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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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 <u>Lancet</u>. 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 <u>Pediatric Research</u> for review and publication.

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 α-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 α-methyl-β-hydroxybutyrate, and a
seemingly smaller excess of α-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-u-^{14}C to CO_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 α-methyl-β-hydroxy-butyrate 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: <u>2</u>

3. PRESUMED ENZYME DEFECT: Valine Transaminase

4. METABOLITES ACCUMULATING: L-Valine

5. CLINICAL FEATURES: Failure to thrive, psychomotor retar-
    dation, 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 <u>Wada et al</u>,
    (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.
    <u>Treatment</u> 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.

Table I-1 (cont.)   2.

1.   PHENOTYPE: hyperLeucine-Isoleucinemia

2.   NUMBER IN FIGURE I-1: <u>11</u>

3.   PRESUMED ENZYME DEFECT: Transaminase

4.   METABOLITES ACCUMULATING: Leucine, Isoleucine

5.   CLINICAL FEATURES: Failure to thrive, mental retar-
     dation, convulsions, retinal degeneration, apparent
     nerve deafness.

6.   COMMENT: First reported by <u>Jeune et al</u>, (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 trans-
     amination demonstrated <u>in vitro</u>. Pyridoxal therapy
     attempted <u>in vivo</u> with no response. Importance of
     disease lies in suggestion that transaminase of C-6
     branched-chain amino acids is specific.

Table I-1 (cont.)   3.

1. PHENOTYPE: Isovaleric Acidaemia, 1) "classical"

2. NUMBER IN FIGURE I-1: <u>3</u>

3. PRESUMED ENZYME DEFECT: Isovaleryl-CoA Dehydrogenase

4. METABOLITES ACCUMULATING: Isovaleric Acid, β-OH Iso-
    valeric Acid, Isovalerylglycine.

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 <u>per se</u> or
    secondary to severe acidosis. Spectrum of severity
    of symptoms varies greatly.

6. COMMENT: First described by <u>Tanaka et al</u>, (1966).
    Amino acid chromatogram entirely normal. Gas chroma-
    tography elicited isovaleric acid, a normal metabolite,
    in abnormal amounts in serum and urine; identification
    confirmed by Mass Spectrometry. Isovalerylglycine also
    found and identified by <u>Tanaka et al</u> (1967). β-hydroxy
    isovalerate found and identified by <u>Tanaka et al</u> (1968).
    Isovalerylglycine formation thought to be detoxification
    mechanism. Can be saturated during ketosis when β-
    hydroxyisovaleric acid appears. Presence of β-hydroxy-
    isovaleric acid suggests a direct hydroxylation reaction

Table I-1 (cont. Isovaleric Acidaemia ...)   4.

i.e. Isovaleryl-CoA $\rightleftarrows$ β-OH Isovaleryl-CoA

If this assumption is correct, then the subsequent

conversion

     β-OH Isovaleryl CoA $\rightleftarrows$ β-CH_3 Crotonyl-CoA

would provide a by pass of the isovaleryl-CoA dehydro-

genase 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.)   5.

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

2.   NUMBER IN FIGURE I-1: <u>15</u>

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 retar-
     dation; no neutropenia.

6.   COMMENT: Ketotic hyperglycinemia was first described by
     <u>Childs et al</u>,(1961) subsequently divided into <u>at least</u>
     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. <u>In vitro</u> work with fibroblasts shows that no
     glycine is converted to hippuric acid in contrast to the
     large amounts of hippurate formed in normals.

Table I-1   (cont.)   6.

1. PHENOTYPE: β-methyl crotonyl glycinuria (β-hydroxy
   isovaleric acidemia), 1) Biotin unresponsive

2. NUMBER IN FIGURE I-1:   <u>10</u>

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 <u>Eldjarn et al</u> 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.

Table I-1   (cont.)   7.

1. PHENOTYPE: β-methyl crotonylglycinuria (β-hydroxy isovaleric acidemia), 2) Biotin responsive.

2. NUMBER IN FIGURE I-1: <u>14</u>

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 <u>Gompertz et al</u> 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.)   8.

1. PHENOTYPE: Methylmalonic Aciduria, 1) B_{12} non-responsive

2. NUMBER IN FIGURE I-1: <u>4</u>

3. PRESUMED ENZYME DEFECT: Methylmalonyl CoA Mutase

4. METABOLITES ACCUMULATING: Methylmalonic acid, propionate,

    (?) 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

    <u>Oberholzer et al</u> (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 (<u>Phansalkar</u>

    <u>et al</u>, 1971). This is a form of ketotic hyperglycinemia.

    Relationship between glycine and branched-chain metabolism

    remains obscure. Vitamin B_{12} is a cofactor for the normal

    mutase apoenzyme. Response to its administration divides

    methylmalonic aciduria into 2 sub-groups. B_{12}-resistant

    form carries a higher mortality. Protein restriction

    tried with some success.

Table I-1   (cont. Methylmalonic Aciduria, 1)...)   9.

MMA has been diagnosed <u>in utero</u> by amniocentesis as well as by actual metabolite detection in maternal urine (Morrow et al, 1970). Leukocytes fail to convert propionate-^{14}C to $^{14}CO_2$ <u>in vitro</u> (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 decarboxylation of α-methylacetoacetate (Menkes, 1966), a metabolite 2 steps removed from the mutation block.

Table I-1   (cont.)   10.

1. PHENOTYPE: Methylmalonic Aciduria   2) B_{12} responsive

2. NUMBER IN FIGURE I-1: <u>5</u>

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_{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 expe-
    riments were done before and during B_{12} therapy.
    <u>In vitro</u> results reflected <u>in vivo</u> observations.
    Clinical prognosis better than in B_{12}-resistant form.

Table I-1 (cont.) 11.

1. PHENOTYPE: Propionic Acidaemia, 1) biotin unresponsive

2. NUMBER IN FIGURE I-1: <u>7</u>

3. PRESUMED ENZYME DEFECT: Propionyl CoA Carboxylase

4. METABOLITES ACCUMULATING: Propionic acid. (?) α-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 <u>Hommes et al</u> 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, 1) ...)   12.

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.)   13.

1. PHENOTYPE: Propionic Acidaemia. 2) biotin responsive

2. NUMBER IN FIGURE I-1: <u>8</u>

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

5. CLINICAL FEATURES: Onset at age 2 days. Lethargy,

    hypotonia, athetoid movements. Ketoacidosis, hyper-

    glycinaemia. Psychomotor retardation. Persistent

    hypotonia.

6. COMMENTS: This variant described by <u>Barnes et al</u> (1970).

    Biotin is a cofactor in the reaction:

$$\text{Propionyl CoA} \xrightarrow{\quad CO_2 \quad biotin \quad} \text{Methylmalonyl CoA}$$
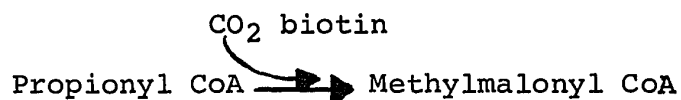
    and when administered to the patient (5 mg for 5 days)

    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.

Table I-1 (cont.)   14.

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, α-keto-isovalerate, L-isoleucine, α-keto βCH$_3$ valerate, L-leucine, α-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, peri-toneal dialysis during acute illness.

Table I-1 (cont.)   15.

