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GRADUATE COLLEGE

CATABOLISM OF ISOLEUCINE BY *PSEUDOMONAS PUTIDA*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

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BY

ROBERT STANLEY CONRAD

Oklahoma City, Oklahoma

1972

CATABOLISM OF ISOLEUCINE

BY PSEUDOMONAS PUTIDA

APPROVED BY

John R. Sokatch

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DISSERTATION COMMITTEE

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LIST OF ABBREVIATIONS

ATP	- adenosine triphosphate
C	- centigrade
CoA, CoASH	- coenzyme A
2,6-DCPIP	- 2,6-dichlorophenolindophenol
2,4-DNP	- 2,4-dinitrophenylhydrazine
g	- gram or gravity
L	- liter
µg	- microgram
mg	- milligram
ml	- milliliter
mM	- millimolar
n	- nano
NAD	- nicotinamide adenine dinucleotide (oxidized)
NADH	- nicotinamide adenine dinucleotide (reduced)
Tris	- tris(hydroxymethyl)aminomethane

THE CATABOLISM OF ISOLEUCINE IN *PSEUDOMONAS PUTIDA*

CHAPTER I

INTRODUCTION

Background

The inability to catabolize branched-chain amino acids became a matter of clinical concern after Menkes *et al.* showed the relationship between this deficiency and progressive familial infantile cerebral dysfunction (23). The so-called "Maple Syrup Urine Disease" that he described is a devastating disease in children. The normal prognosis includes intense multiple neurological manifestations and early death. The characteristic maple syrup odor present in the urine of afflicted children is due to the increased excretion of branched-chain amino acids and their corresponding keto acids. Connelly *et al.* demonstrated that bovine liver tissues normally oxidatively decarboxylated the keto acids of isoleucine, and leucine by means of α-keto-isocaproic; α-keto-β-methylvaleric acid dehydrogenase (3, 5). The keto acid of valine, 2-ketoisovalerate, was metabolized by a separate enzyme. A block in these enzymes could therefore produce elevated cellular levels of branched-chain amino acids and their keto acid analogues. Schulman *et al.* (32) and Goedde *et al.* (11) reported that cultured leukocytes and fibroblasts from maple syrup urine diseased

patients had a deficiency in branched-chain keto acid dehydrogenase activity that was consistent with clinical manifestations. Goedde et al. measured enzyme activity by incubating labelled ketoacids with enzyme. The labelled CO_2 liberated was absorbed on filter paper saturated with KOH and then quantitatively estimated in a liquid scintillation spectrometer. He postulated that children completely lacking decarboxylase activity were homozygous for the mutated allele of the autosomal gene which normally controls the synthesis of branched chain keto acid dehydrogenase. According to his hypothesis heterozygotes would have an intermediate decarboxylase capability and corresponding mild form of disease expression.

Other branched-chain amino acid metabolites have been implicated in related metabolic disorders. Stokke et al. described one patient deficient in methylcrotonyl-CoA carboxylase activity (a leucine catabolic enzyme) (38). Daum et al. found 2-methyl-3-hydroxybutyrate and 2-methylacetoacetate in the urine of one patient suffering from a mild form of maple syrup urine disease (8). Since both of these metabolites are proposed intermediates of isoleucine catabolism past the branched-chain keto acid dehydrogenase step, it is quite likely that genetic lesions in catabolic enzymes specific for isoleucine are responsible for metabolic diseases.

The proposed enzymatic reactions by which P. putida catabolizes isoleucine to acetyl-CoA and propionyl-CoA is shown in Figure 1. Some of these reactions have been previously demonstrated in Pseudomonas. Norton and Sokatch found that L-isoleucine and 2-ketoglutarate were transaminated by branched-chain amino acid transaminase

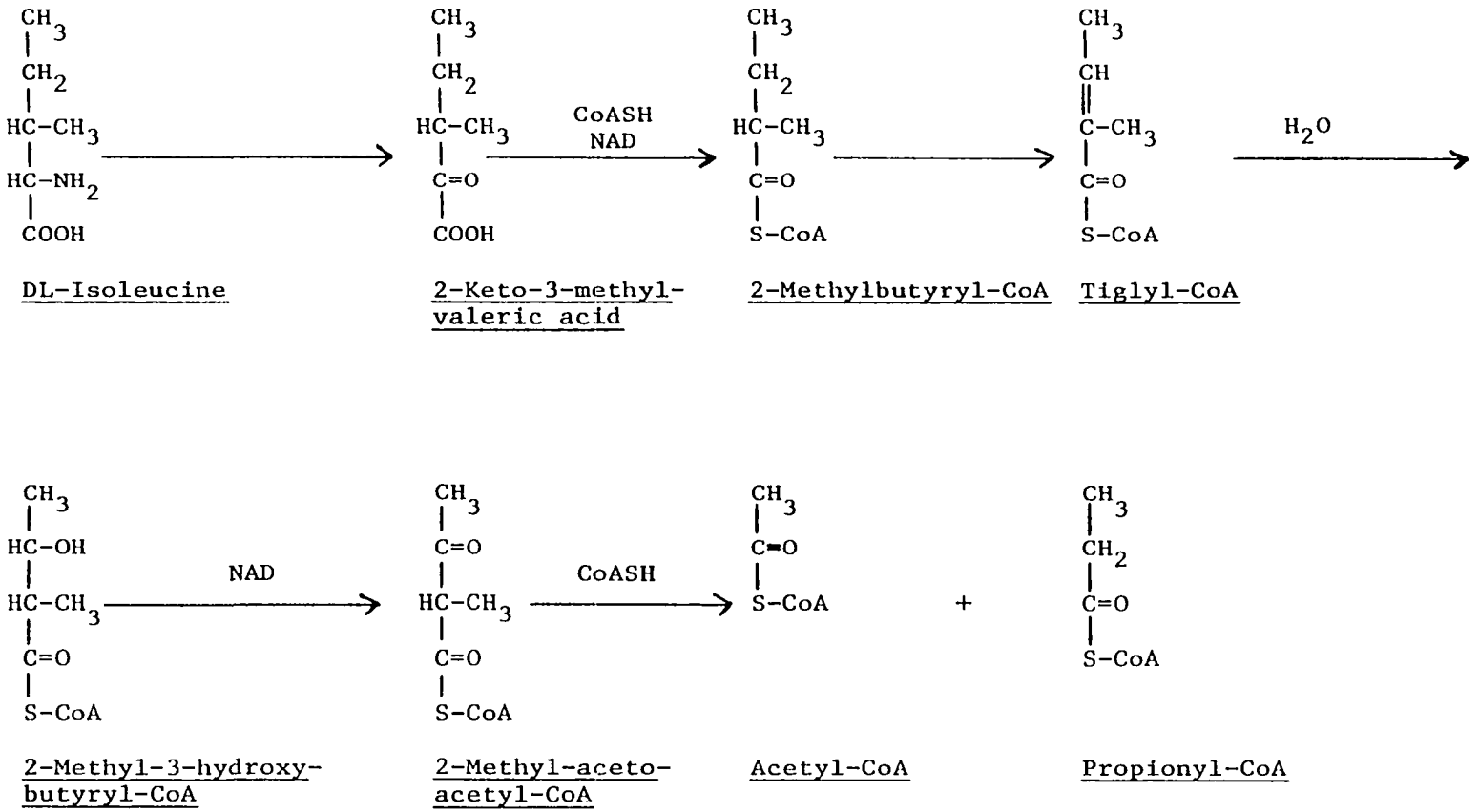

Figure 1. Proposed pathway for isoleucine catabolism in Pseudomonas putida.

(25). Marshall and Sokatch showed that D-isoleucine was deaminated by D-amino acid dehydrogenase (19). Marshall and Sokatch also found that 2-keto-3-methylvalerate was oxidatively decarboxylated to 2-methylbutyryl-CoA by branched-chain keto acid dehydrogenase (20). Branched-chain amino acid transaminase and D-amino acid dehydrogenase catalyze similar reactions on analagous metabolites of isoleucine, valine and leucine.

The catabolism of isoleucine beyond 2-methylbutyrate has been studied only in mammalian tissues. Coon and his associates used 2-methylbutyrate labelled with C^{14} to demonstrate that 2-methylbutyrate undergoes beta-oxidation on the longer carbon chain and cleavage to two and three carbon fragments (6,7). The "acetate" fragment came from carbon 3 and 4 of 2-methylbutyrate. The three carbon acid fragment came from carbons 1, 2 and α-methyl of 2-methylbutyrate. Coon et al. proposed a catabolic pathway for 2-methylbutyrate based on the distribution of radioactivity and the determination of metabolic end products. His studies did not include characterizing the individual enzymes responsible for this metabolic scheme.

Valine and Leucine Catabolism in Pseudomonas

The valine catabolic pathway is shown in Fig. 2. The following enzymes from Pseudomonas have been purified and partially characterized: branched-chain amino acid transaminase, (25), 3-hydroxyisobutyrate dehydrogenase, (Bannerjee, D., personal communication), and methylmalonate semialdehyde dehydrogenase (1).

Marshall and Sokatch demonstrated the presence of branched-chain keto acid dehydrogenase, isobutyryl-CoA dehydrogenase, and

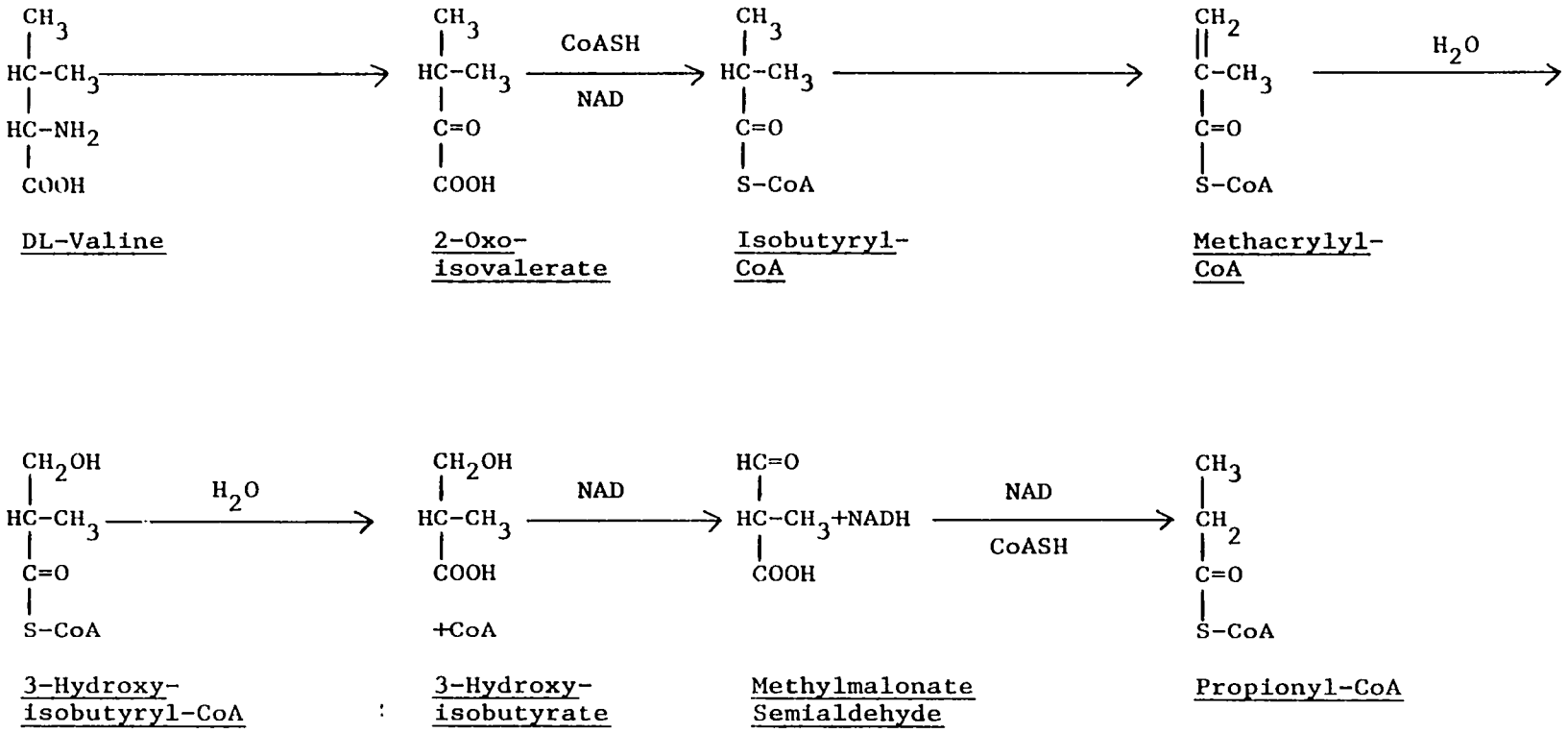

Figure 2. The pathway of valine catabolism in Pseudomonas.

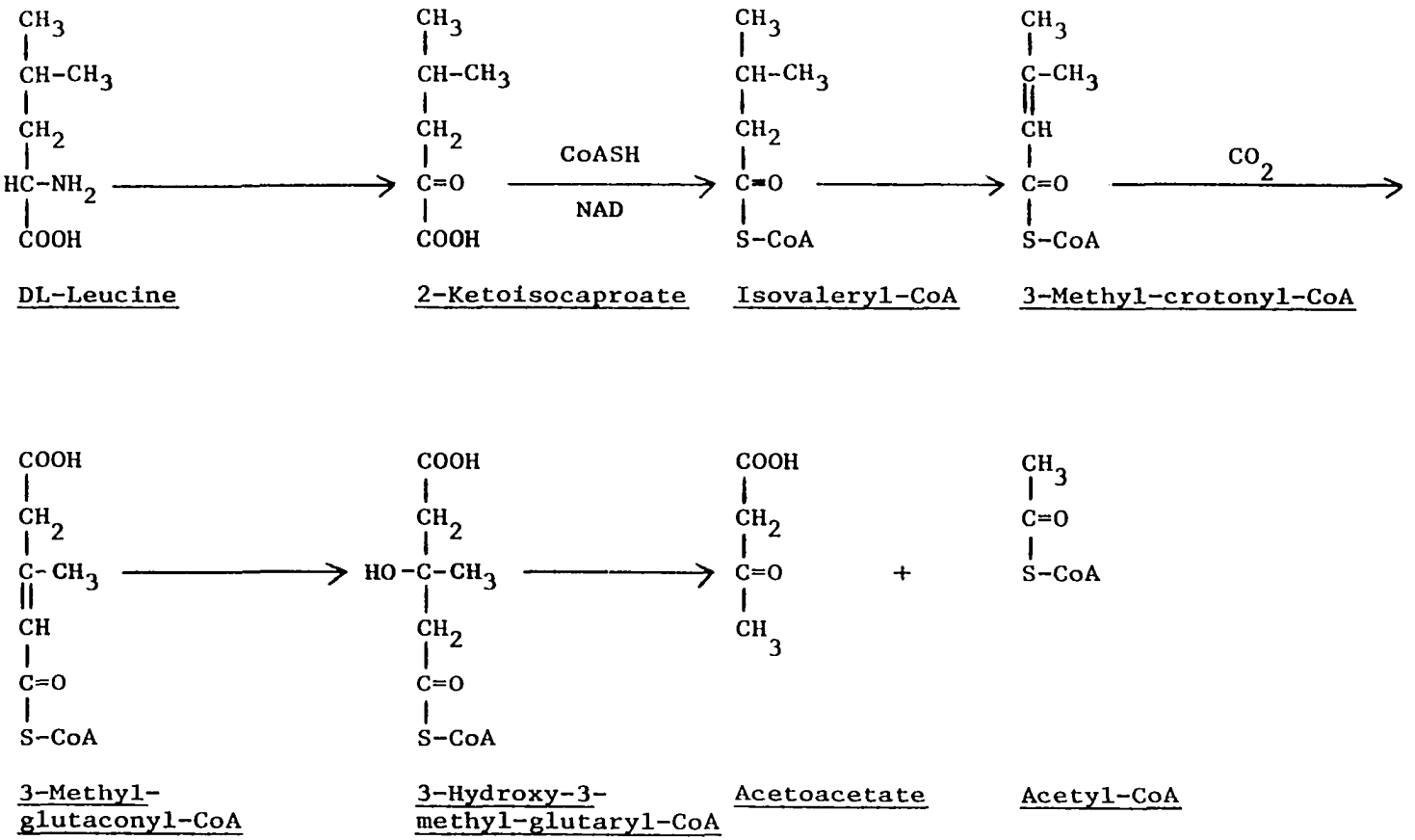

Figure 3. Proposed pathway for leucine catabolism in Pseudomonas putida.

methylacryl-Co hydrase in crude extracts of P. putida (20). Marshall found three separate inductive processes were involved in the valine catabolic pathway. D-amino acid dehydrogenase and branched-chain keto dehydrogenase were induced separately. 3-Hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase were induced coordinately.

The proposed pathway for the catabolism of leucine in P. putida is shown in Figure 3. Leucine is catabolized to isovaleryl-CoA by enzymes common to the catabolism of valine and isoleucine (20, 21). Lynen et al. purified bacterial enzymes responsible for converting 3-methyl-crotonyl-CoA to 3-hydroxy-3-methyl-glutaryl-CoA (18). Stegink and Coon enriched an enzyme from bovine liver that cleaved 3-hydroxyl-3-methyl-glutaryl-CoA to acetyl-CoA and acetoacetate (36). Massey (personal communication) has found that P. putida uses the same pathway to catabolize leucine.

Objective of Research

The purpose of this investigation was to elucidate the pathway used by P. putida to catabolize isoleucine. Isoleucine is catabolized to 2-methylbutyryl-CoA by enzymes common also to the catabolism of valine and leucine (20, 21). It was necessary to demonstrate that the following metabolic reactions took place in P. putida: dehydrogenation of 2-methylbutyryl-CoA, the hydration of tiglyl-CoA, dehydrogenation of 2-methyl-3-hydroxybutyryl-CoA, and the cleavage of 2-methyl-acetoacetyl-CoA.

The selection of P. putida as the experimental organism was based on several factors. Other workers in this laboratory have used

P. putida to study the catabolism of valine and leucine. Using the same species facilitates a comparison of common or similar enzymes in related pathways. The genetics of P. putida have been reasonably well studied which would be advantageous in future regulatory studies. P. putida possesses extraordinary nutritional versatility which makes it possible to study a wealth of catabolic pathways within a single bacterium.

Growth studies were conducted to correlate cell yields with potential energy yields that would be derived from the proposed catabolic pathways of isoleucine, valine, and leucine. Growth rates were used as an indicator of the interaction and antagonism existing between catabolic and anabolic pathways of branched-chain amino acids.

Despite the occurrence of a metabolic disorder, Maple Syrup Urine Disease, the catabolism of isoleucine has been mostly neglected prior to the present study. The use of P. putida as a metabolic model may lead to a greater understanding of the disease process in infants. The elucidation of isoleucine catabolism may therefore yield information directly pertinent to clinical medicine, as well as increasing our basic knowledge of amino acid catabolism.

CHAPTER II

MATERIALS AND METHODS

Culture Methods

Organisms

Pseudomonas putida, PpG2 (ATCC 23287), the species used throughout this investigation, was obtained from Dr. I. C. Gunsalus at the University of Illinois, Department of Biochemistry.

Stock cultures were maintained on 2% tryptose phosphate agar at 4 C until needed. Transfers were made at 4 to 5 weeks intervals.

Media

The basal medium used in this study was previously described by Jacobson (14). The components of this medium included:

Basal G Solution

K_2HPO_4	43.5 g
KH_2PO_4	17.0 g
NH_4Cl	21.4 g
H_2O	to 1 liter

Salts "S" Solution

$MgSO_4 \cdot 7\ H_2O$	39.44 g
$MnSO_4 \cdot 2\ H_2O$	5.58 g

$FeSO_4 \cdot 7\ H_2O$	1.11 g
$NaMoO_4 \cdot 2\ H_2O$	0.48 g
$CaCl_2$	0.33 g
NaCl	0.12 g

One liter of complete medium contained: "Basal G", 100 ml; "Salts S", 10 ml; appropriate amounts of carbon source; distilled water added to bring the final volume to 1 liter. Sterilization of the medium was accomplished by autoclaving without "Salts S" at 121 C for 15 min. Stock solutions of "Salts S" were titrated to pH 1-2 and autoclaved separately. "Salts S" was added to the medium after both had sufficiently cooled to prevent precipitation. Final pH of the complete medium was 6.7-6.9. Organic acids were titrated to neutrality prior to addition to growth medium and were autoclaved together with Basal G and water.

Growth of P. putida

A loopfull of cells from stock culture slants of P. putida was inoculated into 50 ml of 0.3% L-glutamate medium and shaken for 8-10 hours at 30 C. This culture served as the inoculum for other media at the ratio of 1-2 ml of the glutamate grown cells per 100 ml of fresh medium.

Cells used for the preparation of cell free extracts were harvested at late exponential phase. Aeration was provided by a New Brunswick gyrotory water bath shaker (model G 76) maintained at 30 C. Side arm flasks with 50 ml of medium were used to determine growth rates and cell yields. Optical density was read at 660 nm in a Bausch and Lomb Spectronic 20. The proportionality between optical density and

dry weight of bacteria was linear up to 0.70 optical density units, with .01 optical density units being equal to 5.25 μg dry weight of bacteria. Growth rates were calculated by the method of Sokatch (34). The exponential growth rate, R, was defined as the number of generations per unit time and was calculated by the formula:

$$R = \frac{\log_2 N_2 - \log_2 N_1}{t_2 - t_1}$$

The reciprocal of R is the generation time, G, calculated by:

$$G = \frac{1}{R}$$

Preparation of Extracts

Cells were harvested with a Servall RC-2 refrigerated centrifuge at 10^4 x g for 15 min, washed once with cold 0.05 M potassium phosphate buffer, pH 7.5, and resuspended in the same buffer. The cell suspension was chilled in an ice bath and cell free extracts were prepared by sonic oscillation (Branson Sonic Power Co., model 140) at a power setting of 4 for 90 to 120 seconds. Cellular debris was removed by centrifugation of 10^4 x g for 15 min at 4 C. Protein concentration was determined by the method of Warburg and Christian (41).

Synthesis of Substrates and Standards

2-Methyl-3-hydroxybutyrate was synthesized by the method of Blaise and Herman (2). Purity was confirmed by quantitative organic microanalysis performed by Galbraith Laboratories. 3-Hydroxyisobutyrate was synthesized according to the method of Kupiecke and Coon (15). All coenzyme A derivatives were synthesized by methods described by Stadtman (35), which were based on the procedures of Simon and Shemin

(33). These coenzyme A derivatives were lyophilized to remove ethyl ether which caused the non-enzymic reduction of NAD in the presence of cell free extract. The concentration of coenzyme A derivatives was determined by the method of Lipmann and Tuttle (17). The spontaneous hydration of crotonyl-CoA in the presence of glycine buffer had been previously reported by Stern et al. (37).

2,4-Dinitrophenylhydrazone standards were made by a procedure developed during this investigation. The appropriate ketone or aldehyde was added directly to acidic 2,4-dinitrophenylhydrazine solution (15 mM in HCl). The precipitated hydrazones were selectively extracted by ethyl acetate. Hydrazones were dried under vacuum at 45 C. Recrystallization was accomplished in ethyl acetate by the same procedure.

Assay of Enzymic Activity

All enzymes were assayed spectrophotometrically in a Beckman model DU spectrophotometer equipped with a Gilford model 2000 multiple absorbance recorder. The spectrophotometer had thermospacers through which water at 30 C was continuously circulated. All assays were performed at 30 C except transaminase which was at room temperature.

Assays of NAD-linked enzymes were read at 340 nm. The μmolar extinction coefficient of 6.22 was used to calculate the amount of NADH produced (30).

Enzyme assays based on the reduction of 2,6-dichlorophenolindophenol were read at 600 nm. The μmolar extinction coeffient of 21 was used to calculate the amount of dye reduced (37).

D-Amino Acid Dehydrogenase

(EC·1·4·3·3)

D-amino acid dehydrogenase was assayed by the method of Norton and Sokatch (24). The assay mixture contained 50 µmoles of amino acid, extract, 300 µmoles of tris buffer, pH 7.5, 20 µmoles of NaCN, 40 µg of 2,6-dichlorophenolindophenol and water to 3 ml. Reaction was initiated by the addition of substrate. The control cuvette contained all reagents except amino acid. One unit of enzyme activity was that amount of enzyme which caused the reduction of 1 nmole of 2,6-DCPIP per min.

Branched-Chain Amino Acid Transaminase

(EC 2·6·1·6)

Branched-chain amino acid transaminase was measured by the assay of Taylor and Jenkins (39). The standard assay contained 20 µmoles of L-amino acid, 20 µmoles of 2-ketoglutarate (pH 7.0), 100 µmoles of potassium phosphate buffer, pH 8.5 and water to 3.0 ml. The control test tube contained all reagents except amino acid. The assay mixture was equilibrated for 5 min and the reaction was initiated by the addition of extract. After 10 min the reaction was terminated by 1.0 ml of 2,4 dinitrophenylhydrazine reagent. Dinitrophenylhydrazone formation continued for 10 min before the keto acid hydrazones were selectively extracted by ethyl acetate. All extractions were performed with a Vortex test tube mixer. The biphasic mixture was centrifuged and 3.5 ml of the organic phase was transferred to another tube. It was then extracted with 2 ml carbonate solution containing per liter: 5.3 g anhydrous $NaCO_3$, 0.83 g $NAHCO_3$ and 200 g Na_2SO_4. Three ml of

the organic phase were transferred to another tube and 5 ml of petroleum ether and 1.5 ml of 0.1 n Na_2CO_3-0.01 N $NaHCO_3$ were added. One ml of the aqueous phase was removed and added to 2 ml 1N NaOH in a 3 ml cuvette. Color change was read at 440 nm in the DU Spectrophotometer. The control tube minus amino acid was used as the blank.

Under these conditions, 1 µmole of 2-keto-3-methylvaleric acid equaled 0.94 optical density units. One unit of transaminase activity was equal to 1 nmole of 2-keto-3-methylvaleric acid produced per min. Assays for L-leucine transaminase activity differed in that the first extraction utilized cyclohexane rather than ethyl acetate.

Branched-Chain Keto Acid Dehydrogenase

Oxidation of branched-chain keto acids was assayed by the method of Marshall and Sokatch (20). Enzyme activity was measured by NADH production. The standard assay contained 10 µmoles of keto acid, 100 µmoles of phosphate buffer, pH 8.0, 5 µmoles of NAD, 1 µmole of CoA, 10 µmoles of dithiothreitol, extract, and water to 1 ml. The control cuvette contained all reagents except keto acid. Reaction mixtures were equilibrated at 30 C and the reaction initiated by adding extract to both cuvettes. One unit of enzyme activity was that amount of enzyme which caused the production of 1 nmole of NADH per min.

3-Hydroxyisobutyrate Dehydrogenase

(EC 1·1·1·31)

The oxidation of 3-hydroxyisobutyrate was assayed spectrophotometrically by the method of Robinson and Coon (30). The assay

system contained per ml: 100 µmoles of tris buffer, pH 9.2, 10 µmoles of 3-hydroxyisobutyrate, 1 µmole of NAD, extract, and water. The reaction was initiated by the addition of substrate. The control cuvette contained all reagents except substrate. One unit of enzyme activity was defined as 1 nmole of NADH formed per min.

2-Methylbutyryl-CoA Dehydrogenase

The oxidation of 2-methylbutyryl-CoA was assayed by a modification of a method developed by Green et al. for assay of butyryl-CoA dehydrogenase (12). Enzyme activity was followed by measuring the reduction of 2,6-DCPIP. The standard assay mixture contained 100 µmoles of tris buffer, pH 7.4, 20 µg of 2,6-DCPIP, extract, 0.40 µmole of 2-methylbutyryl-CoA, and water to 1 ml. Reaction was initiated by the addition of substrate. The control cuvette contained all reagents except 2-methylbutyryl-CoA. One unit of enzyme caused the reduction of 1 nmole 2,6-DCPIP per min.

2-Methyl-3-Hydroxybutyryl-CoA Dehydrogenase

The assay to detect the oxidation of 2-methyl-3-hydroxybutyryl-CoA was developed during the course of this investigation. Enzyme activity was measured spectrophotometrically by following the production of NADH. The standard assay contained 100 µmoles glycine buffer, pH 9.5, 5 µmoles of NAD, 0.40 µmoles of 2-methyl-3-hydroxybutyryl-CoA, extract, and water to 1 ml. The control cuvette contained all reagents except substrate. The reaction was initiated by the addition of substrate. One unit of enzyme activity was defined as the amount of enzyme which caused the production of 1 nmole NADH per min.

Tiglyl-CoA Hydrase

The assay to measure activity of tiglyl-CoA hydrase also was developed during the course of this investigation. The assay was based on the appearance of 2-methyl-3-hydroxybutyryl-CoA, the hydration product of tiglyl-CoA, which was oxidized to 2-methylacetoacetyl-CoA by 2-methyl-3-hydroxybutyryl-CoA dehydrogenase. The simultaneous induction of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and tiglyl-CoA hydrase permitted an approximation of tiglyl-CoA hydrase activity in crude extracts without addition of other enzymes. However, the reaction rate of enzyme activity of tiglyl-CoA hydrase in crude extracts was limited by the quantity of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase present in the same extract.

The purification of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase made it possible to measure tiglyl-CoA hydrase activity without this rate-limiting feature. The standard assay contained 100 μmoles of glycine buffer, pH 9.5, 5 μmoles of NAD, 0.40 μmole tiglyl-CoA, enzymes (0.20 unit 2-methyl-3-hydroxybutyryl-CoA dehydrogenase added if indicated), and water to 1 ml. The control cuvette contained all reagents except tiglyl-CoA. The assay mixture was equilibrated and the reaction was initiated by the addition of tiglyl-CoA. One unit of enzyme activity was defined as that amount of enzyme which caused the production of 1 nmole NADH per min.

2-Methylacetoacetyl-CoA Cleavage Enzyme

Activity of 3-methylacetoacetyl-CoA cleavage enzyme was measured by a modification of the methods of Stegink and Coon (36)

and Ochoa et al. (26). Acetyl-CoA resulting from the cleavage of 2-methylacetoacetyl-CoA was measured by incorporation into citrate in the coupled enzyme assay. The standard assay contained 200 µmoles tris buffer, pH 8.1, 10 µmoles $MgCl_2$, 5 µmoles dithiothreitol, 1 µmole coenzyme A, 2.5 µmoles NAD, 2.5 µmoles L-malate, enzyme, 0.6 unit malate dehydrogenase, 0.9 unit citrate synthetase, 0.15 µmole 2-methyl-3-hydroxybutyryl-CoA, and water to 1 ml. The control cuvette contained all reagents except substrate. The assay mixture was equilibrated and the reaction initiated by the addition of substrate. The assay proceeded in two phases. In the first phase, 2-methylacetoacetyl-CoA was generated enzymatically by the oxidation of 2-methyl-3-hydroxybutyryl-CoA and cleaved to acetyl-CoA and propionyl-CoA by enzymes of the crude extract. The second phase of the assay measured acetyl-CoA production and was initiated by the simultaneous addition of malate dehydrogenase and citrate synthetase. In Figure 4 is shown a schematic representation of the spectrophotometry involved. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 nmole of NADH per minute after the addition of citrate synthetase and malate dehydrogenase.

3-Hydroxy-3-Methylglutaryl Coenzyme A Cleavage Enzyme

3-Hydroxy-3-methylglutaryl-CoA cleavage enzyme was measured spectrophotometrically by the method of Stegink and Coon (36). Enzyme activity was assayed by NADH production resulting from acetyl-CoA incorporation into citrate by a coupled enzyme assay. The standard assay contained: 200 µmoles tris buffer, pH 8.1, 10 µmoles $MgCl_2$, 2.5 µmoles dithiothreitol, 2.5 µmoles L-malate, 1.4 µmoles NAD, 0.05 mole NADH,

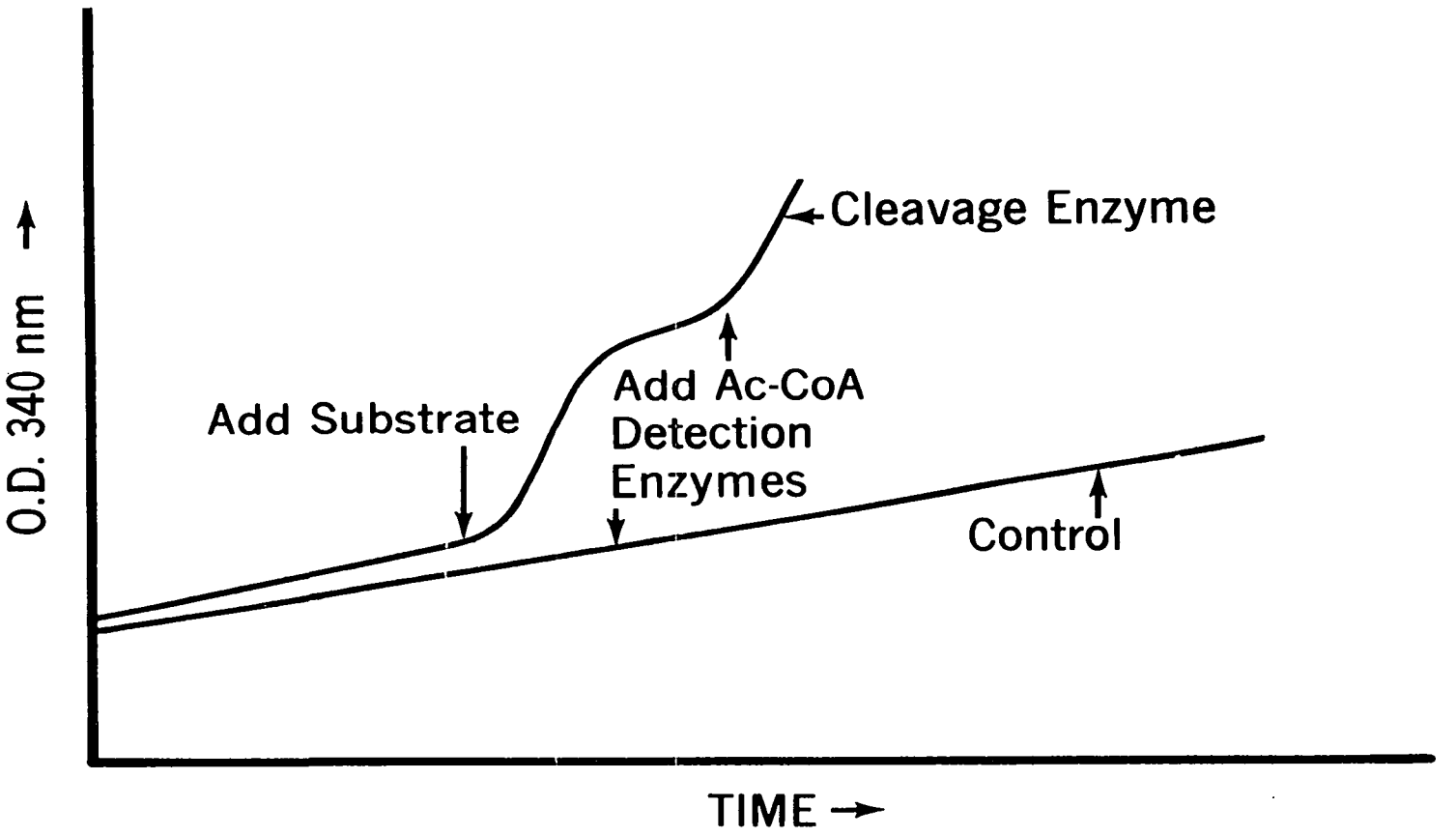

Figure 4. Schematic representation of 2-methylacetoacetyl-CoA cleavage enzyme assay based on the reduction of NAD.

0.6 unit malate dehydrogenase, 0.9 unit citrate synthetase, 0.20 μmole 3-hydroxy-3-methylglutaryl-CoA, extract, and water to 1.0 ml. The reaction was initiated by the addition of substrate after equilibration of other components. Substrate was omitted from the control cuvette. One unit of enzyme activity was defined as 1 nmole of NADH formed per min.

Purification of 2-Methyl-3-Hydroxybutyryl-CoA Dehydrogenase and Tiglyl-CoA Hydrase

Cell free extracts of P. putida grown on 0.3% DL-isoleucine were prepared from 30 g of frozen cells suspended in 30 ml of 0.05 M potassium phosphate buffer, pH 7.5. The cell suspension was sonicated in 10 ml aliquots for 150 sec. Cellular debris was removed from the combined extracts by centrifugation at 10^4 x g for 30 min. Methods of determining protein concentration and enzyme activity have been previously described.

Protein insoluble at 35 to 60% ammonium sulfate saturation was removed by centrifugation at 4 x 10^4 g for 40 min. Calculations from the data of Brenner-Holzach and Staehelin were used to determine the amount of ammonium sulfate to be added (4).

$$g(NH_4)_2\ SO_4 = \frac{(1.77)\ (\text{ml of solution})\ (\text{Sat.2-Sat.1})}{3.54\text{-Sat.2}}$$

(Sat. = Saturation)

The white precipitate was dissolved in 5 ml of 0.05 potassium phosphate buffer, pH 7.5, and dialyzed overnight against 1 liter of the same buffer. All fractions throughout the purification process were kept at 4 C to prevent undue inactivation.

The dialysate was placed on a 25 x 450 mm DEAE

(diethylaminoethyl) cellulose column which had been equilibrated with pH 7.5, 0.05M potassium phosphate buffer. The protein was eluted by the same buffer and 9 ml fractions collected. Fractions containing significant enzyme activity were pooled and used for the succeeding step. The pooled fractions containing 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and tiglyl-CoA hydrase were subjected to isoelectric focusing by the method of Li and Li (16). The electrolytes used gave a pH gradient from 3 to 10. The entire isoelectric column was collected in fractions of 200 drops after constant amperage was obtained, which indicated that all electrolytes, including proteins, were at their isoelectric points.

Fractions containing significant amounts of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase were pooled and subjected to molecular sieving by G-150 Sephadex in a 25 x 450 mm column equilibrated with pH 7.5, 0.05M potassium phosphate buffer. Elution was accomplished with the same buffer.

Disc gel electrophoresis in 7% acrylamide gels was performed by the methods of Davis (9) and Ornstein (27). Protein bands were fixed and stained by 10% trichloroacetic acid containing 0.12% coomassie brilliant blue.

Identification of Product of 2-Methyl-3-Hydroxybutyryl-CoA Dehydrogenase Activity

The enzyme fraction used to determine the end product of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase was enriched from crude extracts by elution from a 25 x 450 mm DEAE-cellulose column by 0.05 M potassium phosphate buffer, pH 7.5. The specific activity was 1,500

(nmoles NADH/min/mg protein). The reaction mixture for native enzyme contained 300 μmoles of glycine buffer, pH 9.5, 25 μmoles of NAD, 0.22 mg protein, 7 μmoles of 2-methyl-3-hydroxybutyryl-CoA, and water to 5 ml. The enzyme control contained the same reagents except the enzyme had been boiled prior to its addition to the reaction mixture. In the other control, enzyme and substrate were both omitted and 2 μmoles of redistilled methyl ethyl ketone were added. Calculations derived from following the activity in a 1 ml aliquot of the total enzyme reaction indicated that 1.8 μmoles of 2-methylacetoacetyl-CoA had been produced after 10 min. The boiled enzyme preparation did not cause a measurable reduction of NAD. After the reaction had reached completion, 0.2 ml of 3.5 N NaOH was added to each tube to hydrolyze coenzyme A derivatives. The solutions were allowed to incubate 1 hr and were then titrated to pH 3.5-4.0 with 1.0 M H_2SO_4. This solution was allowed to sit overnight at room temperature to facilitate the decarboxylation of 2-methyl-acetoacetate to methyl-ethyl ketone. Each tube was then extracted 3 times with equal volumes of ethyl ether. Two ml of acidic 2,4 dinitrophenylhydrazine reagent (15 mM in 2N HCl) was added to the combined ether extracts. Tubes containing 2,4 dinitrophenylhydrazone standards of formaldehyde, methyl ethyl ketone, and acetone were dissolved in ether and treated the same as the other reaction mixtures throughout the remaining procedures. After 1 hr at room temperature with occasional shaking, the solutions were extracted 2 times with 10 ml of 1.0 M H_2SO_4. They were then extracted by carbonate solution (0.1 N $NaCO_3$-.01 N $NaHCO_3$) and water. The organic phase was taken to dryness under nitrogen. The residue was dissolved

in 0.05 ml ethyl acetate, spotted on thin layer plates of activated aluminum oxide G and developed in n-hexane and benzene (1:1). Spots that corresponded to methyl-ethyl ketone 2,4, dinitrophenylhydrazone standard and all other standards, were scraped off, the hydrazone eluted by ethyl acetate, dried by nitrogen and redeveloped by the same chromatographic system. R_{for} was calculated by the method of Denti and Luboz (10).

$$R_{for} = \frac{R_f \text{ of compound hydrazone}}{R_f \text{ of formaldehyde hydrazone}}$$

Under these conditions, R_{for} for the compound hydrazone would be determined by a comparison of the R_f of the compound hydrazone to the R_f of formaldehyde hydrazone which would always have a value of 1.0.

CHAPTER III

RESULTS

Growth

In Table 1 are shown growth rates of P. putida with isomers of branched-chain amino acids, glucose, and glutamate used as the sole source of carbon and energy. Toxic effects of L-leucine have been previously reported in P. putida (21), Escherichia coli (31), and Hydrogenomonas (13). A generation time of 20.4 hours was interpreted to mean that L-leucine was inhibitory to the growth of P. putida (Table 1). L-leucine toxicity in P. putida was partially relieved by L-valine, D-valine, and 2-ketoisovalerate (Table 2). The basis of L-leucine toxicity and the mode of action by which it was relieved were not determined. Leucine toxicity in E. coli was relieved by isoleucine. These experiments used liquid media whereas Martin et al. used plates (21).

Cell yields per mole of substrate are shown in Table 3. The complete oxidation of isoleucine requires 30 electrons (4 electrons per mole oxygen).

$$C_6H_{13}NO_2 + 7\tfrac{1}{2}\ O_2 \longrightarrow 6\ CO_2 + 5\ H_2O + NH_3$$

According to Payne (27), aerobic growth of heterotrophs in a basal medium yields 3.14 µg of dry weight bacteria for every electron

TABLE 1

GROWTH RATES OF P. PUTIDA ON BRANCHED-CHAIN AMINO ACIDS

Growth Substrate (0.3%, W/V)	Growth Rate (Generation/Hr)	Generation Time (Hours)
DL-Isoleucine	0.211	4.7
D-Isoleucine (with allo)	0.196	5.1
L-Isoleucine (allo free)	0.183	5.5
DL-Valine	0.096	10.4
D-Valine	0.088	11.4
L-Valine	0.125	8.0
DL-Leucine	0.300	3.3
D-Leucine	0.266	3.7
L-Leucine	0.049	20.4
D-Glucose	0.833	1.2
L-Glutamate	1.033	0.97

TABLE 2

RELIEF OF L-LEUCINE TOXICITY

Growth Substrates[a]	Growth Rate (Generation/Hr)	Generation Time (Hours)
L-Leucine	.049	20.4
L-Leucine + L-Valine	.093	10.8
L-Leucine + D-Valine	.120	8.3
L-Leucine + 2-Ketoisovalerate	.083	12.0
L-Leucine + L-Isoleucine	.045	22.2
L-Leucine + L-Glutamate	.040	25.0

[a] L-Leucine concentration was 5.0 μmoles per ml; additive concentration was 0.10 μmoles per ml.

TABLE 3

CELL YIELDS OF P. PUTIDA GROWN ON DIFFERENT SUBSTRATES

Growth Substrate (5 µmoles/ml)	Y_{sub} Cell Yield ($g \times mole^{-1}$)
D-Glucose	91.3
D-Isoleucine (with allo)[a] + L-Isoleucine (allo free)	95.7
DL-Isoleucine (4 isomers)	62.1
D-Isoleucine (with allo)	94.3
L-Isoleucine (allo free)	96.3
DL-Leucine	72.4
D-Leucine	65.1
L-Leucine	74.5
DL-Valine	64.0
D-Valine	55.6
L-Valine	67.6

[a] equimolar amounts each isomer

transferred during substrate oxidation. Therefore, the calculated yield per μmole of isoleucine should be 94.2 μg (3.14 x 30), which is very close to the results shown in Table 3 for D- and L-isoleucine.

Cell yields with stereoisomers of branched-chain amino acids were expressed as dry weight of bacteria per mole of substrate (Y_{sub}) (Table 3). Growth on valine produced lower cell yields than did growth on isoleucine and leucine, both of which have one carbon more. Generation times appeared to have little bearing on Y_{sub} as evidenced by similar cell yields in L-leucine (G = 20.4 hr), DL-leucine (G = 3.3 hr), and D-leucine (G = 3.7 hr). Isoleucine and leucine have identical carbon numbers and oxidation states but significantly different cell yields. The reason(s) for this difference is of interest since the theoretical ATP yield obtained by the complete catabolism of isoleucine and leucine are comparable.

Study of comparative cell yields from isoleucine isomers led to the suspicion that one of the four stereoisomers present in commercial DL-isoleucine was nonmetabolizable (both L and D-isoleucine have allo and erythro enantiomers). Similar cell yields were obtained by growth on L-isoleucine (allo free), D-isoleucine (with allo), and a mixture of equal amounts of these two preparations. These data indicated that L-allo-isoleucine was apparently not metabolized by P. putida (Table 3). The problem most likely lies with deamination of the amino acid rather than in the metabolism of the keto acid since D-isoleucine and L-alloisoleucine are both converted to 1-2-keto-3-methylvaleric acid which is readily metabolized by P. putida (22).

Assay of Isoleucine Catabolic Enzymes

2-Methyl-3-Hydroxybutyryl-CoA Dehydrogenase

Coon and Abrahansen (6) and Coon et al. (7) studied the metabolic fate of 2-methylbutyrate in rat liver and pig heart using 2-methylbutyrate labelled in various positions with C^{14}. They were primarily concerned with identifying the intermediates and end products of 2-methylbutyrate catabolism and did not characterize any of the individual enzymes of their proposed pathway. It was demonstrated that in liver tissue, 2-methylbutyrate underwent β-oxidation on the longer carbon chain, followed by cleavage to 2 and 3 carbon fragments. They postulated that one of the intermediates, 2-methyl-3-hydroxybutyryl-CoA, was oxidized to 2-methylacetoacetyl-CoA. It was necessary to establish the existance and properties of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase in order to prove that P. putida catabolized isoleucine via a pathway analagous to that proposed for mammalian tissue. The present investigation was the first demonstration of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, a previously unreported enzyme. A characterization of this enzyme was essential, because it was the first reported enzyme, which was unique to the catabolism of isoleucine. Previously described isoleucine catabolic enzymes were characterized incidental to the study of the catabolism of other branched-chain amino acids.

The existance of a common pathway for early catabolic reactions in the metabolism of branched chain amino acids led us to consider that other similar reactions may have also been catalyzed by common enzymes (20, 21). One likely possibility would have been the

enzyme(s) catabolizing the dehydrogenation of the β-hydroxy derivatives of valine and isoleucine. The results indicated that 2-methyl-3-hydroxybutyryl-CoA dehydrogenase was induced by growth on isoleucine and 3-hydroxyisobutyrate dehydrogenase was induced by valine (Table 4).

2-Methyl-3-hydroxybutyryl-CoA dehydrogenase present in the cell free extract of induced cells was partially characterized. NAD reduction did not proceed in the absence of either substrate or enzyme or in the presence of boiled enzyme. The proportionality between enzyme activity and protein concentration was linear between 0.10 and 0.45 mg protein per ml. The optimal pH for enzyme activity in crude extracts was 9.7. Dehydrogenase activity was completely lost after 6 minutes at 60 C.

2-Methyl-3-hydroxybutyryl-CoA dehydrogenase was purified by the procedures outlined in Table 5. The protein profile of the eluate from the DEAE-cellulose column is shown in Figure 5. Enzyme activity of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and tiglyl-CoA hydrase were inseparable through DEAE-cellulose column chromatography. Both enzymes were among the first proteins eluted from the DEAE-cellulose column (Fig. 5, fractions 14-19). No enzyme activity was found in any of the other protein peaks eluted from the column.

However, dehydrogenase and hydrase were separated by isoelectric focusing with a pH gradient from 3-10 (Fig. 6). The isoelectric point for each enzyme was determined by reading the pH of the fractions exhibiting the highest specific activity. The isoelectric point (pI) of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase was pH 5.80. Alternate tubes of the entire pH gradient were assayed for both

TABLE 4

INDUCTION OF CATABOLIC ENZYMES IN *P. PUTIDA*

Growth Substrate (0.3%, W/V)	Specific Activity[a] (nmoles/min/mg protein)	
	2-Methyl-3-Hydroxy-butyryl-CoA Dehydro-genase	3-Hydroxyisobu-tyrate Dehydro-genase
DL-Isoleucine	125	102
DL-Valine	64	233
DL-Leucine	36	148
L-Glutamate	6	92

[a] Separate experiments

TABLE 5

PURIFICATION OF 2-METHYL-3-HYDROXYBUTYRYL-CoA DEHYDROGENASE OF P. PUTIDA

Fraction	Total Protein (mgs)	Total Units	Specific[a] Activity	Purification (fold)
Crude Extract	5,470	1,250	0.228	1
Ammonium Sulfate	705	543	0.771	3
DEAE Cellulose Pool	66	116	1.758	8
Isoelectric Focusing Pool	5.4	132	24.500	106
G-150 Sephadex Pool	.54	3.5	6.400	28

[a] μmoles/min/mg protein

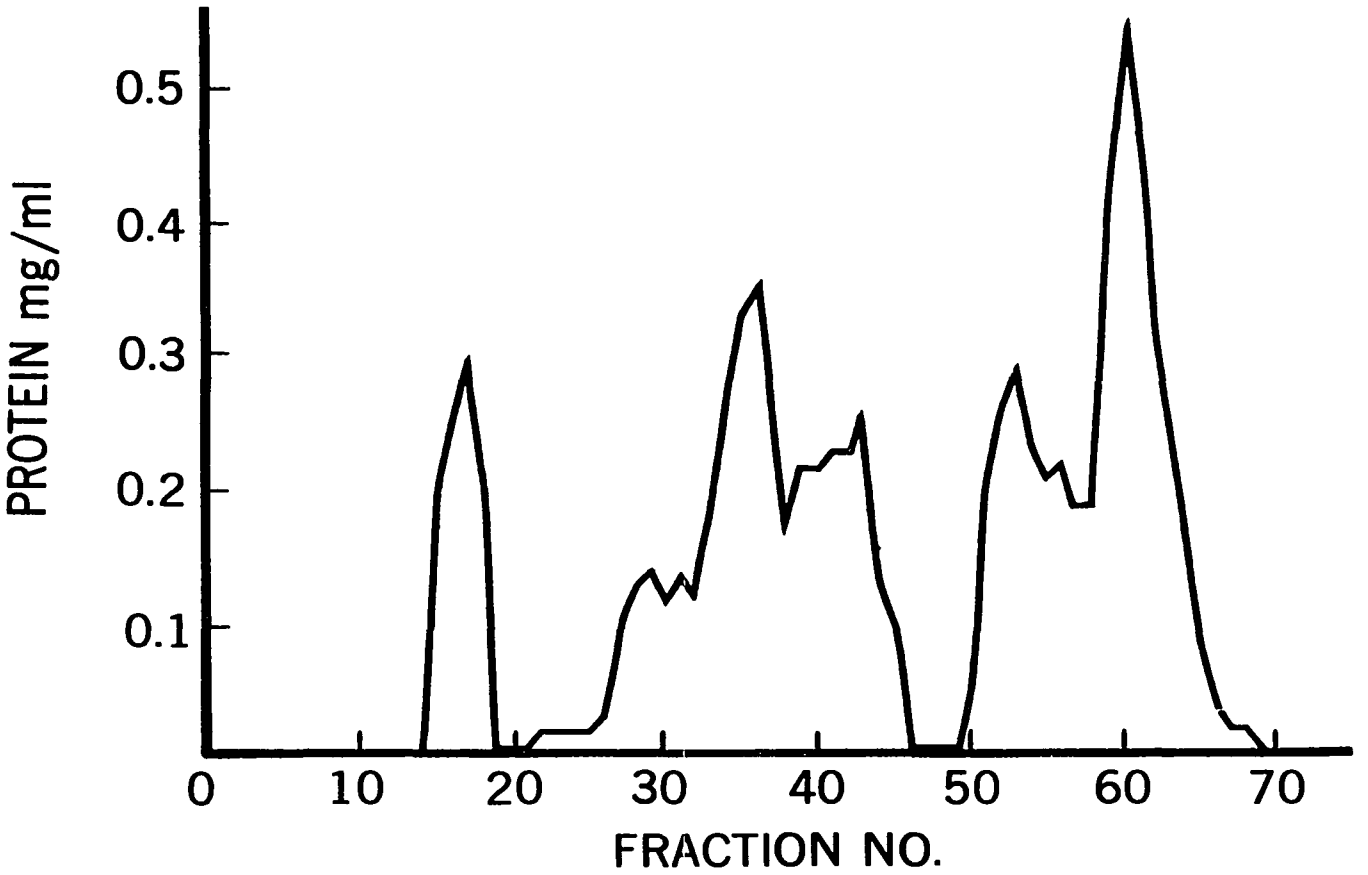

Figure 5. Protein profile of the eluate from DEAE-cellulose column. Proteins were insoluble in 30-50% ammonium sulfate.

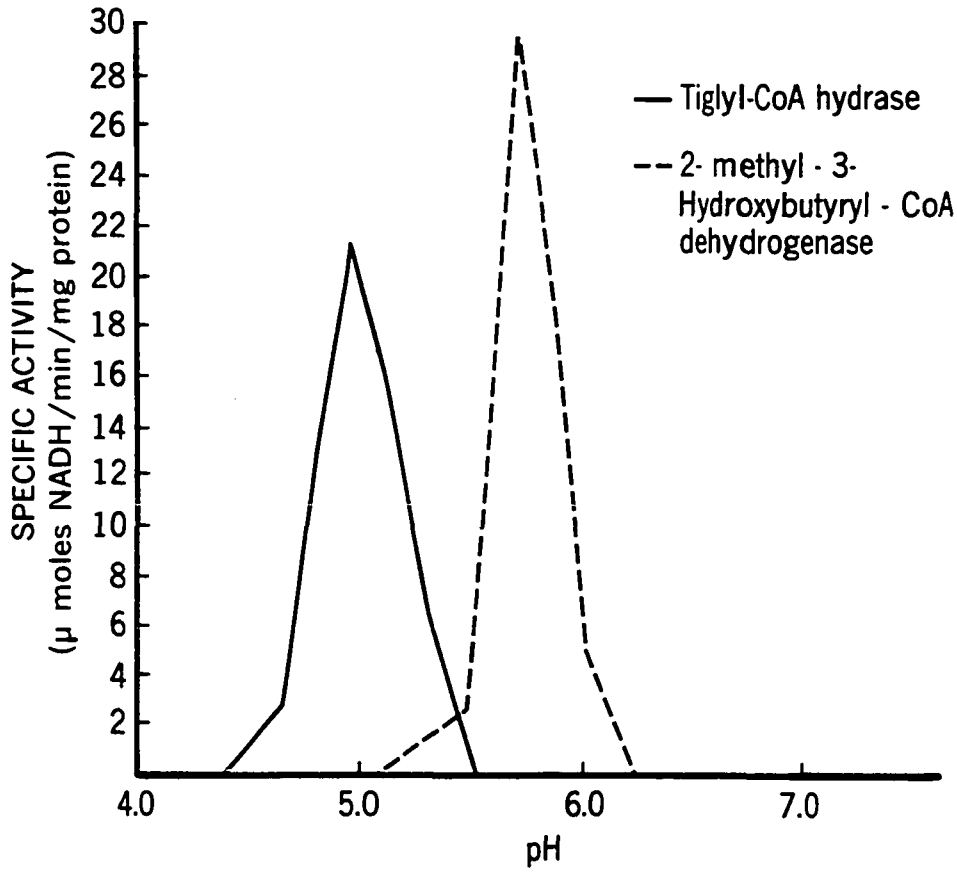

Figure 6. Separation of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and tiglyl-CoA hydrase by isoelectric focusing with pH gradient from 3-10.

Figure 7. Electropherograms of fractions containing 2-methyl-3-hydroxybutyryl-CoA dehydrogenase activity. Fractions were as follows: (A) DEAE-cellulose column chromatography, (B) Isoelectric focusing, (C) Sephadex G-150, and (D) Control.

Figure 8. Electropherograms of partially purified isoleucine catabolic enzymes. (A) 2-Methyl-3-hydroxybutyryl-CoA dehydrogenase, (B) Tiglyl-CoA hydrase, (C) 2-Methyl-3-hydroxybutyryl-CoA dehydrogenase plus tiglyl-CoA hydrase, and (D) Control.

dehydrogenase and hydrase activity. Dehydrogenase precipitated at the isoelectric point retained 30% of its original specific activity after 2 weeks elapsed time as opposed to 5% retention of original specific activity for tiglyl-CoA hydrase under similar conditions. Stained electropherograms illustrate the progressive enrichment of dehydrogenase (Fig. 7).

Tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase were established to be separate enzymes by a comparison of electropherograms containing recombined and separated individual enzymes (Fig. 8).

The optimal pH for enzyme activity of purified dehydrogenase was 9.3. Assays with boiled enzyme at pH 9.5, 10.3, and 10.6 insured that NAD reduction at higher pHs was not proceeding non-enzymatically. The substrate specificity of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (1 protein band in stained electropherograms) was determined by using structurally related analogues of 2-methyl-3-hydroxybutyrate (Table 6). The coenzyme A derivative of the substrate was an absolute prerequisite for enzyme activity. Parallel assays using the free acid form of the substrates were all negative. The highest specific activity was obtained when 2-methyl-3-hydroxybutyryl-CoA was the substrate (7.05 μmoles NADH/min/mg protein). The specific activity with 3-hydroxybutyryl-CoA was 4.02, which indicated that enzyme activity was not limited to branched-chain hydroxy acids. However, the specific activity with 3-hydroxyisobutyryl-CoA was only 0.08, which indicated that the length of the carbon chain was vital. A specific activity of 0.83 when 2-hydroxy-3-methyl-valeryl-CoA was the substrate demonstrated

TABLE 6

SUBSTRATE SPECIFICITY OF PURIFIED 2-METHYL-3-HYDROXYBUTYRYL-CoA DEHYDROGENASE[a]

Substrate[b]	Specific Activity (μmoles/min/mg protein)
2-Methyl-3-Hydroxybutyryl-CoA	7.05
3-Hydroxybutyryl-CoA	4.02
2-Hydroxy-3-Methyl-Valeryl-CoA	.83
3-Hydroxyisobutyryl-CoA	.08
2-Ethyl-2-Hydroxybutyryl-CoA	0
2-Methyl-2-Hydroxybutyryl-CoA	0
2-Hydroxyisobutyryl-CoA	0
3-Hydroxy-3-Methyl-Glutaryl-CoA	0

[a] Each assay used .14 unit of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase.

[b] Enzyme was not active with any free acid. No activity was observed in presence of 2-methyl-3-hydroxybutyrate plus free coenzyme A.

that 2-methyl-3-hydroxybutyryl-CoA dehydrogenase could also catalyze the dehydrogenation of α-hydroxy acids, albeit at a greatly reduced rate. The results from this assay also demonstrated that compounds five carbons long could serve as substrate. No enzyme activity was detected when any other group was attached to the same carbon containing the hydroxy group.

Partially purified enzyme (specific activity of 1.500 nmoles NADH/min/mg protein) was used to identify the end product of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase when 2-methyl-3-hydroxybutyryl-CoA was the substrate. The protocol is described in the Methods section. During this procedure coenzyme A derivatives were hydrolyzed, chemically decarboxylated, and selectively extracted by organic solvents. The 2,4-dinitrophenylhydrazones were prepared and chromatographed by thin layer chromatography. The expected product of enzyme activity, 2-methylacetoacetyl-CoA, would yield methyl-ethyl ketone in this procedure, which was identified by thin layer chromatography (Table 7) by a comparison with appropriate standards. The boiled enzyme control demonstrated that this oxidation was enzymatically catalyzed. The molecular weight of dehydrogenase was not determined but was likely greater than 150,000 daltons since the enzyme was excluded from Sephadex G-150.

Tiglyl-CoA Hydrase

Tiglyl-CoA hydrase was another previously undescribed enzyme whose existence had been predicted by the study of Robinson and Coon on isoleucine catabolism in mammalian tissues (29).

The induction of tiglyl-CoA hydrase paralleled the induction

TABLE 7

THIN LAYER CHROMATOGRAPHY OF 2,4-DINITROPHENYLHYDRAZONES OF 2-METHYL-3-HYDROXYBUTYRYL-CoA DEHYDROGENASE END PRODUCT

2,4-Dinitrophenylhydrazone from	R_{for} [a]
Reaction with Native Enzyme	1.77
Methyl Ethyl Ketone Standard[b]	1.72
Reaction with No Substrate + Methyl Ethyl Ketone	1.66
Acetone Standard	1.22
Formaldehyde Standard	1.00
Reaction with Boiled Enzyme	0

[a] $R_{for} = \frac{R_f \text{ of compound hydrazone}}{R_f \text{ of formaldehyde hydrazone}}$

[b] Literature value as per Denti and Luboz was 1.64.

of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (Table 8). Tiglyl-CoA hydrase was induced by growth on tiglate and isoleucine (Table 9). The data for tiglyl-CoA hydrase in Table 8 was compiled with a rate limiting amount of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase whereas dehydrogenase was present in excess amounts for the induction study outlined in Table 9. Some induction was exerted by growth on 2-keto-3-methyl-valerate, valine, and leucine. It was of interest that cells grown on crotonate were uninduced since crotonate is closely related structurally to tiglate (tiglate has an α-methyl group) and is reportedly metabolized in an analogous fashion (40, 41). The hydration product of crotonate, 3-hydroxybutyrate, was also ineffective as an inducer of either dehydrogenase or hydrase. The hydration of tiglyl-CoA in crude extracts was linear between 0.10-0.30 mg protein per ml.

Tiglyl-CoA hydrase was partially purified concomitantly with the enrichment of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (Table 10). Tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase were inseparable through DEAE-cellulose column chromatography but were resolved by isoelectric focusing (Fig. 6). Two major protein bands were detected by electrophoresis (Fig. 8). