*ISOVALERIC ACIDEMIA: A NEW GENETIC DEFECT OF LEUCINE METABOLISM**

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At present, leucine-induced hypoglycemia and maple-syrup urine disease are the only inborn errors of leucine metabolism recognized in man. In the former disorder, recurrent hypoglycemia is the result of leucine-induced hyperinsulinism.[1] In maple-syrup urine disease there is a block in the oxidative decarboxylation of leucine, isoleucine, and valine resulting in the accumulation of the amino acids themselves as well as their α-keto derivatives.[2] We have recently obtained evidence for a new genetic disorder of leucine metabolism in which there is a defect in the catabolism of leucine resulting in the accumulation of isovaleric acid.

This disease has been observed in two siblings, aged $2^1/_2$ and 4, who since the early months of life had a persistent odor to their breath and body fluids described as "cheesy" or "like sweaty feet." In addition, these children experienced recurrent episodes of vomiting often progressing to metabolic acidosis and either stupor or coma. These episodes were usually precipitated by protein ingestion or intercurrent infections; the children actually had a pronounced aversion to dietary proteins. During attacks of acidosis the peculiar odor of the children usually intensified. Since the odor resembled that of short-chain fatty acids, the blood and urine were analyzed for these acids by gas-liquid chromatography (GLC) and mass spectrometry. These studies resulted in the demonstration of markedly increased amounts of isovaleric acid in the body fluids of these patients and evidence for a block in the utilization of this branched-chain fatty acid. The complete clinical details of this syndrome will be presented in full detail elsewhere.

Materials and Methods.—Patient material: Biochemical studies were performed on blood, urine, and feces of the two patients, B. A. (male, $2^1/_2$ yr) and S. A. (female, 4 yr) during attacks of acidosis and after recovery from such attacks. Comparable determinations were performed on specimens from four adults and four children without known metabolic abnormalities.

Gas-liquid chromatography (GLC): An improved GLC method for short-chain fatty acids was used (detailed description to be published). Serum samples were acidified with 0.2 vol of 1.0 *N* H_2SO_4, then extracted with 20 vol of chloroform-methanol (v/v 2:1), and subsequently filtered. Mixing with 0.2 vol of 0.1 *N* NaOH at 0–5° resulted in the formation of a two-phase system. The upper layer was evaporated to dryness and acidified with o-phosphoric acid and steam-distilled.[3] The evaporated residue of the lower layer was hydrolyzed with 1.0 *N* KOH in methanol-water (v/v 4:1) for 4 hr at 65°, and similarly steam-distilled. Preliminary experiments indicated that virtually all (>99%) of short-chain fatty acids (straight and branched) (C2–C8) recovered were in the upper layer, and greater than 96% of neutral lipids (tributyrin, tricaproin, trioctanoin) were present in the lower layer. Urine specimens were alkalinized, evaporated to dryness, then acidified and steam-distilled. Alkalinized distillates were evaporated to dryness and acidified with aqueous formic acid. Samples were then injected into a Barber-Colman gas chromatograph (model 10) equipped with a hydrogen flame detector. Columns used were glass U tubes packed with Anakrom (80–90 mesh) coated with 25% neopentylglycol adipate (NPGA)–2% o-phosphoric acid (PA), 20% dioctylphthalate (DOP)–5% o-phosphoric acid, or 20% DOP alone. NPGA-PA and DOP-PA columns were used for free fatty acid assay. DOP column was used only for qualitative analysis of the methyl esters. Methyl esters were produced by reacting the fatty acids with a slight excess of etherial diazomethane. Quantitative analysis was done on NPGA-PA columns,

using commercially available standards (Eastman Organic Chemicals, Rochester, N. Y.). Adsorption of short-chain fatty acids on column[4] was negligible under the conditions employed. In preliminary studies it was determined that the extraction procedures, solvent evaporation, and final gas chromatography resulted in approximately 90% recovery of short-chain fatty acids. The recovery of β-methylcrotonic acid was also about 90%.

Mass spectrometry: The mass spectrometer used was an Atlas-Werke model CH4 with a gas chromatographic inlet system, and was operated with an electron ionizing energy of 20 eV. The sample analyzed was the methyl ester of the unknown compound from the urine of B. A. which showed one major peak on GLC. We are indebted to Dr. J. A. McCloskey of the Lipid Research Center, Baylor University, for performing these analyses.