1. PHENOTYPE: Maple syrup urine disease, 2)"intermittent"

2. NUMBER IN FIGURE I-1: <u>6</u>

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 inter-
    current infections, etc. Death reported during some
    attacks. Usually normal mental and physical development.

6. COMMENTS: First described by <u>Morris et al</u> (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.

Table I-1 (cont.)   16.

1.   PHENOTYPE: Maple syrup urine disease, 3) "mild"

2.   NUMBER IN FIGURE I-1: <u>9</u>

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 <u>Schulman et al</u> (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. Treat-
     ment may not be necessary or protein restriction may
     suffice.

Table I-1 (cont.)   17.

1. PHENOTYPE: Maple syrup urine disease, 4) "thiamine responsive"

2. NUMBER IN FIGURE I-1: <u>13</u>

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

4. METABOLITES ACCUMULATING: L-valine, α-OH isovalerate, L-isoleucine, α-OH β-CH$_3$ 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%). Keto acidosis occurs only associated with endogenous catabolic load secondary to infection.

6. COMMENTS: First described by <u>Scriver et al</u> (1971). Striking biochemical features include no ketoaciduria except during acute episodes. The corresponding hydroxy-acids predominate otherwise. Biochemical response to thiamine HCl (20 mg/day); i.e. reduction in excretion of all above metabolites.

Table I-1   (cont.)   18.

1. PHENOTYPE: α-methyl acetoacetic aciduria

2. NUMBER IN FIGURE I-1: <u>12</u>

3. PRESUMED ENZYME DEFECT: α-methylacetoacetic thiolase

4. METABOLITES ACCUMULATING: α-methylacetoacetic acid,
   α-methyl β-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.

6. COMMENTS: First described by <u>Daum et al</u> (1971).
   Relatively benign disease if protein restriction and
   corrective management are practiced. One death and one
   retarded child secondary to severe acidosis known.
   Enzyme deficiency - postulated at thiolase step -
   a presumptive inference from metabolic picture. Marked
   increase in metabolite excretion following isoleucine
   loading. Slight increase after leucine loading.
   Butanone in urine during acidotic episode. Partial
   defect in oxidation of L-isoleucine-^{14}C to $^{14}CO_2$ <u>in vitro</u>.

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-methyl-

crotonate 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 I - 1
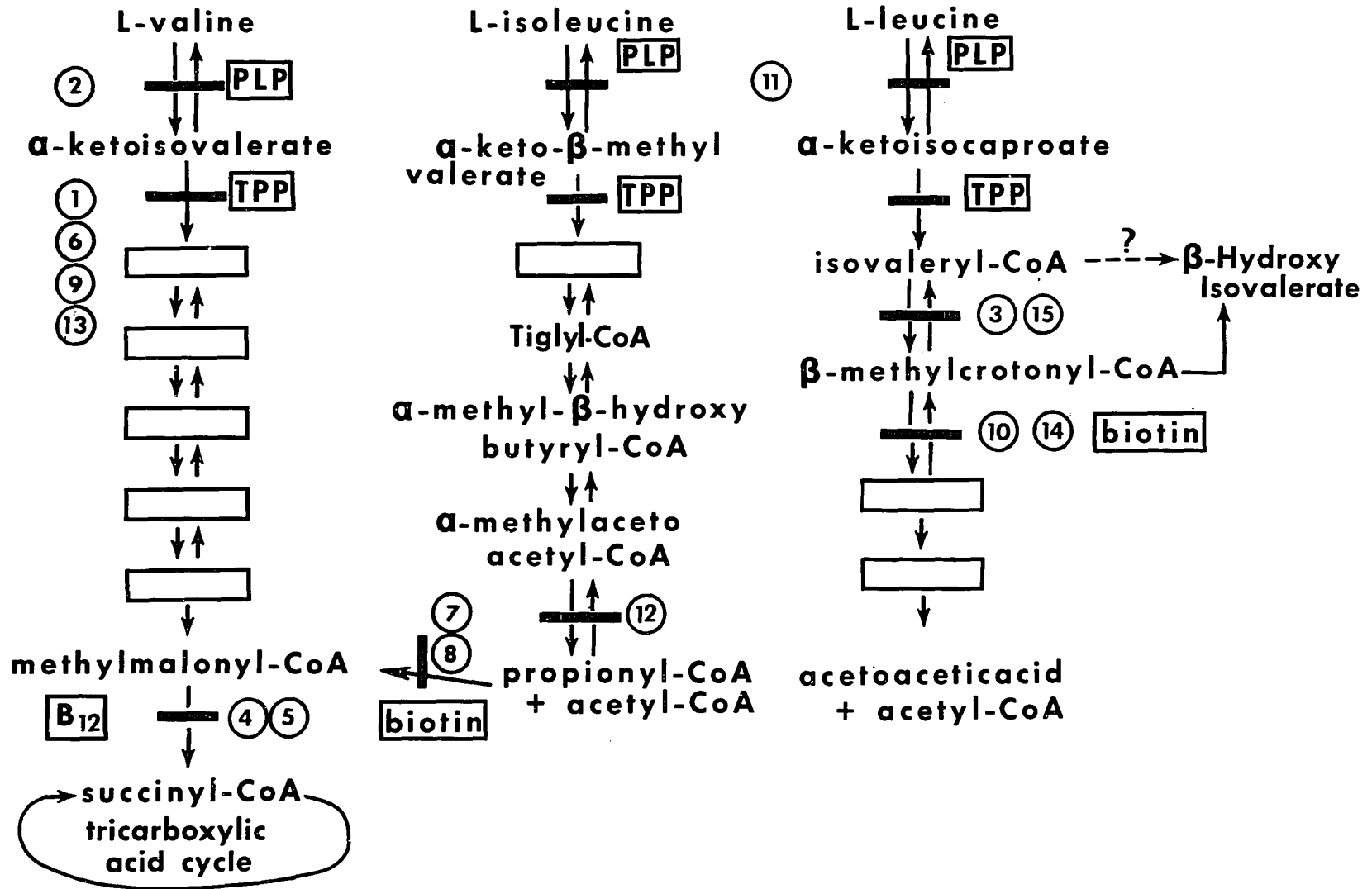
REFERENCES (Section I)

1. Ando, T., Klingsberg, W.D., Ward, A.N., Rasmussen, K.,
    and Nyhan, W.L.: Isovaleric acidemia presenting with
    altered metabolism of glycine. Pediat. Res. 5:478-486
    (1971A).

2. Ando, T., Rasmussen, K., Nyhan, W., Donnell, G.N., and
    Barnes, N.D.: Propionic acidemia in patients with
    ketotic hyperglycinemia. J. Pediat. 78:827-832 (1971B).

3. Barnes, N.D., Hull, D., Balgobin, L., and Gompertz, D.:
    Biotin responsive propionicacidemia. The Lancet ii)
    244-245 (1970).

4. Childs, B., Nyhan, W.L., Borden, M., Bard, L., and
    Cooke, R.E.: Idiopathic hyperglycinemia and hyper-
    glycinuria: a new disorder of amino acid metabolism I.
    Pediatrics 27:522-538 (1961).

5. Dancis, J., and Levitz, M.: Abnormalities of branched-
    chain amino acid metabolism. In Stanbury, J.B.,
    Wyngaarden, D.S., and Fredrickson, D.S., eds., The
    Metabolic Basis of Inherited Disease, 3rd edit.,
    pp 426-439. New York, McGraw Hill Book Co., 1971.

