionyl-CoA is effectively excreted as propionylcarnitine after carnitine supplementation (7–9). Similarly, in the medium chain acyl-CoA dehydrogenase deficiency, enhanced excretion of hexanoylcarnitine and octanoylcarnitine were demonstrated (10). Stanley et al. (11) have demonstrated carnitine deficiency in isovaleric acidemia and suggested increased excretion of acylcarnitine as a possible cause.

In this study, we present the unequivocal identification of isovalerylcarnitine (IVC) and its excretion in isovaleric acidemia and attempt to compare the relative therapeutic utility of L-carnitine with that of glycine in this disorder.

Methods

The materials administered to the patient in this study were glycine and L-leucine, supplied by Ajinomoto, Inc. (Raleigh, NC), and L-carnitine, supplied by Sigma-Tau (Holmdel, NJ). IVG and *n*-valerylglycine were gifts from Dr. R. A. Chalmers (Clinical Research Center, Pediatric Research Group, Harrow, England). Benzoylglycine (BG) and the silylation reagents were obtained from Sigma Chemical Co. (St. Louis, MO), while [$^2H_2$]BG and [$^2H_9$]isovaleric acid (IVA) were supplied by MSD Isotopes Div., Merck Frosst Canada, Inc. (St. Louis, MO). IVC and [$^2H_9$]IVC were synthesized from the free acids using thionyl chloride and from L-carnitine using a procedure described by Bohmer and Bremer (12) for propionylcarnitine. All aqueous solutions were prepared in distilled deionized water, and all other solvents and materials were reagent grade.

The mass spectrometer/datasystem used in this research was a double focusing magnetic sector type, model 7070HS/11-250 (VG Instruments, Inc., Stamford, CT), coupled to a Varian 3700-Series gas

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Received for publication 14 August 1984.

1. *Abbreviations used in this paper:* B/E, magnetic (B) to electric (E) field ratio; BG, benzoylglycine; Co A, coenzyme A; CoASH, free coenzyme A; FAB, fast atom bombardment; GC, gas chromatography; IVA, isovaleric acid; IVC, isovalerylcarnitine; IVG, isovalerylglycine; MS, mass spectrometry; m/z, mass to charge ratio.

J. Clin. Invest.

0021-9738/84/12/2290/6 $1.00
Volume 74, December 1984, 2290–2295

chromatograph (Varian Associates, Palo Alto, CA). For GC/MS analyses of IVG and BG, samples (1–2 μl) were injected in the split mode (1:30 split ratio) onto a fused silica capillary GC column (30 m/DB-1, Durabond J & W Scientific, Rancho Cordova, CA) that was directly coupled to the mass spectrometer's ion source. The conditions were: injector temperature, 250°C, interface, 260°C, ion source, 220°C (Electron Ionization, 70 eV electron energy, 1000 resolution), oven temperature program, 8°C/min from 90–200°C. For IVA analysis, the oven was operated isothermally (90°C). Under these conditions, isovaleric and 2-methylbutyric acids are completely resolved as the trimethylsilane esters. Fast atom bombardment-mass spectrometry (FAB-MS) and constant magnetic (B) to electric (E) field ratio-linked scan analyses were carried out using systems supplied by the manufacturer as previously described (13). Typically, samples (1–2 μl) were dispersed in glycerol (10–20 μl) on the target, which was bombarded with xenon atoms at 6 KeV. The constant B/E ratio-linked scans were recorded oscillographically.

Assay for plasma IVA. The internal standard, [$^2H_9$]IVA (5 nmol), 0.8 ml water, 10 μl concentrated HCl, and sodium chloride (to saturation) were added to the sample (0.1–0.2 ml). The mixture was extracted with ether (2 × 1.0 ml) and the combined extract was concentrated to ~0.1 ml in a stream of nitrogen. Bis(trimethylsilyl)-trifluoroacetamide (BSTFA; 20 μl) was added and the mixture allowed to stand at 20°C for 15 min. Analysis was performed by GC/MS as described above, while monitoring the signals at mass to charge ratio (m/z) 159.08 and 168.14 corresponding to the $(M-15)^+$ ions of IVA and [$^2H_9$]IVA, using the mass spectrometer in selected-ion monitoring mode. These two components were completely resolved and enabled the peak area ratio (m/z, 159/168) to be accurately determined. This ratio was converted into concentration by comparison with a standard curve, derived by adding 0–5 nmol IVA plus 5 nmol [$^2H_9$]IVA into 1-ml aliquots of water, with extraction and analysis as described above. The detection limit was below 1 nmol/ml (0.1 mg/liter).

