A "NEW" DISORDER OF ISOLEUCINE CATABOLISM

by

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"As must be expected, the experiments proceed slowly. At first beginning, some patience is required, but later, when several experiments are progressing concurrently, matters are improved. Every day, from spring to fall, one's interest is refreshed daily, and the care which must be given to one's wards is thus amply repaid. In addition, if I should, by my experiments, succeed in hastening the solution of these problems, I should be doubly happy."

Gregor Mendel
Letter to C. Nägeli
18 April, 1867
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PREFACE

This thesis is submitted according to the newly accepted regulations for thesis style which have been authorized by the Graduate Training Committee of the Biology Department at McGill. The main body of the thesis is written in a form suitable for publication.

The first section of the thesis is a presentation of known disorders of branched-chain amino acid catabolism. The second part is a preliminary report describing a "new" disorder of isoleucine catabolism which has been published in Lancet. This new hereditary disease is the subject of the thesis. The third section describes the "new" disease in detail, emphasizing the genetic and phenotypic aspects, the identification of the unknown metabolites, and the metabolic spectrum of the problem. The significance of the work is discussed. This third part of the thesis has been submitted to Pediatric Research for review and publication.
ACKNOWLEDGEMENTS

This thesis would not be possible without a great deal of help and guidance from many different sources. The longest list I could make would not include everyone and no omission is intentional. The guiding force for the work was Dr. Charles R. Scriver who provided his depth of experience and knowledge, for whose patience and support I am very grateful. Dr. Orval Mamer was kind enough to perform the work on the LKB-9000 Spectrometer and confirmed the initial compound identifications. Dr. Hy Goldman generously grew the skin fibroblast cultures and performed the skin biopsies. I express my gratitude to Dr. Edgard Delvin who taught me many laboratory principles and techniques and whose patience I admire. I thank also Peter Lamm for valuable technical assistance and many philosophical discussions. Indispensable ego support was provided by Carol Clow and Susan Mackenzie. I thank Terry Reade, Ines Koc and Ms Clow again for their assistance in the investigation of the patients and their families.

Finally, I would thank Huguette Ishmael for her long hours and extra efforts in the typing of the manuscript. Her aggravation tolerance level is very high.

The work was carried out during the elective periods provided in the McGill University, Faculty of Medicine curriculum.
ABSTRACT

At least 15 apparently inherited disorders of branched-chain amino acid catabolism are now known; the 12th in chronological order of discovery is the central topic of the thesis. The literature of the 14 other disorders is also reviewed. The present disease is a partial defect of the pathway of isoleucine oxidation beyond the level of oxidative decarboxylation and prior to the oxidation of propionate. The impairment of isoleucine catabolism appears to be situated at the "thiolase" reaction which converts $\alpha$-methylacetoacetyl-CoA to propionyl-CoA and acetyl-CoA.

Two pedigrees (B and M) were investigated in detail. A third (S pedigree) has been brought to our attention for analysis of metabolites in urine but we have not performed additional studies in the latter. Each propositus was ascertained because of intermittent, odorless metabolic acidosis usually precipitated by intercurrent infection. Lethargy and coma occurred frequently during the periods of acidosis. One M sib, also presumably affected, died abroad in such an episode. Symptoms can be ameliorated by a low-protein diet, and careful attention to the management of intercurrent illness.

A large excess of $\alpha$-methyl-$\beta$-hydroxybutyrate, and a seemingly smaller excess of $\alpha$-methylacetoacetate is present at all times in the urine of the three propositi. The M and S propositi also excrete N-tiglylglycine. The amounts of these unusual metabolites increase several fold during
acidosis and after a dietary load of L-isoleucine (75 mg/kg, 3 times daily x 2 days). The urine also contains butanone particularly during acidosis. The amount of propionate, and of glycine and other amino acids in blood and urine, are always normal in our patients. Oxidation of L-isoleucine-\textsuperscript{\textit{u}}\textsuperscript{14}C to CO\textsubscript{2} by cultured skin fibroblasts is about 45 percent of normal in the B propositus.

Studies of family members reveal that presumed, obligate heterozygotes excrete a small excess of \(\alpha\)-methyl-\(\beta\)-hydroxybutyrate at all times; the amount can be increased by L-isoleucine feeding. The condition is apparently inherited in autosomal recessive fashion.
INTRODUCTION

Although Garrod's 1908 Croonian Lecture and his 1909 monograph on Inborn Errors of Metabolism were virtually ignored when they first appeared, his work is now held in great esteem. Through the rare trait alcaptonuria, Garrod developed his concept of the Inborn Error of Metabolism which he defined as a specific, inherited enzyme deficiency at some point in a normal metabolic pathway, behind which metabolites would accumulate. Garrod drew attention to the fact that these conditions tended to be familial, and, with the aid of the new genetic principles elucidated by Mendel and transmitted to scientists in England by William Bateson, it was realized that their appearance in pedigrees conformed to Mendel's laws.

Today, a large number of these enzyme deficiencies are known. An exponential growth of knowledge about the Inborn Errors came as a consequence of a technical application, namely two-dimensional partition chromatography on filter paper with subsequent ninhydrin staining. A large number of Inborn Errors have as their cardinal feature the accumulation of an amino acid and therefore they have lent themselves to "discovery" by chromatography coupled to ninhydrin staining. Indeed for two decades the reporting of new amino acid diseases proceeded exponentially until the pace of discovery abated.

A second, rapid rise in the rate of discovery of inborn errors of metabolism has been seen recently, following the application of gas chromatography and mass spectrometry. It will be seen that "discovery" here is used in its loosest sense
as the number of possible enzyme deficiencies is no doubt very large; the burden falls on the technological advances to provide the biochemical geneticist the tools with which to elicit them.

Many of the conditions presented in Table I-1 are characterized by accumulation of "ninhydrin-negative" compounds. The metabolites of major concern in these diseases are volatile organic acids or derivatives thereof. Thus, gas chromatography has been very useful in identifying these metabolites. The technique is extremely sensitive to even trace quantities and thus identification of secondary metabolites has often been possible.

The branched-chain amino acids, isoleucine, leucine, and valine, have shared the spotlight in this second phase in the growth of knowledge about inborn errors of metabolism. The great majority of metabolites found in their catabolic pathways are volatile organic acids and thus readily lend themselves to study by gas chromatography. Indeed, the large majority of known genetic entities which cause metabolic acidosis in the neonate or the infant are disorders of branched-chain amino acid catabolism. One need only to add the "lactic acidoses", the glycogen storage disease type I, fructose 1,6 diphosphatase deficiency and succinyl-CoA 3-ketoacid-CoA transferase deficiency to the entities presented herein to complete the current differential diagnosis.

It should be noted that the second step in the catabolism of branched-chain amino acids, the decarboxylase reaction, is irreversible. Diseases which affect enzymes above this level yield ninhydrin-positive compounds; those below the irreversible
step yield "GC-detectable diseases".
TABLE I-1

RESUME OF THE HEREDITARY DISORDERS OF BRANCHED-CHAIN AMINO ACID CATABOLISM
1. PHENOTYPE: hyperValinemia
2. NUMBER IN FIGURE I-1: 2
3. PRESUMED ENZYME DEFECT: Valine Transaminase
4. METABOLITES ACCUMULATING: L-Valine
5. CLINICAL FEATURES: Failure to thrive, psychomotor retardation, vomiting, nystagmus, hyperkinesia, muscular hypotonia. EEG normal at 3 months, abnormal at 4 months. No unusual odour.
6. COMMENT: One case known first described by Wada et al, (1963). Important because it suggests some degree of specificity of transaminase enzyme - originally thought to be common at least to all three branched-chain amino acids. No keto-acids found in serum or urine. Abnormal response to L-valine loading. No abnormalities noted in loading with leucine, isoleucine, or methionine. Peripheral leukocyte work done suggesting selective failure of valine transamination. 

Treatment with low valine diet clinically and biochemically successful - vomiting ceased, patient gained weight, serum valine decreased to normal. EEG improved. No reversal of mental retardation, however. Pyridoxal phosphate, a cofactor in the reaction, gave no improvement clinically or biochemically.
1. PHENOTYPE: hyperLeucine-Isoleucinemia
2. NUMBER IN FIGURE I-1: 11
3. PRESUMED ENZYME DEFECT: Transaminase
4. METABOLITES ACCUMULATING: Leucine, Isoleucine
5. CLINICAL FEATURES: Failure to thrive, mental retardation, convulsions, retinal degeneration, apparent nerve deafness.
6. COMMENT: First reported by Jeune et al. (1970) in two sibs who also had type II hyperprolinemia. Leucine and isoleucine concentrations in serum about 2 - 3 times normal; valine concentrations - normal. Excretion of all three - normal. Abnormal response to leucine loading. Partial defect in leucine-isoleucine transamination demonstrated in vitro. Pyridoxal therapy attempted in vivo with no response. Importance of disease lies in suggestion that transaminase of C-6 branched-chain amino acids is specific.
1. PHENOTYPE: Isovaleric Acidaemia, 1) "classical"

2. NUMBER IN FIGURE I-1: 3

3. PRESUMED ENZYME DEFECT: Isovaleryl-CoA Dehydrogenase

4. METABOLITES ACCUMULATING: Isovaleric Acid, β-OH Isovaleric Acid, Isovalerylglutamine.

5. CLINICAL FEATURES: Onset in infancy. Offensive odour about patient resembling sweaty feet. Dietary protein intolerance. Intermittent, severe ketoacidosis, usually associated with febrile infections. During episodes of illness there are more pronounced odour, vomiting, lethargy, hyperactive deep tendon reflexes, intention tremor. Slight psychomotor retardation perhaps secondary to biochemical defect per se or secondary to severe acidosis. Spectrum of severity of symptoms varies greatly.

Amino acid chromatogram entirely normal. Gas chromatography elicited isovaleric acid, a normal metabolite, in abnormal amounts in serum and urine; identification confirmed by Mass Spectrometry. Isovalerylglutamine also found and identified by Tanaka et al (1967). β-hydroxy isovalerate found and identified by Tanaka et al (1968). Isovalerylglutamine formation thought to be detoxification mechanism. Can be saturated during ketosis when β-hydroxyisovaleric acid appears. Presence of β-hydroxyisovaleric acid suggests a direct hydroxylation reaction
i.e. Isovaleryl-CoA ≠ β-OH Isovaleryl-CoA

If this assumption is correct, then the subsequent conversion

β-OH Isovaleryl CoA ≠ β-CH₃ Crotonyl-CoA

would provide a bypass of the isovaleryl-CoA dehydrogenase block. Hypoglycin A, an amino acid found in ackee fruits inhibits isovaleryl-CoA dehydrogenase (α-methyl butyryl-CoA dehydrogenase to a lesser extent). Ingestion produces "Jamaica vomiting sickness", in actual fact a transient isovaleric acidemia.
Table I-1 (cont.)

1. PHENOTYPE: Isovaleric Acidaemia, 2) "hyperglycinemic type"

2. NUMBER IN FIGURE I-1: 15

3. PRESUMED ENZYME DEFECT: Presumed to be same as classical type

4. METABOLITES ACCUMULATING: Isovaleric Acid, Isovaleryl Glycine,
   (also elevation of serum and urinary glycine levels)

5. CLINICAL FEATURES: Intermittent ketoacidosis as for classical type. However, no sweaty feet odour; no retardation; no neutropenia.

6. COMMENT: Ketotic hyperglycinemia was first described by Childs et al, (1961) subsequently divided into at least 3 inborn errors of metabolism being:
   1. Propionic Acidaemia
   2. Methylmalonic Acidaemia
   3. Hyperglycinemic Isovaleric Acidemia

The last variant resembles classical isovaleric acidemic; first described by Ando et al (1971A). β-OH isovaleric acid is not a significant metabolite in this postulated variant. In vitro work with fibroblasts shows that no glycine is converted to hippuric acid in contrast to the large amounts of hippurate formed in normals.
1. PHENOTYPE: β-methyl crotonyl glycineuria (β-hydroxy isovaleric acidemia), 1) Biotin unresponsive

2. NUMBER IN FIGURE I-1: 10

3. PRESUMED ENZYME DEFECT: β-methyl crotonyl-CoA Carboxylase

4. METABOLITES ACCUMULATING: β-methyl crotonyl glycine and β-hydroxy isovaleric acid (in respective 1:4 ratio)

5. CLINICAL FEATURES: Onset in 2nd week. Retarded motor development, hypotonia, muscular atrophy. No ketoacidosis. Mental development, sensory systems normal. Cat's urine odour noted in urine. Chromatography for amino acids, normal. LDH increased, serum creatinine increased.

6. COMMENT: First reported by Eldjarn et al in 1970. β-methylcrotonylglycine also found in urine of both parents and 2 sibs, all of whom are presumed heterozygotes. β-hydroxy isovaleric acid is the dominant metabolite. No ketoacidosis clinically. With dietary treatment, (i.e. leucine restriction), excretion of β-hydroxyisovaleric acid dropped rapidly from 400 → 50 mg/day. β-methylcrotonyl glycine only slightly decreased however. Creatinine excretion decreased markedly. No clinical improvement however and the child died at age 3 months from pneumonia.
1. **PHENOTYPE:** β-methyl crotonylglycinuria (β-hydroxy isovaleric acidemia), 2) Biotin responsive.

2. **NUMBER IN FIGURE I-1:** 14

3. **PRESUMED ENZYME DEFECT:** as for type I

4. **METABOLITES ACCUMULATING:** β-hydroxy isovaleric acid  
β-methyl crotonylglycine, tiglylglycine, β-methyl crotonic acid

5. **CLINICAL FEATURES:** Onset at 5 months. Extensive erythematous skin rash (unresponsive to steroids and nystatin). Severe metabolic ketoacidosis. Cat's urine odour noted in urine. Well nourished. Behaviour abnormal. Dramatic improvement following biotin administration - clinically and chemically.

6. **COMMENT:** First reported by Gompertz et al in 1971. Clinical and chemical features different from biotin unresponsive type in that:

   1. β-methylcrotonylglycine is the predominant abnormal metabolite.
   2. Ketoacidosis is the hallmark of this clinical picture without the hypotonia and muscular atrophy of the biotin unresponsive type.

Anomalous presence of tiglylglycine - isoleucine intermediate. Since β-methylcrotonic acid is an isomer of tiglic acid, one is tempted to suggest enzyme inhibition to account for the apparent lesion of the isoleucine pathway. Biotin treatment (5-10 mg) corrected clinical and biochemical aberrations to normal levels.
Table I-1 (cont.)