Isolation of white blood cells and 1-C^{14} isovaleric acid oxidation studies: White blood cells were obtained from heparinized whole blood by the fibrinogen sedimentation method of Skoog and Beck.[5] Fibrinogen solutions (6%) made up in a modified phosphate buffer[6] were used. After 15 min of sedimentation, the white blood cell containing supernate was removed, and the cells were counted. The final incubation volume was 3.5 ml and contained 3 ml of the white blood cell suspension (1–2 × 10^7 white blood cells), 2.8 μmoles glucose, and 0.36 μmole 1-C^{14} isovaleric (1 μc) (New England Nuclear Corp., Boston, Mass.). The incubation medium was placed in the outer compartment of 25-ml Erlenmeyer flasks which contained a center well. The flasks were capped with rubber serum stoppers and incubated in a Dubnoff shaking incubator at 37° for 90 min. At the end of the incubation, 1 ml of 0.1 *N* KOH was injected into each center well, following which the incubation medium was acidified with 1.0 ml 5 *N* H_2SO_4. After 30 min of shaking at 37°, the KOH was transferred to the outer compartment of another flask and acidified. Liberated $C^{14}O_2$ was collected in hyamine 10-X following 30 min of shaking at 37°. The hyamine was then counted in a Packard liquid scintillation counter.[7]

Results.—In Figure 1 are shown results of a typical serum short-chain fatty acid pattern found in normal subjects as compared to the two patients, B. A. and S. A. It will be noted that in normal fasting subjects the major peak is acetic acid. In contrast, the sera of both patients B. A. and S. A. during a state of mild acidosis revealed huge peaks compatible with isovaleric acid. However, these analyses were carried out on an NPGA-PA column which did not definitely distinguish isovaleric acid from α-methylbutyric acid (Table 1).

Further GLC studies to identify this peak were therefore carried out using different stationary phases. As shown in Table 1 (col. 2), when the samples were chromatographed on a DOP-PA column, the unknown peak had a relative retention time (RRT) of 0.310, with n-caproate as a reference compound. In this system

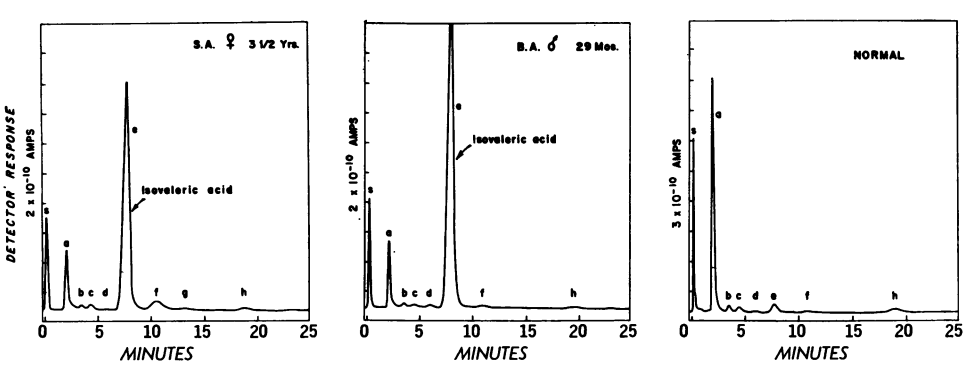

FIG. 1.—Gas chromatograms of serum short-chain fatty acids of the two patients (B. A. and S. A.) during acidosis compared to a normal subject. The column used was a 25% NPGA–2% PA column (6 ft by 1/8 inch). The temperature and inlet pressure were 112° and 14 psi, respectively. Symbols are as follows: *s*, solvent; *a*, acetic; *b*, propionic; *c*, isobutyric; *d*, butyric; *e*, isovaleric; *f*, n-valeric; *g*, β-methylcrotonic; and *h*, n-caproic acid.

TABLE 1

RELATIVE RETENTION TIME OF SHORT-CHAIN FATTY ACIDS AND THEIR METHYL ESTERS

		Relative Retention Time		
		Free Fatty Acids		Methyl esters
	Sample	25% NPGA-2% PA column	20% DOP-5% PA column	20% DOP column
Reference compounds	Acetate	0.101	0.050	0.020
	Propionate	0.181	0.092	0.070
	Isobutyrate	0.228	0.141	0.110
	n-Butyrate	0.305	0.196	0.171
	Pivalate	0.259	0.204	0.143
	Isovalerate	0.405	0.308	0.276
	α-Methylbutyrate	0.405	0.319	0.265
	n-Valerate	0.557	0.450	0.427
	Diethylacetate	0.665	0.643	0.540
	γ-Methylvalerate	0.825	0.789	0.757
	n-Caproate	1.000	1.000	1.000
	n-Hepatanoate	1.763	2.198	—
	n-Octanoate	3.119	4.888	—
	Acrylate	0.254	0.119	0.071
	Crotonate	0.569	0.361	0.325
	β-Methylcrotonate	0.689	0.522	0.622
	Tiglate	0.876	0.658	0.710
Patients	B. A. urine	0.405	0.310	0.276
	B. A. serum	0.405	0.310	—
	S. A. serum	0.405	0.310	—