Specific identification of IVC. An aliquot of urine (0.1–1.0 ml) was acidified (10 μl 6 N HCl) and applied to a cationic exchange column (Dowex-50, 30 × 2 mm). After washing with 0.1 N HCl (two bed volumes) and water (five bed volumes), the acylcarnitines were eluted with 1 N NH_4OH in water:ethanol (4:1; 6 ml). This fraction was lyophilized and treated with urease (1 unit in 1.0 ml water) at 37°C for 30 min, then lyophilized again, and redissolved in 0.01 N HCl (0.1 ml). About 2 μl of this solution, which contained no free IVA as judged by GC/MS of an ether extract, were applied in glycerol (20 μl) to the FAB ion-source target. The remaining sample was base-hydrolyzed (0.2 N KOH, 37°C, 30 min), acidified with 6 N HCl, and extracted with ether (2 × 1.0 ml). The ether extract was concentrated, treated with bis(trimethylsilyl)-trifluoroacetamide (100 μl) for 30 min at 20°C, and then analyzed by GC/MS.

Assay for L-carnitine and acylcarnitine. Serum and urinary L-carnitine and acylcarnitines were determined by the radioenzymatic assay using [^{14}C-l]acetyl-CoA and carnitine acetyltransferase as described previously (14).

Specific assay for IVC. A sufficient volume of the internal standard solution ([$^2H_9$]IVC in distilled deionized water, 1 g/liter) was added to an aliquot of urine (100–500 μl) to provide approximately the same molar concentration as the total acylcarnitine determined by radioenzymatic assay. This mixture was then purified as described above for the specific identification of IVC. Direct measurement of the ratio of MH^+ ion intensities for IVC and [$^2H_9$]IVC (m/z, 246/255) was accomplished by high resolution FAB-selected-ion monitoring analysis (13). This ratio was converted into molar concentration using a calibration curve derived by standard addition of IVC to aliquots of normal urine, to which a fixed amount of [$^2H_9$]IVC was added, then applying the extraction and analytical procedures identical with those described for the patient's urine. Some of the purified urine extracts were base-hydrolyzed (0.1 N KOH, 37°C, 0.5 h) and the liberated free fatty acids extracted into ether and analyzed by GC/MS. By measuring the ratio of signals for these free acids using the mass spectrometer and following the calibration procedure described above for IVA, a second, independent determination of the IVC concentration was afforded.

Assays for IVG and hippurate (BG). The internal standards, *n*-valerylglycine, and [$^2H_2$]BG (40 μl each in methanol solution [1 g/liter]) to an aliquot of the patient's urine (50–100 μl). After addition of water (0.5 ml), 6 N HCl (10 μl), and sodium chloride (0.5 g), the solution was extracted with ether (2 × 1.0 ml). The ether was evaporated and the residue was treated with *N*-(t-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA; 100 μl) for at least 15 min at 20°C, and then analyzed by GC/MS. The ratios of signals corresponding to $[M-57]^+$ ions were determined for the IVG/*n*-valerylglycine pair and for the BG/[$^2H_2$]BG pair. These ratios were converted into concentration by calibration of response ratio vs. concentration in the manner described for the IVC assay (above). If the ratio of signals from the sample urine was outside the calibration range, the analysis was repeated using either a different volume of urine or a different quantity of internal standard.