6. Daum, R.S., Mamer, O.A., Lamm, P., and Scriver, C.R.:
    A "new" disorder of isoleucine catabolism. The Lancet ii)
    1289-1290 (1971).

7. Eldjarn, L., Jellum, E., Stokke, O., Pande, H., and
    Waaler, P.E.: β-hydroxyisovaleric aciduria and β-methyl-
    crotonylglycinuria: a new inborn error of metabolism.
    The Lancet ii) 521-522 (1970).

8. Gompertz, D., Bau, D.C.K., Storrs, C.N., Peters, T.J., and Hughes, E.A.: Localization of enzymic defect in propionicacidemia. The Lancet i) 1140-1143 (1970).

9. Gompertz, D., Draffan, G.H., Watts, J.L., and Hull, D.: Biotin-responsive β-methylcrotonylglycinuria. The Lancet ii) 22-24 (1971).

10. Hommes, F.A., Kuipers, J.R.C., Elema, J.D., Jansen, J.F., and Junxis, J.H.P.: Propionicacidemia: a new inborn error of metabolism. Ped. Res. 2:519-524 (1968).

11. Menkes, J.H.: Idiopathic hyperglycinemia: isolation and identification of three previously undescribed urinary ketones. J. Peds. 69:413 (1966).

12. Menkes, J.H., Hurst, P.L., and Craig, J.M.: A new syndrome: progressive familial infantile cerebral dys-function associated with an unusual urinary substance. Pediatrics, 14:462-466 (1954).

13. Morris, M.D., Lewis, B.D., Doulan, P.D., and Harper, H.A.: Clinical and biochemical observations on an apparently non-fatal variant of branched-chain ketoaciduria (maple syrup urine disease). Pediatrics 28:918-923 (1961).

14. Morrow, G. III, Mellman, W.J., Barness, L.A. and Dimitrov, N.V.: Propionate metabolism in cells cultured from a patient with methylmalonic acidemia. Ped. Res. 3: 217-219 (1969).

15. Morrow, G. III, Schwartz, R.H., Hallock, J.A., and Barness, L.A.: Prenatal detection of methylmalonic acidemia. J. Pediat. 77:120-123 (1970).

16. Oberholzer, V.G., Levin, B., Burgess, E.A., and Young, W.F.: Methylmalonic aciduria. An inborn error of metabolism leading to chronic metabolic acidosis. Arch. Dis. Child. 42:492-504 (1967).

17. Phansalkar, S.V., Nortom, P.M., Holt, L.E. Jr., and Snyderman, J.E.: Amino acid relationships: the effect of a load of leucine on the metabolism of isoleucine. Proc. Soc. Exp. Biol. and Med. 134:262-263 (1970).

18. Rosenberg, L.E., Lilljeqvist, A.C., and Hsia, Y.E.: Methylmalonic aciduria. An inborn error leading to metabolic acidosis, long-chain ketonuria and inter-mittent hyperglycinemia. New Eng. J. Med. 278:1319-1322 (1968a).

19. Rosenberg, L.E., Lilljeqvist, A.C., and Hsia, Y.E.: Methylmalonic aciduria: metabolic block localization and vitamin B_{12} dependency. Science 162:805-807 (1968b).

20. Rosenberg, L.E.: Disorders of propionate, methylmalonate and vitamin B_{12} metabolism. In Stanbury, J.B., Wyngaarden, J.B., and Fredrickson, D.S., eds., The Metabolic Basis of Inherited Disease, 3rd edition, New York, McGraw Hill Book Co. pp 440-458 (1971).

21. Schulman, J.D., Lustberg, T.J., Kennedy, J.L., Museles, M., and Seegmiller, J.E.: A new variant of maple syrup urine disease (Branched chain ketoaciduria). Am. J. Med. 49:118-124 (1970).

22. Scriver, C.R., Mackenzie, S., Clow, C.L., and Delvin, E.: Thiamine-responsive maple-syrup urine disease. The Lancet i) 310-312 (1971).

23. Scriver, C.R. and Rosenberg, L.E.: Disorders of branched-chain amino acid metabolism. Phila., W.B. Saunders Ltd (in press).

24. Rosenberg, L.E. and Scriver, C.R.: Amino Acid Metabolism. In Duncan's Textbook on Diseases of Metabolism, 6th ed., Bondy, P. ed. pp.366-515, Saunders, Phila. 1969.

25. Tada, K., Wada, Y., and Arakawa, T.: Hypervalinemia. Am. J. Dis. Child. 113:64-67 (1967).

26. Tanaka, K., Budd, M.A., Efron, M.L., and Isselbacher, K.J.: Isovalericacidemia: a new genetic defect of leucine metabolism. Proc. Nat. Acad. Sci. (U.S.) 56:236-242 (1966).

27. Tanaka, K., and Isselbacher, K.J.: The isolation and identification of N-isovalerylglycine from urine of a patient with isovalericacidemia. J. Biol. Chem. 242: 2966-2972 (1967).

28. Tanaka, K., Orr, J.C., and Isselbacher, K.J.: Identification of β-hydroxyisovaleric acid in the urine of a patient with isovaleric acidemia. Biochim. Biophys. Acta 152:638-642 (1968).

29. Tanaka, K., Miller, E.M., and Isselbacher, K.J.: Hypoglycin A: A specific inhibitor of isovaleryl CoA dehydrogenase. Proc. Nat. Acad. Sci. (U.S.) 68:20-24 (1971).

30. Wada, Y., Tada, K., Minagawa, A., Yoshida, T., Morikawa, T., and Okamura, T.: Idiopathic valinemia: probably a new entity of inborn errors of valine metabolism. Tohoku J. Exper. Med. 81:46-55 (1963).

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—α-methylacetoacetate 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) [1] and second (decarboxylation) [2-5] steps in its catabolism. The new disorder affects the sixth step, responsible for conversion of α-methylacetoacetate 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 intercurrent 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

caloric 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.[6] 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.
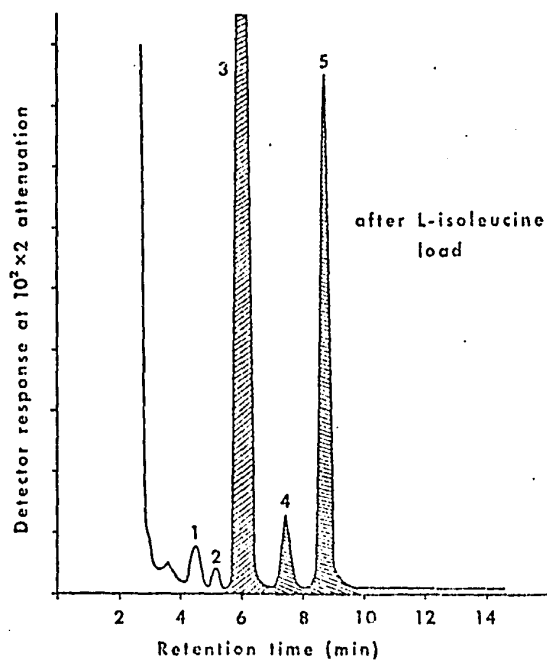
Tracing of gas-liquid chromatogram of trimethyl-silyl (T.M.S.) derivatives from urine of proband after a dietary load of L-isoleucine.
Peaks 1 and 2, minor normal metabolites; 3, α-methyl-β-hydroxybutyrate; 4 and 5, α-methylacetoacetate. Peaks 4 and 5 represent T.M.S. derivatives of the cis and trans forms of the α-M.A.A. enol. Hatching indicates an abnormal amount of the substance.