1. PHENOTYPE: Methylmalonic Aciduria, 1) B₁₂ non-responsive
2. NUMBER IN FIGURE I-1: 4
3. PRESUMED ENZYME DEFECT: Methylmalonyl CoA Mutase
4. METABOLITES ACCUMULATING: Methylmalonic acid, propionate, (7) α-methyl acetoacetic acid, butanone, hexanone. Glycine levels usually increased.
5. CLINICAL FEATURES: Onset early in life. Intermittent severe ketoacidosis. Developmental retardation. Recurrent infections (50%). Osteopetrosis (50%). Early death (80% - 100%). Neutropenia (50%). Hypoglycemia (40% - only during ketoacidosis). Hyperglycinemia (85%). Urine has"mousy" odour (Rosenberg, 1971).
6. COMMENT: Methylmalonic aciduria first described by Oberholzer et al (1967). Defect is in conversion of MMA CoA to succinyl CoA. MMA excretion greatly augmented by protein loading and after isoleucine, valine, methionine, or threonine loading; leucine loading does not augment MMA excretion. In propionyl CoA carboxylase deficiency, however, leucine loading does elicit ketoacidosis in the patient, presumably by induction of isoleucine catabolism (Phansalkar et al, 1971). This is a form of ketotic hyperglycinemia. Relationship between glycine and branched-chain metabolism remains obscure. Vitamin B₁₂ is a cofactor for the normal mutase apoenzyme. Response to its administration divides methylmalonic aciduria into 2 sub-groups. B₁₂-resistant form carries a higher mortality. Protein restriction tried with some success.
MMA has been diagnosed in utero by amniocentesis as well as by actual metabolite detection in maternal urine (Morrow et al, 1970). Leukocytes fail to convert propionate-\textsuperscript{14}C to \textsuperscript{14}CO\textsubscript{2} in vitro (Morrow et al, 1969). The label was recovered in MMA pool. Mutase activity in liver measured to be near 0. Long chain ketonuria (Rosenberg, 1968) thought to be due to deacetylization of \textalpha-methylacetocetate (Menkes, 1966), a metabolite 2 steps removed from the mutation block.
1. PHENOTYPE: Methylmalonic Aciduria 2) B\textsubscript{12} responsive
2. NUMBER IN FIGURE I-1: 5
3. PRESUMED ENZYME DEFECT: (?) as for type 1 or (?) defect in 5'-deoxyadenosyl cobalamin synthesis
4. METABOLITES ACCUMULATING: as for type 1
5. CLINICAL FEATURES: as for type 1.
6. COMMENTS: Response to parenteral B\textsubscript{12} first demonstrated by Rosenberg et al (1968). Diminished MMA excretion from approximately 1000 mg/24 hr to approximately 200 mg/24 hr was noted. Complementing peripheral leukocyte experiments were done before and during B\textsubscript{12} therapy. \textit{In vitro} results reflected \textit{in vivo} observations. Clinical prognosis better than in B\textsubscript{12}-resistant form.
Table I-1 (cont.)

1. PHENOTYPE: Propionic Acidaemia, 1) biotin unresponsive
2. NUMBER IN FIGURE I-1: 7
3. PRESUMED ENZYME DEFECT: Propionyl CoA Carboxylase
4. METABOLITES ACCUMULATING: Propionic acid. (\(\alpha-CH_3\))
   acetoacetic acid, butanone, pentanone, hexanone, long
   chain fatty acids (\(C_{2n-1}\)), serum glycine levels increased
5. CLINICAL FEATURES: Wide clinical spectrum. Hommes' (1968)
   patient died at age 5 days in spite of treatment after onset immediately
   after birth; this patient had severe ketoacidosis, hypotonia, normal glycine. Gompertz' (1970)
   patient died at age 8 days while undergoing vigorous therapy for acidosis. Hypotonia, areflexia, and leukopenia present. Propionate levels extremely high.
   Hyperglycinemia present. Ando et al (1971B) presented 3 patients
   a) VB aged 13 months with hyperglycinemia,
   b) KH aged 5 years with several mild ketotic episodes
      associated with hyperglycinemia - easily correctable.
      Normal intelligence and development.
   c) CE aged 4 1/2 years with physical and mental retardation. Hyperglycinemia.
   No unusual odours noted in any of the patients.
6. COMMENTS: First described by Hommes et al in 1968.
   First patient described did not have an elevated serum glycine, all subsequent patients did exhibit elevated glycine (at least intermittently) and thus this condition is classified with the ketotic hyperglycinemias. Wide clinical spectrum may indicate several diseases (different loci) or genetic heterogeneity (same loci). Defective
Table I-1 (cont. Propionic Acidaemia, l) ...

oxidation of propionate in vitro demonstrated. A selective deficiency of Propionyl CoA carboxylase has been demonstrated in liver, in leukocytes, and in fibroblasts. Gas chromatography demonstrates elevated propionate levels and increased levels of odd-numbered long-chain fatty acids (latter 2° to erroneous incorporation of propionate instead of acetate into fatty acid syntheses ?) Treatment by protein restriction and early aggressive management of acidosis.
Table I-1 (cont.)

1. PHENOTYPE: Propionic Acidaemia. 2) biotin responsive

2. NUMBER IN FIGURE I-1: 8

3. PRESUMED ENZYME DEFECT: as for biotin unresponsive or perhaps the enzyme at fault is the apoenzyme-biotin ligase.

4. METABOLITES ACCUMULATING: as for biotin unresponsive type


6. COMMENTS: This variant described by Barnes et al (1970). Biotin is a cofactor in the reaction:

\[
\begin{align*}
\text{CO}_2 \text{ biotin} &
\rightarrow \text{Propionyl CoA} \rightarrow \text{Methylmalonyl CoA}
\end{align*}
\]

and when administered to the patient (5 mg for 5 days) produces the following effects.

1) decreased propionic acidemia following oral isoleucine loading
2) abolition of long chain ketonuria
3) improved clinical status.

Isoleucine loading did not provoke ketosis before or after biotin treatment, perhaps implying a partial block in this variant.
1. PHENOTYPE: Maple syrup urine disease, 1) "classical"
2. NUMBER IN FIGURE I-1: 1
3. PRESUMED ENZYME DEFECT: "Branched chain amino acid decarboxylase" <2% of normal activity.
4. METABOLITES ACCUMULATING: L-valine, \(\alpha\)-keto-isovalerate, L-isoleucine, \(\alpha\)-keto \(\beta\)CH\(_3\) valerate, L-leucine, \(\alpha\)-keto-isocaproate.
5. CLINICAL FEATURES: Onset in first week of life, areflexia, cyanotic episodes, seizures, abnormal EEG, severe retardation, ketoacidosis, coma, death, odor like maple syrup.
6. COMMENTS: The first of the "organic acidurias" described by Menkes in 1954. Keto-acid excretion is continuous. The deficient enzyme is complex, involving 3 apoenzymes, and several cofactors (Mg\(^{++}\), thiamine pyrophosphate, coenzyme A, lipoic acid, and DPN). Treatment: drastic restriction of branched chain amino acid intake, peritoneal dialysis during acute illness.
Table I-1 (cont.)

1. PHENOTYPE: Maple syrup urine disease; 2) "intermittent"
2. NUMBER IN FIGURE I-1: 6
3. PRESUMED ENZYME DEFECT: "branched-chain amino acid decarboxylase" - 2-15% of normal activity
4. METABOLITES ACCUMULATING: as for classical type
5. CLINICAL FEATURES: Healthy, except during "attacks" which resemble the clinical picture of the classical trait and are usually provoked by high protein intake or intercurrent infections, etc. Death reported during some attacks. Usually normal mental and physical development.
6. COMMENTS: First described by Morris et al (1961). Trait may represent a genetic compound rather than a classic homozygote for a pair of mutant alleles. Alternatively the dehydrogenase reaction is sufficiently complex to allow heterogeneity at different loci.
1. PHENOTYPE: Maple syrup urine disease, 3) "mild"

2. NUMBER IN FIGURE I-1: 9

3. PRESUMED ENZYME DEFECT: "branched-chain amino acid decarboxylase" (2-15% of normal activity)

4. METABOLITES ACCUMULATING: as for classical type

5. CLINICAL FEATURES: Symptoms may not appear until many years after birth. Patient found incidentally because of investigation of mental retardation (IQ = 76).