Case report. This 4 1/12-yr-old black male was the 7 lb., 14 oz. product of an uncomplicated pregnancy, delivery, and neonatal period. He was well until 1 yr of age when he was hospitalized because of vomiting, diarrhea, and dehydration. He was hospitalized again for a week at the ages of 15 mo and 3 yr for the same complaints. Each episode was followed by a brief period of alopecia. The patient was characterized as a poor eater, developmentally delayed, physically weak, and unable to keep up with his peers. At 3 yr, 9 mo, he was referred to Duke University Medical Center in an unresponsive state following a 5-d period of vomiting and diarrhea. He was obtunded and dehydrated, but responded to pain. There was no odor. Admission chemistries included normal serum sodium, potassium, and chloride; CO_2, 9 mmol/liter; blood-urea nitrogen, 88 mg/dl; creatinine, 2.7 mg/dl; and glucose, 172 mg/dl. Admission hemoglobin was 10.5 g/dl and hematocrit, 32%; leukocyte count was 5,100/mm^3; plasma ammonia, 128 μg/dl, and serum salicylate, 5.5 mg/dl. During the course of hospitalization, alopecia and pancytopenia developed (hemoglobin, 7.7 g/dl; hematocrit, 24%; leukocyte count, 900/mm^3; and platelets, 18,000/mm^3). By discharge, leukocyte count was 4,700; platelets, 43,500; and hemoglobin was 7.8. Organic acid analysis by GC/MS revealed large quantities of IVG and 3-hydroxyisovalerate in the urine. The patient was placed on a protein-restricted diet (1.5 g/kg/d) until admission to the Clinical Research Unit. At that time, he had mild alopecia and both expressive and receptive language delay. There was weakness in both abdominal and proximal hip musculature and exaggerated balance reactions, which were delayed compared with normal children at the same age.

Results

The patient was studied while on dietary restriction of protein (1.5 g/kg/d). He had not previously received any glycine or carnitine supplementation. His plasma amino acids were decreased and there was no hyperglycinemia. The pretreatment plasma free carnitine was 5.2 μM ($n = 9$) compared with

Table I. Plasma and Urinary Carnitine Status before Treatment

	Total	Free	Short-chain acylcarnitines	Long-chain acylcarnitines
Plasma (μM)				
Patient (n = 9)	13.4±2.6	5.2±2.0	5.8±1.8	2.3±0.5
Normal	46.1±10.0	36.7±7.6	5.7±3.5	3.7±1.5
Urine ($\mu mol/g$ *creatinine*)				
Patient (n = 4)	36±2.3	4.4±3.5	31.7±2.5	—
Normal	125.1±74.7	51.3±40.1	73.7±39.5	—
Urine (μmol/24 h)				
Patient	6.7	0.9	5.8	—
Normal	217.2±78.4	135.0±73.6	81.2±20.7	—

All values are given as mean±SD.

normal value of 36.7 μM, while the acylcarnitine level was within the normal range (Table I). Similar data have been reported for three other patients with this disorder by Stanley et al. (11). The low plasma total carnitine was apparently due to reduction in available free carnitine. Similarly, the mean urinary free carnitine was greatly reduced to 4.4 compared with the normal value of 51.3±40.1 nmol/mg creatinine, and the 24-h excretion of carnitine before treatment was well below the normal range.

The excreted acylcarnitines were purified by ion exchange chromatography and analyzed by fast atom bombardment-mass spectrometry (FAB-MS). The only significant signal above m/z 200 not attributable to the glycerol matrix was at m/z 246, corresponding to the protonated molecular species of IVC (Fig. 1 *A*). The fragmentation pattern of this ion (Fig. 1 *B*) was identical with that of synthetic IVC (Fig. 1 *C*), but the possibility that the isomeric 2-methylbutyrylcarnitine was also present could not be ruled out by this procedure. However, base hydrolysis of the purified acylcarnitine fraction yielded only IVA plus a little acetic acid, as judged by GC/MS analysis of the ether extract, indicating that the acylcarnitine was predominantly IVC. Normal children excrete mostly acetylcarnitine (15). Selected urine samples from the patient were analyzed by these methods throughout the procedures described below and revealed that IVC was always the dominant acylcarnitine excreted. The intact IVC was assayed in some of these samples by isotope dilution FAB-MS, using [$^2H_9$]IVC as the internal standard. The values obtained were the same (within 15%) as those derived from the short-chain acylcarnitine radioenzymatic assay. Therefore, the radioenzymatically derived quantitative data for short-chain acylcarnitine was considered equivalent to an assay for IVC for the purpose of the studies on this patient.

A series of studies was performed on the patient in the Clinical Research Unit, with informed consent. The following paragraphs describe the procedures and their results in the sequence in which they were carried out. An interval of 48 h was allowed between each procedure to enable return to base line.