3

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-methylaceto-acetate, 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-hydroxybutyr-ate 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 acetoacetate compound in our patient.

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Although a catabolic pathway for isoleucine has been described,[7] 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 the reaction in animal tissues,[7] 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.

REFERENCES

1. Jeune, M., Collombel, C., Michel, M., David, M., Guibaud, P., Guerrier, G., Albert, J. Annls Pédiat. 1970, 17, 85.
2. Dancis, J., Levitz, M., Westall, R. G. Pediatrics, Springfield, 1960, 25, 72.
3. Dancis, J., Hutzler, J., Rokkones, T. New Engl. J. Med. 1967, 276, 84.
4. Schulman, J. D., Lustberg, T. J., Kennedy, J. L., Museles, M., Seegmiller, J. E. Am. J. Med. 1970, 49, 118.
5. Scriver, C. R., Mackenzie, S., Clow, C. L., Delvin, E. Lancet, 1971, i, 310.
6. Mamer, O. A., Crawhall, J. C., Tjoa, C. S. Clinica chim. Acta, 1971, 32, 171.
7. Robinson, W. G., Bachhawat, B. N., Coon, M. J. J. biol. Chem. 1956, 218, 391.

AN INHERITED DISORDER OF ISOLEUCINE CATABOLISM

CAUSING ACCUMULATION OF α-METHYLACETOACETATE AND

α-METHYL-β-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 α-CH_3 acetoacetyl CoA is split into its products acetyl CoA and propionyl CoA. The block causes accumulation of α-CH_3 acetoacetic acid, its immediate precursor α-CH_3 β-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 bicar-
bonate and electrolyte solutions. The clinical picture
improved within two days. A diagnosis of metabolic acidosis
secondary to accidental ingestion of a poison was enter-
tained 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 signi-

ficant 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 meta-

bolic 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 preci-

pitated 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 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 elec-
tive 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[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 (Scriver et al, 1968[2]).

Ketone Analysis.

Urinary and plasma ketones were analysed as their 2,4-DNPH derivatives after the method of Menkes[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% CO_2 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-[14]C (final conc. 0.4 mM) using L-leucine-U-^{14}C and Sodium Succinate-U-^{14}C for control. The reaction was stopped by the addition of 50 µl H_2SO_4. CO_2 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 chromato-
graphy 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
($\alpha-CH_3-\beta-OH$-butyrate) was synthesized. Its mass spectrum
was identical to that of the unknown (Figure 2B). Further-
more, the synthetic compound cochromatographed with the
unknown further confirming its identification. $\alpha-CH_3-\beta-OH$
butyrate is a metabolite in the catabolic pathway of isoleucine.

Urine was also collected between episodes of metabolic
acidosis; all patients excreted $\alpha-CH_3-\beta-OH$ 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 α-methyl acetoacetic acid (α-CH$_3$ acetoacetate). This compound is apparently present in smaller quantities than α-CH$_3$-β-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 (α-keto-β-methyl valeric acid), its derivatives, or α-methylbutyric acid. β-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-CH_3-β-OH butyrate or α-CH_3 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

$[H^+]$ 44 nEq/L., pCO_2 31 mm Hg, $[HCO_3-]$ 18 mEq/L., Base Excess -7.1 mEq/L.

<u>FAMILY STUDIES</u>

<u>B pedigree</u>

Random daytime urine samples were collected from the brothers and parents of the propositus and were analysed for the presence of α-CH$_3$-β-OH-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 α-CH$_3$-β-OH-butyric acid. Normal subjects do not have this response. Neither the base line levels of excretion nor the increases after loading in the parents were as great as in the propositus.

<u>M pedigree</u>

A brother of the propositus excretes abnormal amounts of tiglylglycine, α-CH$_3$-β-OH-butyrate and α-CH$_3$-acetoacetate as well as butanone during acidosis. Two other sibs and both parents excrete a lesser excess of α-CH$_3$-β-OH-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 α-CH$_3$-β-OH-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}C to $^{14}CO_2$ 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 accumu-
lation 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-CH_3-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-CH_3-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 creati-
nine, 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 meta-
bolism. It is possible that in the presence of impaired meta-
bolism 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 $\alpha\text{-}CH_3\text{-}\beta\text{-}OH\text{-}$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-CH$_3$-acetoacetate and α-CH$_3$-β-OH-
butyrate favor the latter as the quantitatively major subs-
tance accumulating in the probands. The metabolic data
however favor an enzyme block at the thiolase step, converting
α-CH$_3$-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 situa-
tion 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-CH$_3$-aceto-
acetate 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 tiglyl-
glycine 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 bio-
chemical 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 consanguinous, 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

1. Mamer, O.A., Crawhall, J.C. and Tjoa, S.S.: The identi-
   fication of urinary acids by coupled gas chromatography-
   mass spectrometry. Clin. Chim. Acta 32:191 (1971).

2. Scriver, C.R. Davies, E., Lamm, P.: Accelerated selec-
   tive short column chromatography of neutral and acidic
   amino acids on a Beckman-Spinco Analyzer, modified for
   simultaneous analysis of two samples. Clin. Biochem.
   1:179 (1968).

3. Menkes, J.H.: Idiopathic hyperglycinemia: isolation and
   identification of three previously undescribed urinary
   ketones. J. Peds. 69:413 (1966).

4. Fomon, S.J.: Infant Nutrition. Philadelphia, W.B.
   Saunders Co. (1967).

5. Hillman, R.E., Feigin, R.D., Tenenbaum, S.M., Keating, J.P.:
   Defective isoleucine metabolism as a cause of the "ketotic
   hyperglycinemic" syndrome (Abstract). Ped. Res. 6: 395/
   135 (1972).

6. Gompertz, D., Draffan, G.H., Watts, J.L., Hull, D.:
   Biotin responsive β-methylcrotonylglycinuria. Lancet 2:22
   (1971).

7. Nyhan, W.L., Ando, T., Rasmussen, K., Nadlington, W.,
   Kilroy, A.W., Cottom, D., Hull, D.: Tiglicaciduria in
   propionicacidemia. Biochem. J. 126: 1035 (1972).

8. Robinson, W.G., Bachhawat, B.K., Coon, M.J.: Tiglyl
   coenzyme A and α-methylacetoacetyl coenzyme A, interme-
   diates in the enzymatic degradation of isoleucine.
   J. Biol. Chem. 218:391 (1956).

9. Lowry, O.H., Rosebrough, M.R., Farr, A.L. and Randall, R.J.:
   Protein measurement with the folin phenol reagent. J. Biol.
   Chem. 193:265 (1951).