6. COMMENTS: First described by Schulman et al (1970). A variant of the grave clinical picture of types I and II, α-keto β-methyl valerate provides the quantitative bulk of keto acid formation in contrast to type I where α-keto isocaproate is the predominant keto acid. Treatment may not be necessary or protein restriction may suffice.
1. **PHENOTYPE:** Maple syrup urine disease, 4) "thiamine responsive"

2. **NUMBER IN FIGURE I-1:** 13

3. **PRESUMED ENZYME DEFECT:** Branched-chain amino acid decarboxylase

4. **METABOLITES ACCUMULATING:** L-valine, α-OH isovalerate, L-isoleucine, α-OH β-CH₃ valerate, L-leucine, α-OH isocaproate.

5. **CLINICAL FEATURES:** Onset in early infancy. Main clinical manifestation is psychomotor retardation. EEG diffusely abnormal. Hypoglycemia present (52-86 mg%). Ketoacidosis occurs only associated with endogenous catabolic load secondary to infection.

6. **COMMENTS:** First described by Scriven et al (1971). Striking biochemical features include no ketoaciduria except during acute episodes. The corresponding hydroxyacids predominate otherwise. Biochemical response to thiamine HCl (20 mg/day); i.e. reduction in excretion of all above metabolites.
Table I-1 (cont.)

1. PHENOTYPE: \(\alpha\)-methyl acetoacetic aciduria

2. NUMBER IN FIGURE I-1: 12

3. PRESUMED ENZYME DEFECT: \(\alpha\)-methylacetoacetic thiolase

4. METABOLITES ACCUMULATING: \(\alpha\)-methylacetoacetic acid, \(\alpha\)-methyl \(\beta\)-hydroxy butyric acid, tiglyl glycine, butanone (propionate normal).

5. CLINICAL FEATURES: Post-natal onset variable in time. Intermittent, severe, recurrent metabolic acidosis (may cause death) usually precipitated by febrile episodes. Good response to protein restriction. No physical or mental retardation unless chronic acidosis uncontrolled. Abnormal EEG and ataxia during acidosis.

LEGEND

Figure I-1: Metabolism of branched-chain amino acids.

Known disorders of branched-chain amino acid metabolism are shown by a number representing the order in which the respective traits have been reported. The metabolites which accumulate in body fluids in the various conditions are named; other metabolites in the pathways are shown by open boxes. Tiglate and β-methylcrotonate have been recovered in urine as their glycine conjugates, tiglyl glycine and β-methylcrotonyl glycine respectively. The presumed direct conjugation reactions are not shown.
**FIGURE 1**

- **L-valine**
  - α-ketoisovalerate
  - α-keto-β-methyl valerate
  - methylmalonyl-CoA
    - B₁₂
    - succinyl-CoA
      - tricarboxylic acid cycle

- **L-isoleucine**
  - α-ketoisovalerate
  - α-keto-β-methyl valerate
  - methylmalonyl-CoA
    - B₁₂
    - succinyl-CoA
      - tricarboxylic acid cycle

- **L-leucine**
  - α-ketoisocaproate
  - isovaleryl-CoA
    - β-Hydroxy isovalerate
  - β-methylcrotonyl-CoA
    - acetoacetic acid + acetyl-CoA
      - biotin
    - acetyl-CoA
      - propionyl-CoA
        - biotin + acetyl-CoA
      - succinyl-CoA
        - tricarboxylic acid cycle
REFERENCES (Section I)


PART II

A "NEW" DISORDER OF ISOLEUCINE CATABOLISM

The B pedigree was the first to come to our attention and, at the time of the following publication, to our knowledge, was the only pedigree with the trait to be described. The following is a preliminary report, briefly describing the identification of the metabolites as well as some of the initial metabolic studies. Since then, the M and S pedigrees have been ascertained with the subsequent identification of the additional metabolite as well as the additional cases to be reported in Part III.
A "NEW" DISORDER OF ISOLEUCINE CATABOLISM

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Summary

A disorder of isoleucine catabolism was found in a child with intermittent metabolic acidosis. The block occurs at the stage of propionate synthesis, and two intermediates—γ-methylacetooacetate and α-methyl-β-hydroxybutyrate—accumulate. The condition is apparently inherited.

INTRODUCTION

This report introduces another, apparently hereditary, disorder of isoleucine catabolism. Earlier reports have described several forms of impaired isoleucine oxidation at the first (transamination) and second (decarboxylation) steps in its catabolism. The new disorder affects the sixth step, responsible for conversion of α-methylacetooacetate to propionate. As with many of the other inborn errors of branched-chain aminoacid catabolism, the presenting clinical feature in our proband was recurrent severe metabolic acidosis.

CASE-REPORT

The proband is the 6-year-old son of two unrelated Dutch parents. He was breast-fed through much of his infancy, and his first admission to hospital was at 17 months of age, when he became comatose following an upper respiratory infection accompanied by fever and anorexia. Severe metabolic acidosis was demonstrated which gradually disappeared as the intermittent infection subsided and corrective intravenous therapy was given. Two further admissions at 34 and 48 months of age for a similar course of events are recorded. Organic aciduria was then diagnosed. Thereafter, three mild transitory episodes of metabolic acidosis were effectively countered in the 5th year of life by dietary management, comprising low-protein intake (2 g. per kg. per day) and maintenance of an adequate
calorie intake during the illness. The physical and mental development of the proband are normal. Two other siblings and both parents are healthy.

METHODS

Organic acids were extracted from urine by the method of Mamer et al.¹ and the trimethylsilyl derivatives were examined on an LKB-9000 coupled gas-chromatography/mass-spectrometry unit. L-isoleucine, L-leucine, and L-valine were given separately by mouth, each for 6 consecutive doses, at meal-time (75 mg. per kg. per dose) over a 2-day period. Urine was collected in 8-hour periods before, during, and after the loading tests. Urine samples were obtained, at random and after isoleucine loading, from family members, and examined for organic acids.

Retention time (min)

Detector response of 10⁻² attenuation

Tracing of gas-liquid chromatograms of trimethylsilyl (T.M.S.) derivatives from urine of proband after a dietary load of L-isoleucine.

Peaks 1 and 2, minor normal metabolites; 3, a-methyl-α-hydroxybutyrate; 4 and 5, a-methylacetacetate. Peaks 4 and 5 represent T.M.S. derivatives of the cis and trans forms of the a-M.A.A. enol. Hatching indicates an abnormal amount of the substance.
RESULTS

Urine obtained from the proband during an episode of metabolic acidosis contained large amounts of three unusual silylated derivatives. The excreted amounts of these substances were relatively small between attacks of acidosis, but could be greatly augmented if the dietary intake of L-isoleucine was specifically increased (see accompanying figure). The "unknown" compounds were shown by gas-liquid chromatography and mass spectrometry to be α-methyl-β-hydroxybutyric acid and α-methylacetoacetate, the latter appearing as two silylated derivatives. The concentration of isoleucine and other aminoacids, in plasma and urine, was normal at all times.

The parents and one sib had elevated levels of α-methyl-β-hydroxybutyric acid in their urine; excretion was further increased after L-isoleucine feeding. Normal subjects excrete only trace amounts of this substance.

DISCUSSION

Our patient had a severe metabolic acidosis, which is the common mode of presentation for patients with a hereditary disorder of branched-chain aminoacid metabolism. Perhaps because the isoleucine content of human milk is lower than that of cow's milk, and because no intercurrent infection apparently provoked a catabolic episode early in life, the first clinical episode of acidosis and coma was delayed until late infancy. When the underlying metabolic disease was identified, a diet restricted in protein content but adequate in calories could be prescribed, particularly during periods of intercurrent infectious illness. This regimen clearly ameliorated the frightening episodes of acidosis and mental disorientation.

The major, unusual organic acids in the patient's urine were α-methyl-β-hydroxybutyric acid and α-methylacetoacetate. The presence of either compound in more than trace amounts is abnormal, and in this patient suggests an impairment in their conversion to propionate. Since interconversion of α-methylacetoacetate and α-methyl-β-hydroxybutyrate occurs by an oxidation/reduction step and is analogous to the interconversion of the normal ketone bodies, β-hydroxybutyrate and acetoacetate, this may explain the predominance of the butyrate derivative over the acetacetate compound in our patient.
Although a catabolic pathway for isoleucine has been described, the authenticity of the final conversion of the tiglyl-CoA through α-methyl-β-hydroxybutyryl-CoA and α-methylacetoacetyl-CoA to propionyl-CoA has not been confirmed in man. That this is the pathway was assumed by analogy with a similar reaction in animal tissues, and because of the close parallel to β-oxidation of straight-chain fatty acids in man. The accumulation of α-methyl-β-hydroxybutyrate and α-methylacetoacetate in the patient now confirms that these two substances are formed during isoleucine oxidation. The informative trait which offered this insight appears to be another autosomal-recessive inborn-error of metabolism.

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REFERENCES

AN INHERITED DISORDER OF ISOLEUCINE CATABOLISM
CAUSING ACCUMULATION OF α-METHYLACETOACETATE AND
α-METHYL-Δ-HYDROXYBUTYRATE, AND INTERMITTENT
METABOLIC ACIDOSIS

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INTRODUCTION

During the last decade, 18 disorders of organic acid metabolism have been described, 15 of which involve catabolism of the branched chain amino acids - valine, leucine and isoleucine (Figure III-1). We believe the patients to be presented illustrate a "new" disorder of isoleucine catabolism involving a partial block at the thiolase step in which $\alpha$-CH$_3$ acetoacetyl CoA is split into its products acetyl CoA and propionyl CoA. The block causes accumulation of $\alpha$-CH$_3$ acetoacetic acid, its immediate precursor $\alpha$-CH$_3$ $\beta$-OH butyric acid, and a conjugate, tiglylglycine. As with many other inborn errors of branched chain amino acid catabolism, the presenting clinical feature was severe, recurrent, metabolic acidosis. This report details the case histories of patients from two separate pedigrees along with the investigations carried out to elicit the nature of the biochemical defect.
CASE REPORTS

"B" Pedigree

Patient B, III, 2 is the propositus who led us to the discovery of the disease. He was born in 1965 after a normal gestation and delivery. He was breast fed until 6 months of age and was well until 22 months when he was first admitted to the Montreal Children's Hospital after two days of fever, rhinorrhea, and poor appetite. On the day of admission, there was profuse vomiting and diarrhea. The child was semi-comatose and moderately dehydrated; the liver was 1 cm below the costal margin. Deep tendon reflexes were brisk and symmetrical but plantar reflexes were upgoing. The respiratory rate was 38/minute and blood gas studies revealed a profound metabolic acidosis (Table III-1). Chemical analyses of blood were normal for Na, K, Cl, glucose, lactate, BUN, alkaline phosphatase, and SGOT. An electrocardiogram revealed prolongation of the QT interval. An electroencephalogram revealed a mild, diffuse disturbance. He was treated with intravenous bicarbonate and electrolyte solutions. The clinical picture improved within two days. A diagnosis of metabolic acidosis secondary to accidental ingestion of a poison was entertained because the boy lived on a dairy farm where many chemicals were available.

The child presented again with the same clinical picture when 34 months old. He had been well until 4 days before, when he had developed an upper respiratory tract infection followed by vomiting, hyperventilation, and irritability; he
was semi-comatose. The blood pressure was 90/68, pulse 140 and regular, temperature 102, respirations 60/min. A sweetish odour was noted to the breath and metabolic acidosis was demonstrated (Table I). On examination there were no significant neurological signs. Urinalysis showed minimal proteinuria and strong acetonuria. The excretion of urinary porphyrins and amino acids was normal. After treatment with an intravenous bicarbonate and electrolyte solution, the boy recovered quickly.

The child was again admitted in coma when 48 months old after a two-day illness with sore throat and fever. In the day prior to admission, he vomited 15 times. A macular rash attributable to rubella was noted on his body. Profound metabolic acidosis was again documented. Routine chemistries were all normal. Plasma and urinary amino acids were again found to be within normal limits. The Rothera test for "acetone" in plasma and urine was strongly positive. He was again treated for acidosis.

Another admission with similar signs and symptoms occurred when the patient was 58 months of age. On this occasion, referral to our group led to the discovery of large amounts of a substance (α-methyl β-hydroxybutyric acid) in his urine; this compound is present only in trace amounts in normal urine. There were two subsequent admissions for mild acidosis precipitated by intercurrent febrile illness (Table III-1). The diagnosis of a disorder of isoleucine catabolism encouraged the use of a low protein diet as well as early management of incipient acidosis; the family were counselled concerning
the nature of the illness. The child has been well since and physical and mental growth have remained normal. The parents are Dutch immigrants to Canada and are not related. The propositus is the first to be affected within the pedigree to our knowledge.  

"M" Pedigree  

The "M" family came to Canada in 1971, having emigrated from Chile. Patient M, VII, 5 is the propositus. The parents of this pedigree are related via a common great-great-great-great-great ancestor (see below).  

The propositus, (PATIENT M, VII, 5) was born in 1967 after a 34 week gestation when Cesarian section was carried out for "severe toxemia". She weighed 2400 g at birth and was apparently well until 1 year of age when she was admitted to hospital with fever, hematemesis, and bloody diarrhea. She was discharged after 1 week. She developed as a thin child "who never liked either milk or meat". The child came to the Montreal Children's Hospital at 4 years of age on several occasions with vomiting and acetonuria. She was referred to our group at which time a substance with the identical retention time and mass spectrum of the "unknown" material excreted by the patient in the "B" pedigree was identified. The child again presented with vomiting and acetonuria at age 5 years. Preventive management was carried out on this occasion with dimenhydrinate and protein restriction, and the crisis resolved in 24 hours. Physical and mental growth are normal.
PATIENT M, VII,3 was born in 1962 after a normal gestation. Delivery was by footling breech presentation after elective induction of labour. She was apparently well until age 12 months when she was admitted to hospital with fever, vomiting, and hyperventilation. Coma, hematemesis, and bloody diarrhea ensued and a sweetish odour to the breath was noted. In spite of therapy, she remained unconscious, convulsed repeatedly, and died on the third hospital day. PATIENT M, VII, 4 was born in 1963. His gestation was normal but, because of feto-pelvic disproportion, delivery was performed under general anesthetic. He was breast fed until 4 months of age when he began refusing the nipple; bottle feedings were refused as well. Continued milk feedings were only possible after persistent offerings. At 12 months, he was admitted to a hospital with a short history of fever, hematemesis, and diarrhea. Within hours he had lapsed into a coma which endured 3 days. Convulsions were noted during this period. At 15 months, a similar episode occurred. At 36 months he was admitted to another hospital for similar reasons. After 4 days of coma, his condition slowly improved. Two months later he was readmitted for another episode. During this last admission, he suffered a cardiac arrest but was resuscitated. About 6 more acidotic episodes have occurred, all of lesser severity. At age 8 years, the family moved to Montreal when the child came to our attention. Examination indicates that he has suffered psychomotor damage probably as a sequel to the comatose episodes.
MATERIALS AND METHODS

Identification of Organic Acids.

Organic acids were extracted from urine by the method of Mamer et al\textsuperscript{(1)}. Silylated ether extracts (1-2 µl) were analysed on an LKB-9000 Gas Chromatograph - Mass Spectrometer coupled unit. Column conditions were: 6' x 1/4" glass column packed with SE-30 Ultraphase on Chromosorb W (HP) 80/100 mesh (Pierce Chemical Company, Rockford III) flash heater at 275°C, temperature programming 80°C to 280°C at 4°/minute with no delay, flow rate of Helium 30 ml/min. The separator operated at 285°C with an ion source temperature of 290°C. Ionizing energy and current were 70 eV and 120 µA respectively. Scan time was 4 seconds between m/e 60 and 600. Acceleration potential was 3.5 KV. After identification of the unknown material, all subsequent investigation was carried out with a Hewlett-Packard #402 Gas Chromatograph operated under identical conditions.

Amino Acid Analysis.

Quantitative analysis of amino acids in plasma (1 vol.) deproteinized with sulfosalicylic acid (3% w/v) (5 vol.), was performed on a Beckman-Spinco Amino Acid Analyzer modified for rapid analysis of branched chain amino acids (Scriven et al, 1968\textsuperscript{(2)})

Ketone Analysis.

Urinary and plasma ketones were analysed as their 2,4-DNPH derivatives after the method of Menkes\textsuperscript{(3)}. Aliquots of urine were saturated with NaCl and alkalinized with 1N NaOH to pH 10.5. Urinary ketones were then extracted three times into ether. The ether extract was evaporated to
approximately 25% of original volume following which it was treated with a solution containing 2,4 DNPH. The resultant mixture was chromatographed on Silica Gel sheets (Eastman Kodak Company, Rochester, N.Y.). Neutral hydrazones (i.e. ketones) appeared as yellow spots highly visible under ultra violet light. The solvent used was chloroform - hexane (1:1).

Amino Acid Loading Studies.

Three were undertaken, one each with L-valine, L-leucine and L-isoleucine. The purity of each amino acid used in these studies was demonstrated by chromatographic analysis. The amino acid was given six times, with meals, 75 mg/kg each dose. Each period of dietary loading lasted two days. Two days were allowed between periods so that excretion of organic acid derivatives could return to baseline levels. All urine was collected in 8-hour periods throughout the loading studies and analysed for acetone and organic acids. The excretion of metabolites was compared with pre-load control samples.

Partition Chromatography.

Partition chromatography of urine extracted into ether after acidification (as for Gas Chromatography) was carried out in an ascending system of butanol, acetic acid and water (12:3:5) on Whatman's #1 chromatography paper. Staining was with bromocresol green (50 mg in 100 ml butanol and 1N NaOH).

In Vitro Fibroblast Studies.

Fibroblasts were cultured from skin biopsy explants and incubated at 37°C in Eagle's minimum essential medium,
containing 12% fetal calf serum under an atmosphere of 5% CO₂ in air. Cultures once established were rinsed three times with cold saline, and buffered with 5 ml. TRIS-buffer pH 7.4, following which the cells were scraped with a rubber policeman and placed into a Warburg Flask. Incubation for one hour was carried out in the presence of unlabelled L-isoleucine and L-isoleucine-U-¹⁴C (final conc. 0.4 mM) using L-leucine-U-¹⁴C and Sodium Succinate-U-¹⁴C for control. The reaction was stopped by the addition of 50 µl H₂SO₄. CO₂ was collected on filter paper soaked in 1M KOH for one hour with gentle shaking; the efficiency of this method is 59 ± 4 percent. The paper was dried and placed in 0.4 ml hyamine to which was added 10 ml scintillation fluid. The paper was then counted on a Nuclear Chicago Unilux Scintillation Counter. Protein determination was carried out after the method of Lowry et al(9).
RESULTS

Identification of α-methyl-β-hydroxy butyric acid.

Gas chromatographic analysis of the urines obtained during episodic acidosis of the various patients revealed that the largest unusual peak had a retention time of approximately 7.6 minutes in the LKB-9000 GC system (Figure 2A). Initial mass spectrographic evidence suggested the substance was methylmalonic acid but partition chromatography failed to confirm this identification. Further mass spectrographic analysis of the trimethylsilylated derivatives revealed a mono-methylated, mono-hydroxylated compound with a four carbon aliphatic chain back bone. A compound fitting these requirements, namely α-methyl-β-hydroxy butyric acid (α-CH$_3$-β-OH-butyrate) was synthesized. Its mass spectrum was identical to that of the unknown (Figure 2B). Furthermore, the synthetic compound cochromatographed with the unknown further confirming its identification. α-CH$_3$-β-OH butyrate is a metabolite in the catabolic pathway of isoleucine.

Urine was also collected between episodes of metabolic acidosis; all patients excreted α-CH$_3$-β-OH butyrate at all times, but there was less of the compound between episodes of acidosis, as judged by peak size per milligram of urinary creatinine.

Identification of α-methyl acetoacetic acid.

Analysis of the urine of B and M propositi yielded a second abnormal substance appearing as 2 peaks with retention time of 9 and 10.4 minutes respectively on the LKB-9000 GC system (Figure III-3A). This compound was also analysed by
mass spectrometry (Figure III-3B). When synthesized, it cochromatographed with the "unknown" in urine supporting the identification of $\alpha$-methyl acetoacetic acid ($\alpha$-CH$_3$ acetoacetate). This compound is apparently present in smaller quantities than $\alpha$-CH$_3$-\(\beta\)-OH butyrate most of the time as judged by relative peak size. Enol formation during silylation yields two peaks which are seen on gas chromatography.

**Identification of N-tiglylglycine.**

The M propositus excreted an additional abnormal substance in the urine with retention time of 15.6 minutes on the LKB-9000 system. When this compound was analysed by mass spectroscopy, synthesized, and cochromatographed, the identification of N-tiglylglycine was achieved (Figure 4). It is excreted at all times in some probands and intermittently in others and its excretion is consistently augmented during acidotic interludes.

**Identification of other organic acids.**

Propionate levels in the urine of B and M probands were within normal limits at all times, and there was no evidence for excessive accumulation of the keto acid of isoleucine ($\alpha$-keto-$\beta$-methyl valeric acid), its derivatives, or $\alpha$-methylbutyric acid. $\beta$-hydroxybutyric acid was identified in the patients' urines in large amounts when they were acidotic. This compound was not present in significant amounts during non-acidotic periods. Hippuric acid was present in urine in large amounts in the M probands.

**Urinary ketones.**

Analysis of urine obtained during periods of metabolic
acidosis and following loading with isoleucine revealed acetone and butanone in significant quantities (Figure III-5). Pentanone and hexanone were not present in urine at any time. **Amino acid loading studies.**

The propositus of the B pedigree was studied in more detail by means of loading tests. An L-valine load by mouth did not augment the excretion of α-CH₃-β-OH butyrate or α-CH₃ acetoacetate. L-leucine loading caused a small increase in the excretion of these compounds. L-isoleucine produced a dramatic increase in the excretion of both isoleucine metabolites (Figure III-6). Blood gas analysis performed concurrent with the isoleucine load revealed modest metabolic acidosis during the load; the most abnormal serum values which occurred on the second day in the loading period were

\[
[H^+] \text{ 44 nEq/L., } pCO_2 \text{ 31 mm Hg, } [HCO_3^-] \text{ 18 mEq/L., } Base \text{ Excess -7.1 mEq/L.}
\]
**FAMILY STUDIES**

**B pedigree**

Random daytime urine samples were collected from the brothers and parents of the propositus and were analysed for the presence of \( \alpha-\text{CH}_3-\beta-\text{OH}-\text{butyrate} \). This metabolite was present in the urine of both parents and one sib in small but abnormal amounts. The other sib did not excrete the compound in detectable amounts. Addition of L-isoleucine into the diet (75 mg/kg at each meal for two days) caused a significant increase in the excretion of \( \alpha-\text{CH}_3-\beta-\text{OH}-\text{butyric acid} \). Normal subjects do not have this response. Neither the baseline levels of excretion nor the increases after loading in the parents were as great as in the propositus.

**M pedigree**

A brother of the propositus excretes abnormal amounts of tiglylglycine, \( \alpha-\text{CH}_3-\beta-\text{OH}-\text{butyrate} \) and \( \alpha-\text{CH}_3-\text{acetoacetate} \) as well as butanone during acidosis. Two other sibs and both parents excrete a lesser excess of \( \alpha-\text{CH}_3-\beta-\text{OH}-\text{butyrate} \) in the urine.

On the basis of the phenotypic observations, we have tentatively assigned homozygous status to those individuals with a large abnormal excretion of one or more metabolites in the isoleucine pathway. Individuals who excrete a small excess of \( \alpha-\text{CH}_3-\beta-\text{OH}-\text{butyrate} \) only are assigned heterozygous status. The proposed inheritance of genotypes in these two pedigrees is indicated in Figure III-7. The possibility that the mutant allele is different in the two families has not been excluded.
FIBROBLAST STUDIES

Cultured skin fibroblasts from the B propositus grew well and showed no morphological abnormalities in culture. The metabolic potential of B cells was studied in preliminary fashion. The ability to oxidize isoleucine-U-\(^{14}\text{C}\) to \(^{14}\text{CO}_2\) was impaired in the mutant cells in comparison with control cells grown to the same density and harvested at the same time after subculture (Table III-2). There was no defect in the oxidation of succinate or of leucine. These observations coupled with the metabolic studies in vivo, placed the defect of isoleucine oxidation in the pathway at some point beyond decarboxylation but prior to the oxidation of propionyl-CoA. The effect of temperature upon isoleucine catabolism was determined in vitro because of the frequent clinical correlation between fever and acidotic episodes. Incubations at 40°C and at 42°C did not depress isoleucine oxidation below that observed at 37°C in the cultured fibroblasts.
DISCUSSION

The suspicion that our patients had organic aciduria was initiated by the recurrent acidotic episodes without obvious cause. The clinical evidence seems to suggest that this "new" metabolic aberration need not of its own accord be harmful, but that the severe (uncompensated) acidotic episodes which the patient is likely to experience can be severe enough to cause mental retardation or even death. Patients B, III, 2 and M, VII, 5 have developed normally although they appear to be homozygous for the mutant allele. On the other hand, patient M, VII, 4 is retarded mentally after a series of severe acidotic episodes one of which was complicated by cardiac arrest. Patient M, VII, 3 died during an acidotic episode.

The clinical onset of disease was late in the first year of life or thereafter in our patients. All the children were breast fed and, because the isoleucine content of human milk is considerably lower than that of cow milk (Fomen, 1967) (4), the patients may have been protected from metabolite accumulation and precipitation of acidosis. Intolerance to high protein intake is apparent in all of the patients and each has avoided episodes of illness by restricting protein intake after the diagnosis was established.

α-CH₃-β-OH-butyrate was detected in urines at all times in affected probands, the amounts being increased during periods of acidosis. Quantitation of this and the other metabolites has been hampered by the lack of pure reference standards. A method of synthesis to yield pure α-CH₃-β-OH-butyrate has now been accomplished by one of us (OM) which
has allowed some preliminary quantitation. During acidosis the excretion of this compound in urine exceeds 1 g/g creatinine, while at other times it is in the range of 250 to 1000 mg/g creatinine in the presumed homozygotes. The precipitating stimuli for acidosis and coma are unknown. Each episode was associated with febrile illness at which time the expected protein catabolism could release large amounts of endogenous isoleucine from tissues stores. Our in vitro studies with cultured fibroblasts do not reveal evidence for a temperature-sensitive abnormality of isoleucine metabolism. It is possible that in the presence of impaired metabolism the further input of isoleucine during febrile catabolic episodes causes accumulation of metabolites which in turn further inhibit isoleucine oxidation. Hillman (1972)\(^5\) has described a disorder of isoleucine metabolism which resembles the present condition and in which hyperglycinemia was also observed. He suggested that tiglic acid accumulation causes hyperglycinemia in these patients. There was no hyperglycinemia in our patients on any occasion. Whether tiglic acid inhibits isoleucine oxidation has not yet been investigated.

The metabolite apparently present in greatest amounts in the urine is \(\alpha-\text{CH}_3-\beta-\text{OH}-\text{butyrate}\), while \(\alpha-\text{CH}_3-\text{acetoacetate}\) is apparently present in smaller but abnormal amounts. Tiglylglycine, when present, yields the smallest abnormal peak. These estimates are based on relative peak size and they assume that equimolar amounts of the three metabolites would yield approximately the same peak size on the GC chromatogram,
which is likely because of their similar chemical structure. The relative peak sizes of α-CH₃-acetoacetate and α-CH₃-β-OH-butyrate favor the latter as the quantitatively major substance accumulating in the probands. The metabolic data however favor an enzyme block at the thiolase step, converting α-CH₃-acetoacetyl-CoA to propionyl-CoA and acetyl-CoA. The apparently greater accumulation of a metabolite other than the substrate of the enzyme requires explanation. The situation may be analogous to the metabolic interrelations of the "ketone bodies", β-OH-butyrate and acetoacetate, where the equilibrium between them favors formation of the butyrate derivative. Alternatively a minor pathway of α-CH₃-acetoacetate metabolism may exert its influence on the accumulated metabolite in the mutant state and remove it; the excess of butanone in our patients indicates at least one route by which this is occurring. Since the enzymes which interconvert tiglic acid and the two other metabolites are reversible, accumulation of tiglic acid presumably reflects a new steady-state between the three steps of isoleucine oxidation in our patients. We assume that formation of tiglylglycine is a detoxifying mechanism to rid the organism of this highly toxic organic acid. For reasons which are not yet clear tiglylglycine is also present in abnormal amounts in β-methylcrotonyl-glycinuria of the biotin responsive type (Gompertz et al, 1971)⁶). The non conjugated tiglate derivative is present in abnormal amounts in propionicacidemia (Nyhan et al, 1971)⁷).

Although a catabolic pathway for isoleucine has been described (Figure 1), the authenticity of the final steps
of this pathway involving the conversion of tiglyl CoA through to acetyl CoA and propionyl CoA have not been confirmed in human tissues. It was presumed by Robinson et al(8) that the reactions proceed in the manner shown because of the close parallel with β-oxidation of fatty acids as well as by some indirect experimental evidence. The findings in our patients suggest that the pathway as it is known also carries out the final stages of isoleucine oxidation in man but final conclusions will rest on specific studies in vitro.