Leucine alone. Leucine, 25 mg/kg (0.19 mmol/kg), failed to produce any untoward clinical response and there was no detectable increase in plasma IVA, in contrast to the patient

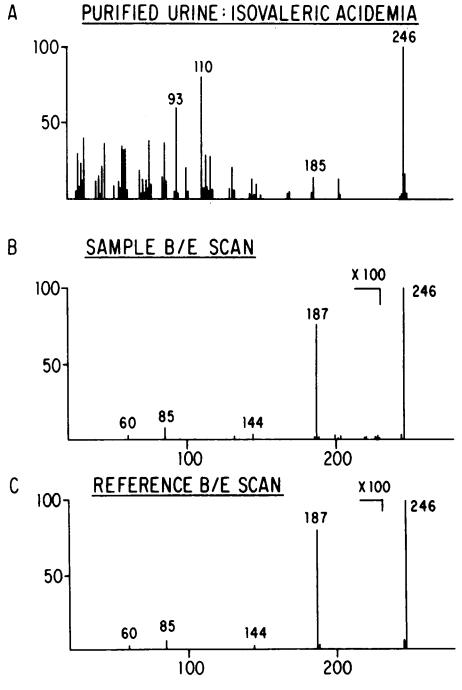

Figure 1. Identification of IVC in admission urine of a patient with isovaleryl-CoA dehydrogenase deficiency. (*A*) FAB mass spectrum of partially purified urine after urease treatment, showing prominent signal at m/z 246 corresponding to the protonated molecule. (*B*) Fragmentation pattern of the m/z 246 ion in urine obtained by constant B/E ratio-linked scan. (*C*) Fragmentation pattern of the m/z 246 ion from synthetic IVC.

studied by Yudkoff et al. (5) following the same dose of leucine. Therefore, he was given 50 mg/kg (0.38 mmol/kg) leucine. Vomiting commenced after 1 h and persisted for 3 h, but he required no therapy and had no further discomfort or nausea. Plasma IVA was not detectable prior to this challenge but rose to 2.7 mg/liter (26.5 μM) at 2 h and gradually decreased to 1.1 mg/liter at 8 h after ingestion. Urinary IVG increased from 8.8 to 21.5 μmol/mg creatinine at 2 h and was associated with a decrease in BG excretion from 4.2 to 1.2 μmol/mg creatinine at the same time (Fig. 2 *A*). Urinary IVC increased slightly, from 0.02 to 0.15 μmol/mg creatinine, by 4 h following the leucine load.

Leucine plus glycine. Since an oral dose of leucine at 0.38 mmol/kg produced measurable clinical and chemical responses, this amount was administered with glycine at 2 mmol/kg (150 mg/kg). There was no apparent clinical response. However, plasma IVA rose to 3.9 mg/l (38.9 μM) after 2 h and declined to 2.3 mg/liter after 4 h. Fig. 2 *B* illustrates the response of IVG excretion, rising from 8.5 to 34.9 mmol/mg creatinine in 3 h. BG did not decrease in the presence of glycine supplementation as had occurred with leucine alone. IVC increased very slightly from the low level of 0.03 to 0.08 μmol/mg creatine by 5 h.

Leucine plus carnitine. Leucine (0.38 mmol/kg) was given with L-carnitine (2 mmol/kg) orally. Again, there was no nausea, vomiting, or other clinical response. Plasma IVA was undetectable after 2 h and returned to the prechallenge level of 1.9 mg/liter after 6 h. As shown in Fig. 2 *C*, excretion of IVG did not significantly change during the 24-h period of this study, remaining between 10–12 μmol/mg creatine. Similarly, there was no significant alteration in the excretion of BG. However, IVC increased dramatically, from 0.03 to 23.7 μmol/mg creatinine, by nearly 800-fold.

L-carnitine alone. The patient was given L-carnitine, 100 mg/kg (0.62 mmol/kg), orally without exogenous glycine or leucine (Fig. 2 *D*). Again, plasma free IVA actually decreased from 1.4 to 0.1 mg/l by 2 h, and then, rose to 2.1 mg/l by 4 h. Prior to administration of L-carnitine, IVG excretion was 10.5 μmol/mg creatinine. In <1 h, the level had decreased to 8.1; by 4 h, it had reached a nadir of 4.6 μmol/mg creatinine; and by 9 h, gradually returned to control levels. There was a concomitant increased excretion of IVC rising abruptly from a level of 0.04 to 6.0 μmol/mg creatinine in <4 h with a peak level of 7.0 μmol/mg creatinine just under 7 h. Associated with this inverse relationship of decreasing IVG and increased IVC was a significant rise in hippurate from 1.0 to 4.0 μmol/mg creatinine, which followed the temporal sequence of IVC excretion.

The total amounts of IVG, hippurate, and IVC excreted in the 24-h periods are presented in Table II. There were four pretreatment or intermediate 24-h urine collections. For these, the mean excretion in micromoles (±SD) was 1605±722 for IVG, 193±97 for BG, and 6±1 for IVC (the acylcarnitine fraction was totally IVC). In terms of patient weight, the mean±SD in micromoles per kilogram per 24 h were 98±44 for IVG, 12±5 for BG, and 0.4±0.1 for IVC. An additional excretion of 111 μmol IVC during the 48-h rest period following the leucine plus carnitine experiment is not shown in the table.

Since discharge, the patient has been treated with L-carnitine (25 mg/kg/6 h). He was not given glycine supplementation because the level of isovaleryl-CoA elimination as

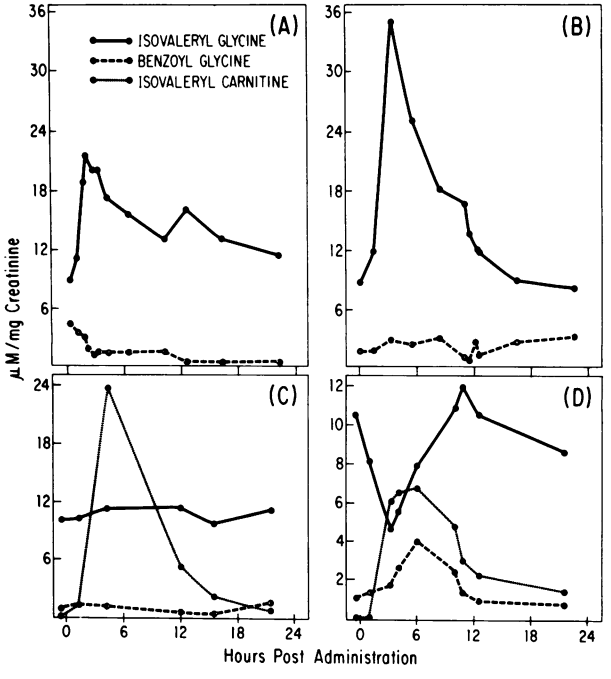

Figure 2. Urinary excretion of IVG, BG, and IVC in a patient with isovaleryl-CoA dehydrogenase deficiency in response to (*A*) leucine (0.38 mmol/kg); (*B*) leucine (0.38 mmol/kg) plus glycine (2 mmol/kg); (*C*) leucine (0.38 mmol/kg) plus carnitine (2 mmol/kg) and (*D*) carnitine (0.62 mmol/kg).

Table II. Excretion of Acylglycines and IVC in a Patient with Isovaleric Acidemia

Procedure	IVG	BG	IVC
Leucine	2,710* (165)‡	222 (14)	11 (0.7)
Leucine + glycine	4,036 (246)	615 (38)	13 (0.8)
Leucine + carnitine	2,379 (145)	240 (15)	1,170 (71)
Carnitine	1,457 (89)	371 (23)	736 (45)
Pretreatment	1,605±722	193±97	6±1
(n = 4; mean±SD)	98±44‡	12±6	0.4±0.1

* μmol/24 h.
† μmol/kg per 24 h.

IVC was equivalent to that of IVG following glycine supplementation. Clinically, he has done quite well except for one notable occasion when the carnitine treatment was interrupted. After 4–5 d without carnitine, he became acutely ill and required hospitalization. Treatment then consisted of intravenous glucose and oral L-carnitine (50 mg/kg/6 h). He recovered and was alert within 24 h. Previously, pretreatment hospitalizations had required several days of fluid support to achieve clinical recovery. Clinical examination after 6 mo of carnitine therapy revealed no evidence of muscle weakness. There was marked improvement in coordination and locomotion skills, which are now only slightly delayed. Although language skills have also improved, there is still marked delay compared with normal.

Discussion

Isovaleryl-CoA is produced inside the mitochondrion by oxidative decarboxylation of 2-oxo-isocaproate. This step requires ATP and free coenzyme A (CoASH). Deficiency of isovaleryl-CoA dehydrogenase in this disease then results in an accumulation of isovaleryl-CoA within the mitochondrion. This toxic acyl-CoA can then either be conjugated with glycine to form IVG and exit the mitochondrion, or be conjugated to L-carnitine as IVC. Both metabolites are excreted in the urine and are associated with the beneficial effect of restoring intramitochondrial free CoA levels. Thus, when carnitine is available, isovaleric acidemia in addition to propionic acidemia (9), methylmalonic aciduria (8), and the medium-chain acyl-CoA dehydrogenase deficiency (10), is characterized by elimination of a short- or medium-chain acyl-CoA compound as a specific acylcarnitine. The carnitine acyltransferases provide an effective pathway for detoxification and elimination of those compounds which include isovaleryl-CoA, propionyl-CoA, hexanoyl-CoA, and octanoyl-CoA.

In previous studies (4–6), glycine administration, either as a bolus or as chronic therapy, is associated with a pronounced increase in excretion of IVG. Yudkoff et al. (5) also demonstrated an increase in hippurate (BG) excretion. In the present study, both IVG and BG excretion increased significantly compared with administration of leucine alone (Table II). Although benzoyl-CoA is a better substrate than isovaleryl-CoA for the mitochondrial glycine-*N*-acylase (16), the relative amount of isovaleryl-CoA could account for the lack of stimulation of BG synthesis when leucine is administered alone as suggested earlier (4). However, when glycine is supplemented and IVG is formed, CoASH is made available for the formation of benzoyl-CoA, the rate limiting step of BG synthesis (17). It is worth noting that patients with this disorder, including the case discussed here, often present with elevated plasma salicylate when acutely ill. Benzoate is a competitor for glycine and as Krieger and Tanaka (4) have pointed out, salicylate also would compete and therefore be contraindicated along with dietary sources of benzoate or hydroxybenzoate.

Administration of a 2 mmol/kg bolus of L-carnitine with leucine produced major changes. IVC excretion increased from a pretreatment level of 7 μmol/24 h to 1170 μmol (Table II) and was sustained for an additional 48 h, producing a total excretion of 1281 μmol of IVC from that single dose. By comparison, glycine at the same dose produced excretion of isovaleryl-CoA as IVG of 1326 μmol above that produced by leucine alone. BG excretion was also elevated significantly. During the load with leucine and carnitine, BG excretion was essentially unchanged, while IVG was increased relative to the pretreatment level (Table II). However, this increase was considerably less than that observed with leucine alone, suggesting competitive formation of IVC. Furthermore, carnitine alone produced a marked decrease in IVG formation that was quantitatively accounted for by the increase in IVC (Fig. 2 *D*). In addition, this transformation was apparently effective in making CoASH more available for BG synthesis. These results support the thesis that high levels of isovaleryl-CoA compete effectively for glycine in the mitochondrion with benzoate. When the level of isovaleryl-CoA is reduced and CoASH is available, benzoate is effectively converted into hippurate (BG).

In summary, this study has demonstrated that in isovaleric acidemia, when adequate carnitine is available, a new metabolite, IVC, is excreted in large amounts. The use of carnitine therapy in isovaleric acidemia appears to be as effective as glycine therapy in the removal of isovaleryl-CoA and is more effective in reducing plasma IVA. By increasing the availability of CoASH, glycine conjugation is also enhanced. IVC formation is not significantly enhanced by glycine supplementation, and renal loss does not appear to account for the initially diminished levels of free carnitine in this patient. Treatment of isovaleric acidemia with carnitine alone overcomes the diminished amount of carnitine. Further, carnitine is utilized in another detoxification pathway via the carnitine acyltransferase system, producing IVC. These observations, coupled with the encouraging progress of the patient, suggest that it is reasonable to treat isovaleric acidemia with carnitine, although the possible advantages of concomitant glycine supplementation warrant further investigation.

Acknowledgments

We are especially grateful to Dr. L. Harris (Fayetteville, NC) for referring this patient, to Dr. R. A. Chalmers (Clinical Research Centre, Harrow, UK) for supplying the isovalerylglycine and valerylglycine standards and to Sigma Tau (Rome, Italy) for a generous supply of L-carnitine. We further appreciate the excellent technical assistance of Ms. W. Boone, Ms. D. Gale, Mr. R. Wynne, and Ms. D. Sanders.

This study was supported in part by a grant from the National Reye's Syndrome Foundation (Bryan, OH), the Reye's Syndrome Research Fund—S.F.A. (Duke University Medical Center), and the

RR-30 General Clinical Research Centers Program Division of Research Resources (National Institutes of Health).

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