TABLE III-I

BLOOD CHEMISTRIES IN PROPOSITUS OF B PELIGREE

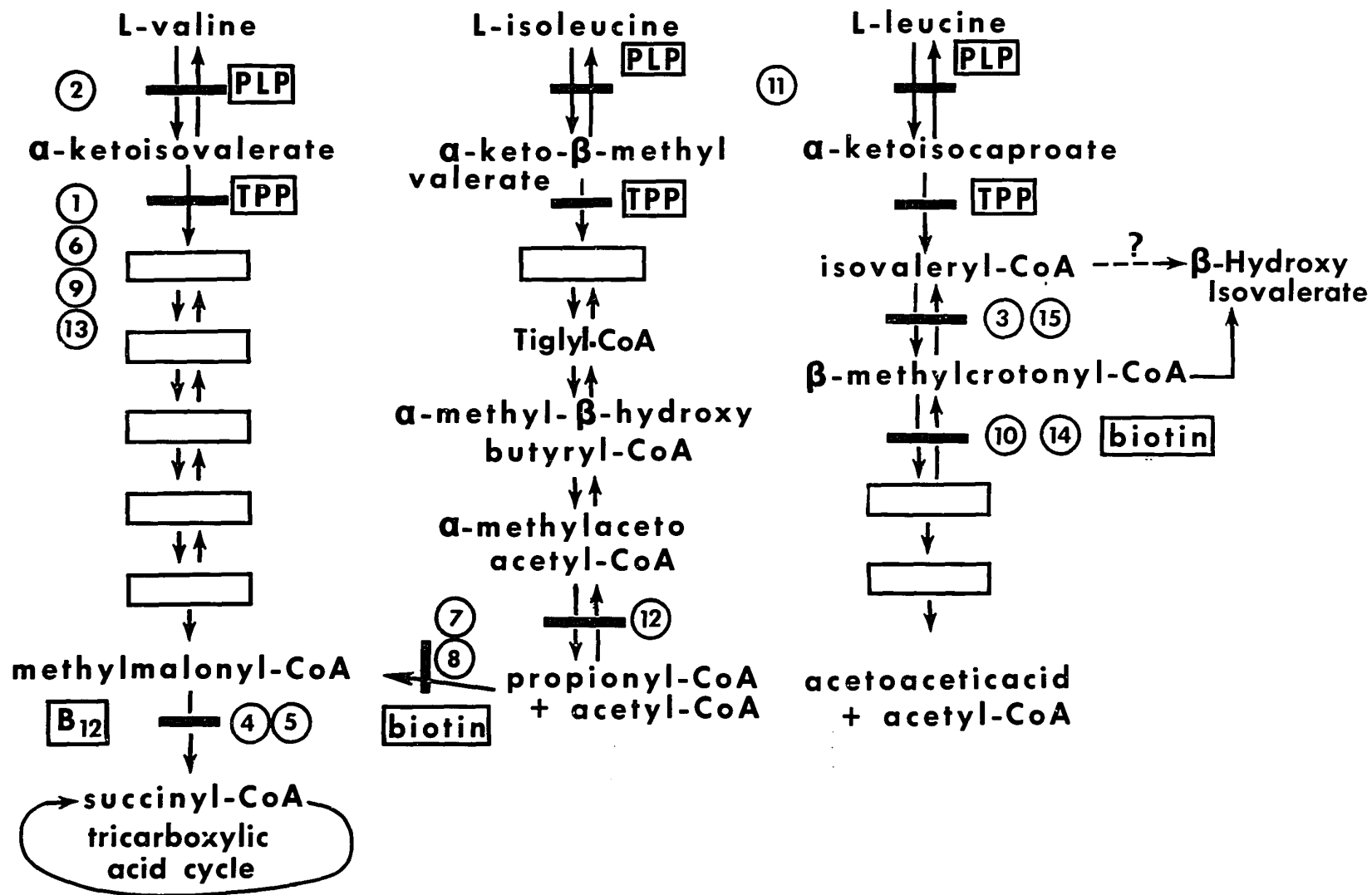| AGE	ADMISSION TEMPERATURE (°C)	pCO_2	pH	HCO_3-	CLINICAL STATE	CLINICAL DIAGNOSIS	COMPOUND
1 yr 5 mo.	38.3	28.2	6.99	8.0	Comatose	Acidosis Secondary to Poison Ingestion	?
2 yrs 10 mo.	38.9	23.1	7.33	14.8	Semi-Comatose	Acidosis Secondary to Phenol Poisoning	?
4 yrs	38.3	19	6.95	9.0	Comatose	Acidosis Secondary to Rubella	++++
4 yrs 10 mo.	38.3	25	7.33	19	Alert	Methyl-Malonic Aciduria	++++
4 yrs 10 mo.	38.9	31	7.36	18	Alert	Methyl-Malonic Aciduria	+++
5 yrs	37.2	40	7.44	26.4	Alert	New Disease	+

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 sympto-
matic management of subsequent episodes.

FIGURE III - 1

L-valine

② ⟶⟵ PLP

α-ketoisovalerate

① ⟶⟵ TPP

⑥
⑨
⑬

methylmalonyl-CoA

B₁₂ ── ④⑤

⟶ succinyl-CoA
tricarboxylic
acid cycle

L-isoleucine

⟶⟵ PLP

α-keto-β-methyl
valerate

⟶⟵ TPP

Tiglyl-CoA

α-methyl-β-hydroxy
butyryl-CoA

α-methylaceto
acetyl-CoA

⑦ ⟶⟵ ⑫
⑧

biotin

propionyl-CoA
+ acetyl-CoA

L-leucine

⟶⟵ PLP ⑪

α-ketoisocaproate

⟶⟵ TPP

isovaleryl-CoA ---?--→ β-Hydroxy
Isovalerate

③ ⑮

β-methylcrotonyl-CoA

⑩ ⑭ biotin

acetoaceticacid
+ acetyl-CoA

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 β-methyl-

crotonate have been recovered in

urine as their glycine conjugates,

tiglyl glycine and β-methylcrotonyl

glycine respectively. The presumed

direct conjugation reactions are not

shown. The reaction presumably

affected in the patients reported

here is #12.

FIGURE III – 2 a/b
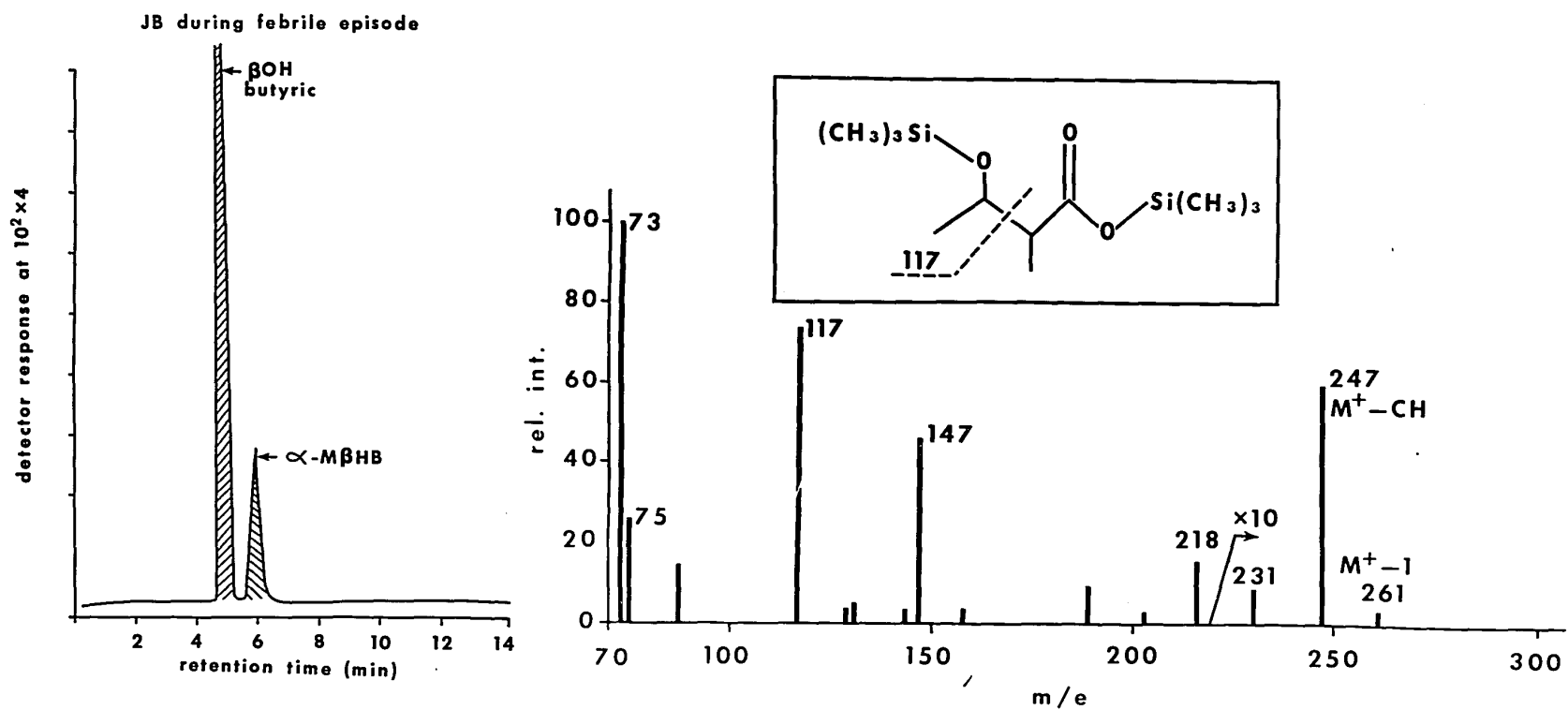
Figure III-2A: Gas-Liquid chromatogram of tri-
methyl-silylated (TMS) derivatives
in urine from proband B,III,2
during a febrile episode.
This chromatogram shows 2 significant
peaks. β-hydroxybutyric acid, which
is a "normal ketone body"; α-methyl-
β-hydroxybutyric acid (α-MβHB), the
abnormal metabolite. 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.

Figure III-2B: Mass spectrum of α-methyl-β-hydro-
xybutyric acid, bis-TMS derivative.
Significant ions are m/e 247, re-
presenting loss of a TMS methyl group
by the molecular ion (m/e 262), m/e
147 assigned to a rearrangement ion
characteristic of compounds incorpo-
rating 2 or more TMS groups, and m/e
117 indicative of hydroxyl substitu-
tion on the next-to-terminal carbon
atom.

FIGURE III - 3 a/b
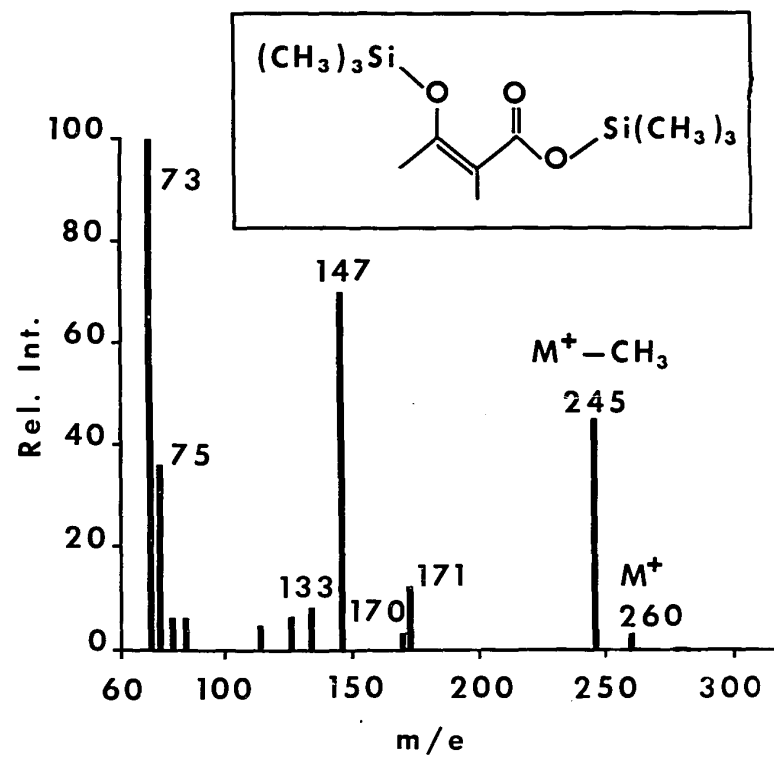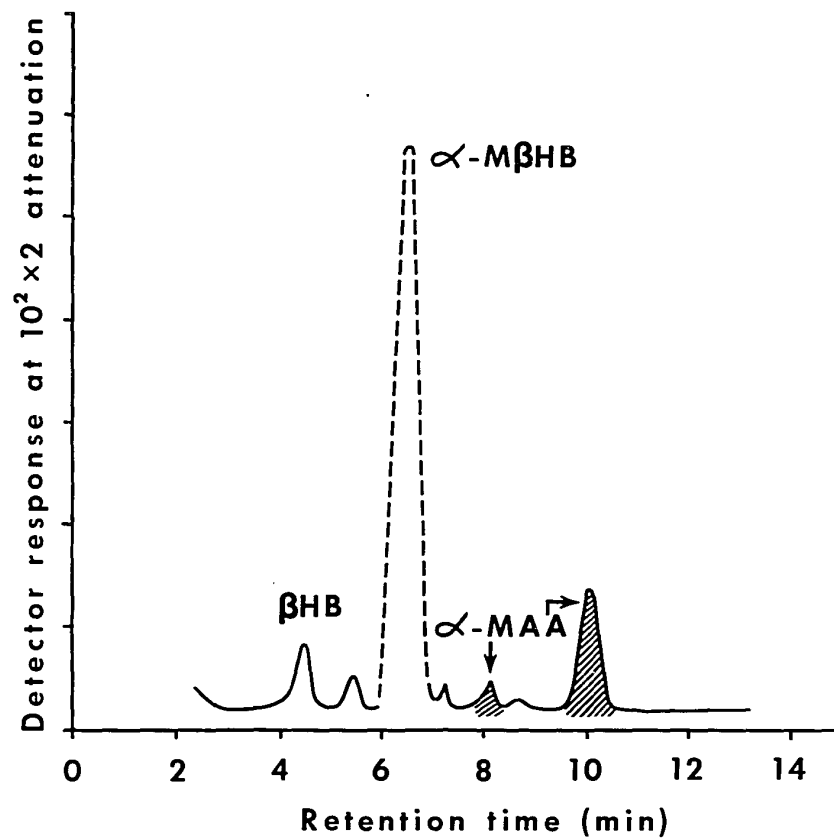
Figure III-3A: Gas-liquid chromatogram of TMS

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-methylaceto-
acetic 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 intra-
conversion involving the double bond
and the carbonyl group.

FIGURE III - 4
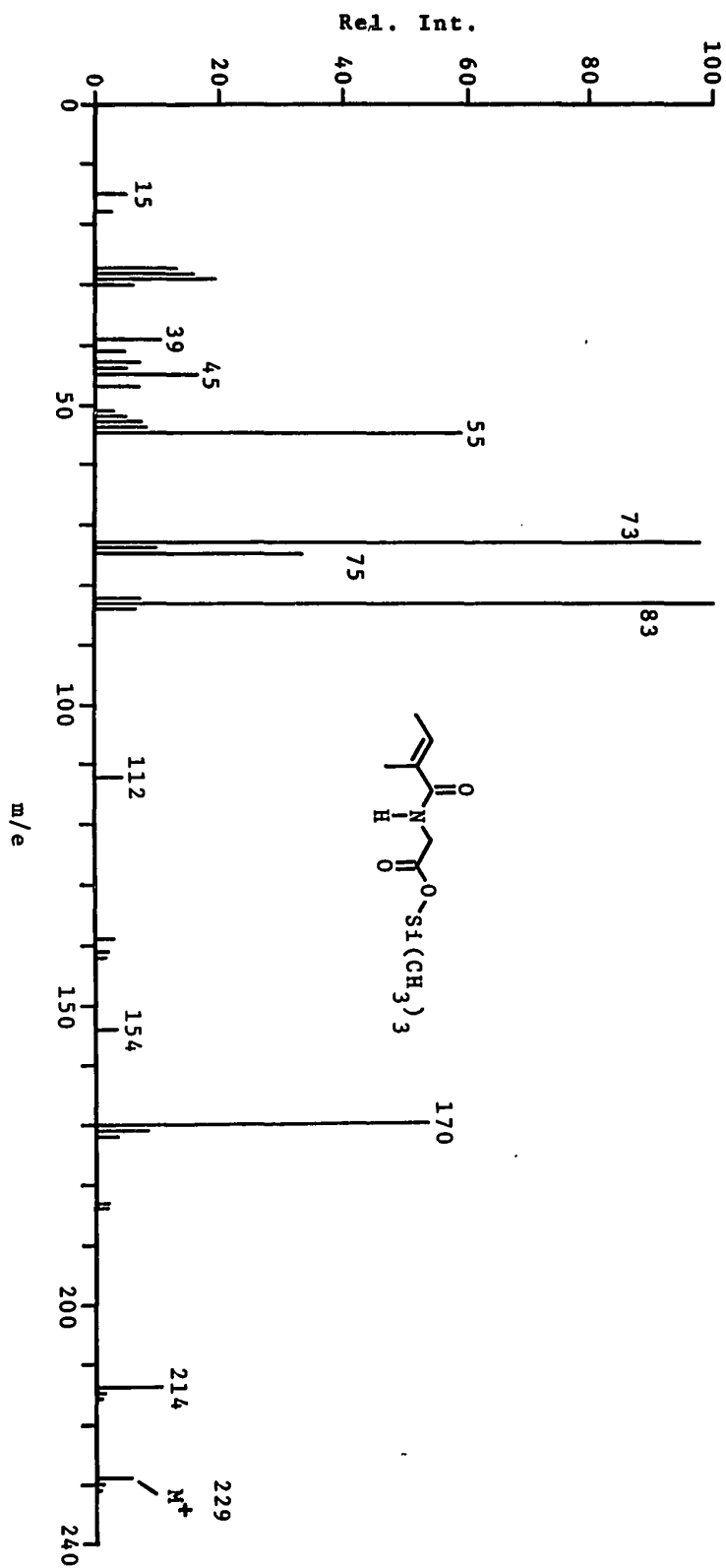
LEGEND

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 conjuga-

tion of free tiglic acid, a derivative

of isoleucine, with glycine. The peak

at m/e 83 represents the tiglyl frag-

ment and a molecular ion at m/e 229
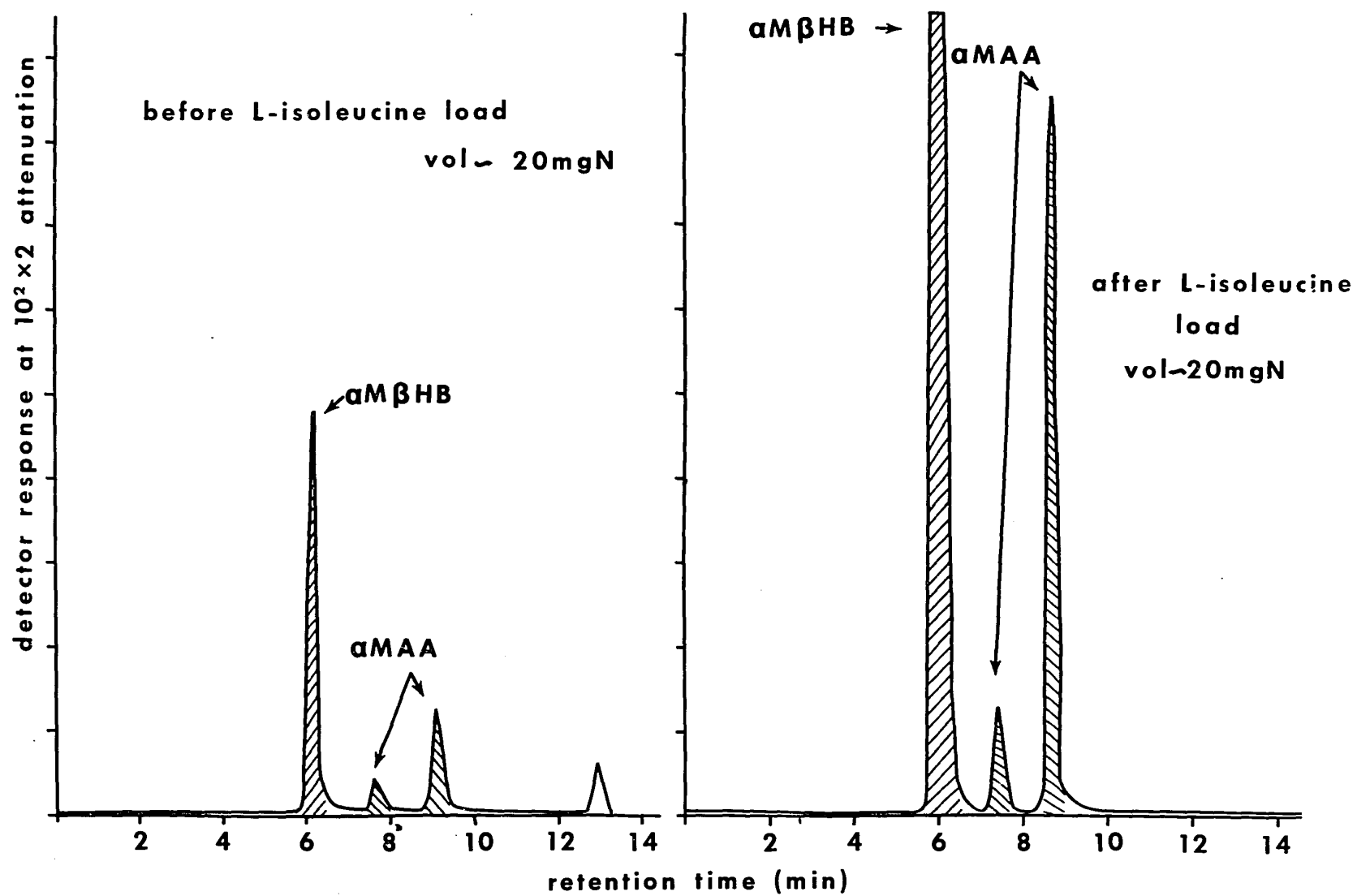
is evident.

FIGURE III-5

JB   MEK.   RM
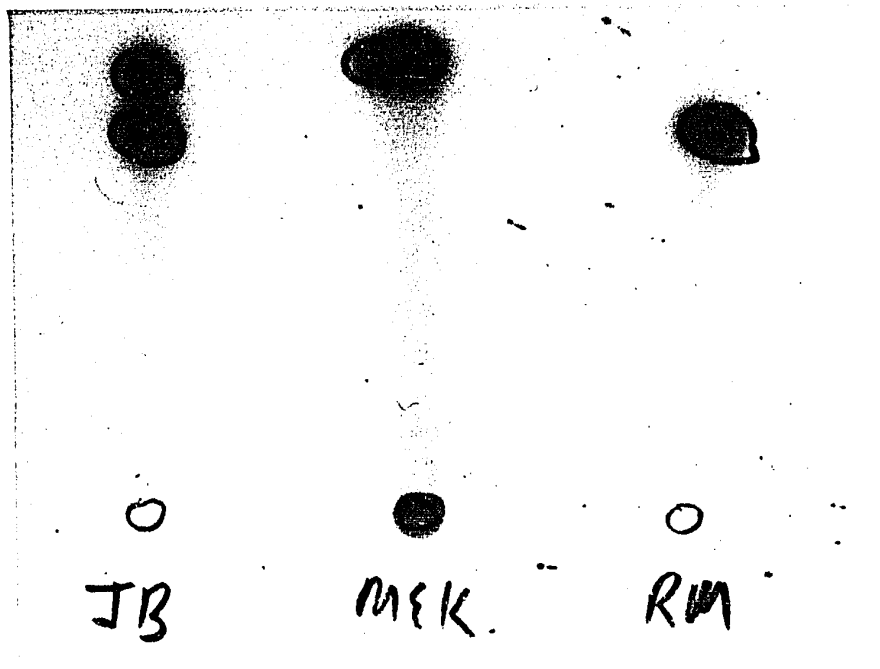
Figure III-5:   Thin-layer chromatogram on silica

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 stan-

dard.   The unknown substance in

the patient's urine also co-chroma-

tographed 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

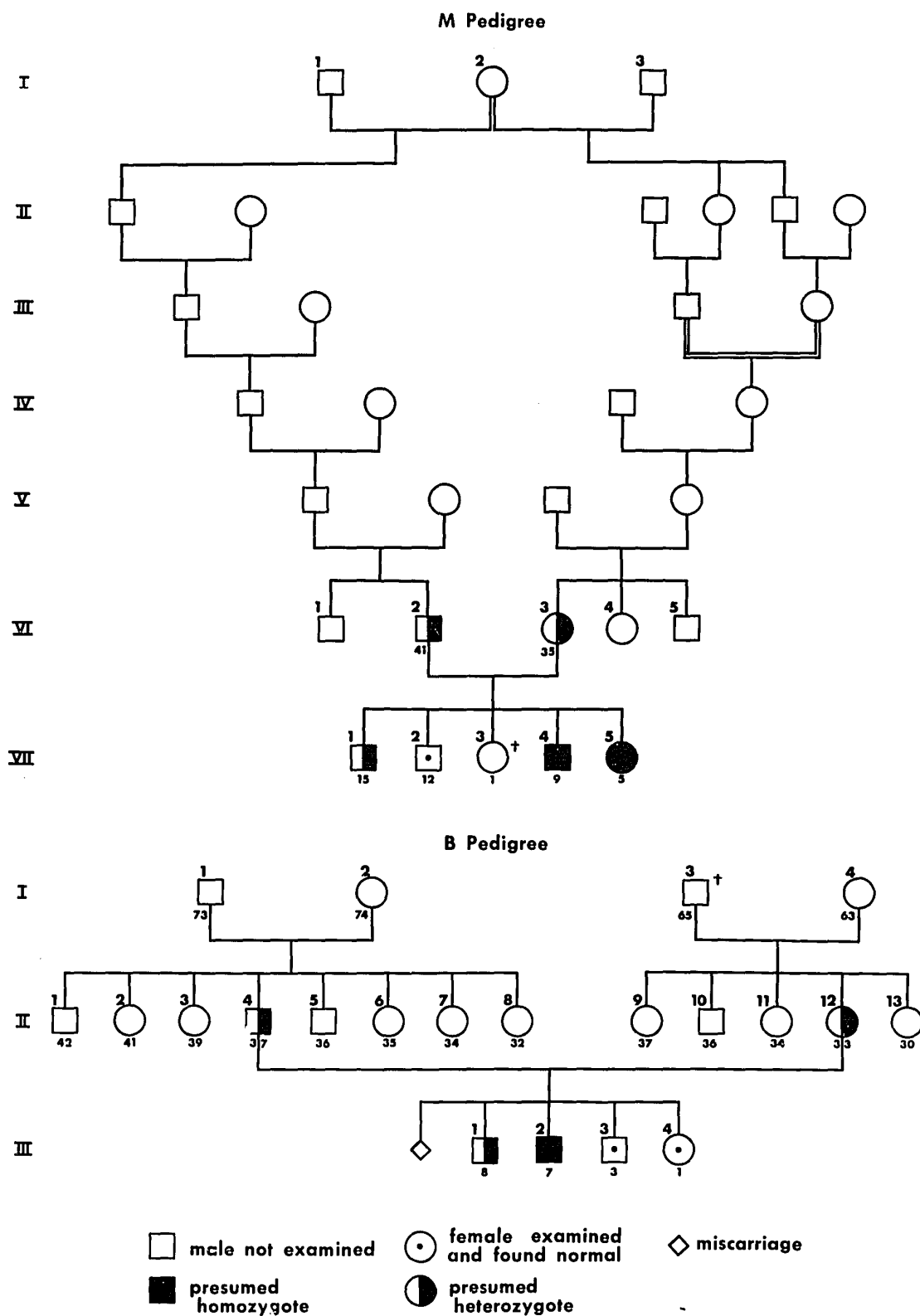exhibited similar characteristics.

FIGURE III – 6

detector response at $10^2 \times 2$ attenuation

before L-isoleucine load

vol ~ 20mgN

αMβHB

αMAA

αMβHB →

αMAA

after L-isoleucine

load

vol~20mgN

retention time (min)

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 III-II

METABOLISM OF ^{14}C LABELLED SUBSTRATE

	L-ileu (ul) (A)	L-ileu (ul) (B)	L-leu (ul)	Succinate (1,4-^{14}C)
Distribution of ^{14}C in,				
CO_2	44	46	113	106
Sol. extract	89	131	170	126
Protein	127	94	94	52

TABLE III-II

Metabolism of ^{14}C Labelled Substrate.

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 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 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.