For the NPGA-PA, DOP-PA, and DOP columns the temperature of the columns and inlet pressure were, respectively, as follows: 112°, 14 psi; 80°, 18 psi; and 70°, 14 psi. Column size was 6 ft by 1/8 inch, except the DOP column which was 8 ft by 1/8 inch. Data are expressed with n-caproate as a reference compound and with its retention time considered as 1.000. For abbreviations, see text.

the RRT for isovaleric acid was 0.308, and for α-methylbutyric acid was 0.319. Methyl esters were also prepared of the steam distillates of the urine from B. A. and analyzed on a 20 per cent DOP column. The standard methyl ester of isovaleric acid and that of the fatty acid isolated from urine both had an RRT of 0.276, while that of α-methylbutyric acid was 0.265.

A further demonstration that the GLC peak was isovaleric acid, rather than α-methylbutyric acid, was obtained from mass spectrometric analysis of the peak. The results are shown in Figure 2.

The small molecular peak at m/e 116 excluded, on a molecular weight basis, anything but the methyl ester of a pentanoic acid. This allowed four possibilities: (1) isovaleric acid, (2) n-valeric acid, (3) α-methylbutyric acid, and (4) pivalic acid. Pivalic acid and n-valeric acid were excluded by the data in Table 2. The base peak at m/e 74, known to be $[CH_2{=}\overset{\displaystyle OH}{\overset{|}{C}} - OCH_3]^+$ produced by 2,3 cleavage and rearrangement of a hydrogen atom[8, 9] and the absence of a peak at m/e 88, excluded α-methylbutyric acid. The latter compound has an intensive peak at m/e 88 and a small peak at 74 because of the methyl substitution at the 2 position.[8, 10] The GLC and mass spectrometry data taken together therefore clearly identified the abnormal metabolite as isovaleric acid.

It is noteworthy that only trace amounts of β-methylcrotonic acid were detected. Furthermore, the lower chloroform layer of the serum extracts after saponification contained no isovaleric acid, indicating neutral glycerides containing this acid were not present.

Quantitative studies on the sera of the two patients indicated that during an

attack of acidosis, the serum isovaleric acid level in B. A. was 30,500 μg/100 ml compared to a mean fasting level of 58 ± 22 μg/100 ml in normal individuals (Table 2). A level of 6320 μg/100 ml was obtained from S. A. during an attack associated with mild acidosis and lethargy. In both individuals the serum isovaleric acid levels declined as the attack of acidosis and lethargy cleared, but their baseline fasting level of isovaleric acid was still about 2–3 times normal. The serum levels of other C-3 to C-6 fatty acids were only minimally elevated during the attacks of acidosis as compared to the increase in isovaleric acid. Isovaleric acid analysis on feces revealed no significant elevations. α-Ketocaproic acid was measured by the methods of Dancis[11] and Cavallini,[12] and only trace amounts were detected in the urine at the time isovaleric acid was present in excess. Paper chromatographic examination of serum and urine for amino acids[13] during periods of exacerbation and remission revealed no significant quantitative or qualitative changes; specifically, no increases in leucine were detected.

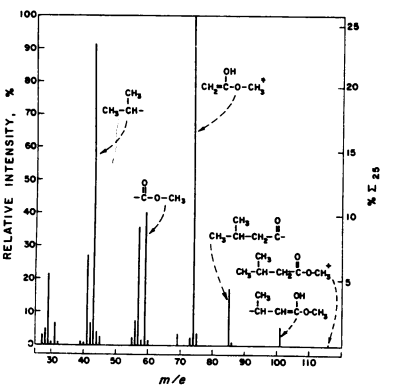

FIG. 2.—Mass spectrum of methy ester of the "unknown" peak from B. A.'s urine. Experimental conditions and the interpretation are described in the text.

Since isovaleric acid is a normal metabolite of leucine and in view of the striking history of protein intolerance, loading tests with L-leucine were carried out (Fig. 3). With an oral dose of 100 mg L-leucine/kg, serum isoveric acid levels increased as much as 200-fold in these subjects, and some ataxia and incoordination appeared but no appreciable acidosis occurred. In normal individuals, these oral loading tests produced only negligible alterations in serum isovaleric acid levels.

Evidence in support of the concept that the accumulation of isovaleric acid is due to block in its further metabolism was obtained with *in vitro* studies on the patients' white blood cells. The data in Table 3 indicate that the oxidation of 1-C^{14} isovaleric acid was markedly depressed in the two patients compared to normal subjects. These results occurred both with cells obtained during an attack of acidosis as well as in the recovery phase.

Discussion.—The biochemical observations on two siblings presented above provide evidence for a new genetic disorder of leucine metabolism. These children have a metabolic defect in the catabolism of isovaleric acid which results in marked

TABLE 2

SHORT-CHAIN FATTY ACID CONTENT OF SERUM (μG/100 ML)

	Clinical state	Propionic	Iso-butyric	n-Butyric	Iso-valeric	n-Valeric	β-Methyl-crotonic	n-Caproic
B. A.	Acidosis	126	78	91	30,500	99	60	230
	Recovery	98	33	27	183	35	10	157
S. A.	Acidosis	48	79	28	6,320	252	45	56
	Recovery	78	41	39	290	78	—	231
Controls								
Adults (4)		25	28	10	94	22	5	44
± SD		±3	±2	±0	±29	±4	±1	±7
Children (4)		28	27	12	58	4	—	16
± SD		±7	±10	±11	±22	±3	—	±10

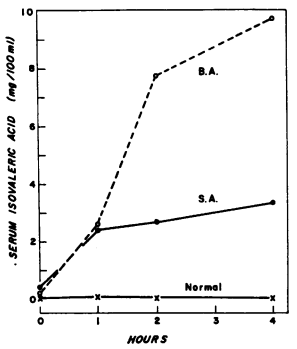

FIG. 3.—Serum isovaleric acid levels after oral L-leucine (100 mg/kg) in the patients as compared to mean of five normal controls.

accumulation of this short-chain fatty acid in the blood and urine. As might be predicted from the biochemical pathway of leucine catabolism shown in Figure 4, both protein ingestion and exogenous leucine loading resulted in marked increases in their serum isovaleric acid levels.

The sequential steps in catabolism of leucine have been well outlined in mammalian tissues (Fig. 4).[14] The initial steps common to leucine and the other branched-chain amino acids (isoleucine and valine) involve transamination of the amino acids with α-ketoglutarate as an acceptor leading to the formation of the respective α-keto acids. This is followed by an irreversible oxidative decarboxylation to the corresponding acyl-CoA derivatives. In the case of leucine these two reactions involve the formation of α-ketoisocaproic acid and subsequently the production of isovaleryl-CoA (Fig. 4, reactions *I* and *II*).

In the congenital defect known as "maple-syrup urine" disease, a block at the level of reaction *II* results in the accumulation of the three branched-chain amino acids and their α-keto derivatives.[2] In the present cases, the absence of elevated blood and urine levels of the branched-chain amino acids and the α-keto derivatives clearly differentiates their metabolic defect from that described in "maple-syrup urine" disease.

The marked accumulation of isovaleric acid in blood and urine, together with the demonstrated impairment in the ability of the patients' white blood cells to convert 1-C^{14} isovaleric acid to $C^{14}O_2$, indicates a defect in the catabolism of this short-chain fatty acid. The absence of increased β-methylcrotonic acid levels during periods of isovaleric acid elevation suggests that the site of the block is at the level of the conversion of isovaleryl-CoA to β-methylcrotonyl CoA (Fig. 4, reaction *III*). Additional support for this concept comes from data on biotin-deficient rats in which β-methylcrotonyl CoA carboxylase is known to be depressed.[15, 16] In such animals we have found a pronounced accumulation of β-methylcrotonic acid as well as of isovaleric acid in the serum, the ratio of these values being approximately 1.[17] The fact that leucine and α-ketoisocaproic acid were not increased in the serum of

TABLE 3

1-C^{14}-ISOVALERIC ACID OXIDATION TO $C^{14}O_2$ *in vitro*

Subjects	$C^{14}O_2$ liberated (dpm/10^7 cells)
Normals (6)	5350 ± 1390 (SD)
Patients	
B. A. during acidosis	300
" recovery phase	880
S. A. during acidosis	330
" recovery phase	870

Conditions of the incubation system are described in text. To each flask 1 μc of 1-C^{14} isovalerate was added as substrate (0.1 μmole/ml). Because of the high isovalerate concentrations in the serum of the patients during acidosis, the final isovalerate concentration in the medium was analyzed by GLC. The normal flasks had minimal endogenous isovalerate, and the concentration was 0.1 μmole/ml. For B. A. and S. A., the concentrations during acidosis were 1.6 and 0.25 μmole/ml, respectively; during the recovery phase they were 0.12 and 0.124 μmole/ml, respectively.

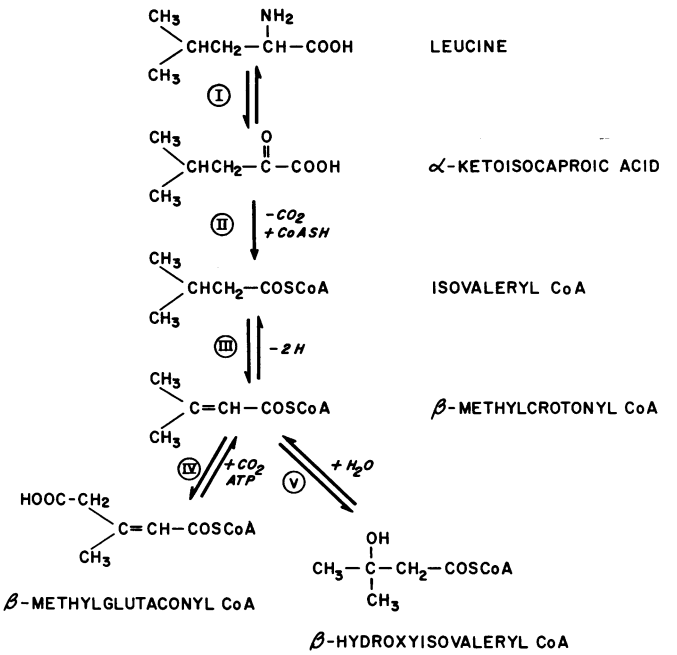

FIG. 4.—Scheme of the early steps in the pathway of leucine catabolism.

the patients would appear to be explained by the fact that the decarboxylation of α-ketoisocaproic is an irreversible step (Fig. 4, reaction *II*).

The oxidation of isovaleryl-CoA to β-methylcrotonyl CoA is considered to be analogous to the corresponding reaction with straight-chain fatty acids.[18] In the present study the absence of a significant increase of other short-chain fatty acids such as n-butyric and n-caproic, known to be dehydrogenated by green acyl CoA dehydrogenase,[19] suggests that isovaleryl-CoA may be oxidized by a separate acyl CoA dehydrogenase. This dehydrogenase may be specific for isovaleryl-CoA since in these patients there was no accumulation of α-methylbutyric acid and isobutyric acid, the corresponding metabolites of the isoleucine and valine pathways. This is in contrast to the enzyme system catalyzing reaction *II* which appears to be involved in the metabolism of all three branched-chain amino acids.

There are few published observations which relate to the potential toxicity of short-chain fatty acids. However, it is noteworthy that when leucine loading tests were performed in these patients, the serum isovaleric acid levels increased and definite neurologic manifestations occurred. Several groups of investigators have shown that short-chain fatty acids have a narcotic effect on the nervous system of experimental animals[20] and that their administration to animals produces electroencephalographic changes.[21] At the cellular level, studies with C-8 and C-10 fatty acids have demonstrated them to be potent inhibitors of oxidative phosphorylation,[22, 23] to inhibit P^{32} incorporation into various chemical fractions,[24] and to induce mitochondrial swelling.[25] The relevance of these observations to the metabolic and neurologic derangement seen in patients with isovaleric acidemia still remain to be elucidated.

Summary.—A new genetic defect of leucine metabolism has been described, in which there occurs a marked accumulation of isovaleric acid, a catabolite of leucine. Two siblings studied with this hereditary disease were found to have an intolerance to protein and to develop recurrent episodes of metabolic acidosis, with stupor or coma. They have mild mental retardation and an unusual body odor which is due to the elevated levels of isovaleric acid in their body fluids. When these children were given oral leucine, the blood isovaleric acid levels increased greatly and neurologic changes developed. The metabolism of 1-C^{14}-isovaleric acid to $C^{14}O_2$ by white blood cells of these patients was found to be markedly impaired. On the basis of the present evidence, it is postulated that the metabolic block in this disorder may be in the conversion of isovaleryl CoA to β-methylcrotonyl CoA.

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