The pedigree studies indicate that the trait which affects isoleucine catabolism is inherited, apparently in autosomal recessive fashion. Probands are presumed homozygotes but heterozygotes, who are otherwise healthy, show some biochemical features of the trait. Whether the mutant allele is the same in B and M pedigrees is unknown and whether the trait(s) described here are identical to that reported in abstract by Hillman (1972)(5) is also not known. We presume that the M probands have inherited a pair of rare but similar mutant alleles because of the consanguinity in this pedigree. However, the B pedigree which to the best of our knowledge is not consanguinuous, may contain a dissimilar pair of mutant alleles at the same genetic locus which when paired produce a genetic compound with an apparently homozygous phenotype. Specific evaluation of the enzyme defect and the appropriate kinetic studies in vitro may discern the extent of genetic heterogeneity in this trait.
REFERENCES


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LEGEND

TABLE III-I

Summary of Hospitalizations of Patient B,III,2.

Note the lateness of onset of clinical disease in this patient, not requiring hospitalization until 1 year, 5 months of age. Note also the severity of acidosis on the first 3 admissions as opposed to the last 3. Some insight into the child's condition was gained between the third and fourth admissions allowing for preventive as well as earlier symptomatic management of subsequent episodes.
Figure III-1: Metabolism of branched chain amino acids.
Known disorders of branched-chain amino acid metabolism are shown by a number representing the order in which the respective traits have been reported. The metabolites which accumulate in body fluids in the various conditions are named; other metabolites in the pathways are shown by open boxes. Tiglate and $\beta$-methylcrotonate have been recovered in urine as their glycine conjugates, tiglyl glycine and $\beta$-methylcrotonyl glycine respectively. The presumed direct conjugation reactions are not shown. The reaction presumably affected in the patients reported here is #12.
JB during febrile episode

\[
\begin{align*}
&\text{BOH} \\
&\text{butyric} \\
\end{align*}
\]

\[
\begin{align*}
&\text{(CH}_3\text{)}_3\text{Si} \\
&\text{M}^{+} - \text{CH} \\
\end{align*}
\]

retention time (min)

rel. int.

\[
\begin{align*}
&75 \\
&73 \\
&117 \\
&147 \\
&218 \times 10 \\
&231 \\
&247 \\
&261 \\
\end{align*}
\]

\[
\begin{align*}
&70 \\
&100 \\
&150 \\
&200 \\
&250 \\
&300 \\
\end{align*}
\]
LEGEND

Figure III-2A: Gas-Liquid chromatogram of trimethyl-silylated (TMS) derivatives in urine from proband B,III,2 during a febrile episode. This chromatogram shows 2 significant peaks. β-hydroxybutyric acid, which is a "normal ketone body"; α-methyl-β-hydroxybutyric acid (α-MβHB), the abnormal metabolite. Note that the retention time as shown was obtained on the Hewlett-Packard Instrument; this system of analysis is slightly different from that quoted in the text which was derived from the LKB-9000 Gas Chromatograph-Mass Spectrometer.
LEGEND

Figure III-2B: Mass spectrum of α-methyl-β-hydroxybutyric acid, bis-TMS derivative. Significant ions are m/e 247, representing loss of a TMS methyl group by the molecular ion (m/e 262), m/e 147 assigned to a rearrangement ion characteristic of compounds incorporating 2 or more TMS groups, and m/e 117 indicative of hydroxyl substitution on the next-to-terminal carbon atom.
Figure III-3A: Gas-liquid chromatogram of TMS derivatives in urine from proband B,III,2 during a mild acidotic episode. This chromatogram shows the retention times of the 2 silylated isomers of α-methyl-acetoacetate (α-MAA), and the relative position of α-MβHB. Minor technical differences again account for the discrepancy between times shown and those mentioned in the text.
Figure III-3B: Mass Spectrogram of α-methylacetoacetic acid (α-MAA). This compound is a 5-carbon unsaturated carboxylic acid. There is a methyl group substitution at the number 2 (α) position and a carbonyl ("keto") group at position number 3 (β). Two peaks occur because of enol intramolecular conversion involving the double bond and the carbonyl group.
LEGEND

Figure III-4: Mass spectrogram of tiglyl glycine. This compound was present in the urine of patients in the M pedigree. It is presumably formed by conjugation of free tiglic acid, a derivative of isoleucine, with glycine. The peak at m/e 83 represents the tiglyl fragment and a molecular ion at m/e 229 is evident.
LEGEND

Figure III-5: Thin-layer chromatogram on silica gel of urinary ketones as 2,4-DNPH derivatives. J3 is proband B,III,2, RM is a normal control. MEK is the 2,4-DNPH derivative of methyl ethyl ketone, used as the butanone standard. The unknown substance in the patient's urine also co-chromatographed with MEK when the latter was added to the patient's urine. The butanone derivative on the silica gel sheet is yellow in visible light and lavender in ultraviolet light. The patient's unknown spot exhibited similar characteristics.
before L-isoleucine load
time: 20mgN

after L-isoleucine load
time: 20mgN

FIGURE III-6

detector response at 10^2 x 2 attenuation

Retention time (min)

2 4 6 8 10 12 14

αMβHB

αMAA

αMβHB

αMAA
Figure III-6: Response to isoleucine loading. The gas-liquid chromatogram on the left was derived from a urine specimen prior to the "chronic" isoleucine load (see text). On the right is a chromatogram derived from a urine taken in the immediate 24 hours following the last dose. Note the dramatic increase in peak size of the abnormal metabolites associated with isoleucine feeding.
FIGURE III - 7

M Pedigree

B Pedigree

- male not examined
- female examined and found normal
- miscarriage
- presumed homozygote
- presumed heterozygote
Figure III-7: Relevant pedigrees for the M and B propositi (M, VII, 4 and B, III, 2 respectively). Assignment of the presumed genotype is based on the biochemical phenotype, determined by GC analysis of urine samples. Presumed homozygotes excrete large amounts of α-MβMB and α-MAA (and also N-tiglylglycine in the M pedigree); presumed heterozygotes excrete a small excess of α-MβMB only. L-isoleucine feeding augments excretion of the unusual metabolites.
<table>
<thead>
<tr>
<th></th>
<th>L-ileu (ul)</th>
<th>L-ileu (ul)</th>
<th>L-leu (ul)</th>
<th>Succinate (1,4-(^14)C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution of (^14)C in,</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CO(_2)</td>
<td>44</td>
<td>46</td>
<td>113</td>
<td>106</td>
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<tr>
<td>Sol. extract</td>
<td>89</td>
<td>131</td>
<td>170</td>
<td>126</td>
</tr>
<tr>
<td>Protein</td>
<td>127</td>
<td>94</td>
<td>94</td>
<td>52</td>
</tr>
</tbody>
</table>
Simultaneous experiments using mutant fibroblasts from patient B,III,2 and normal fibroblasts were carried out as described in the Materials and Methods. The data presented represent the number of counts obtained in the mutant cell experiments as a percentage of the counts obtained from the control cell. The most striking differences are in oxidation of L-isoleucine-U-\(^{14}\)C where mutant cells were only able to carry out these catabolic reactions at slightly less than \(\frac{1}{2}\) the "normal" rate. Incorporation of isoleucine into protein is presumed from the data to proceed at a normal rate (i.e. virtually 100\%). The soluble extract (i.e. that remaining after protein precipitation) reflects the accumulation of metabolites behind the presumed block. The metabolism of leucine and succinate to \(\text{CO}_2\) is normal in our patients fibroblasts. The apparent increase in the soluble fractions after experimentation with these substances is not easily accounted for.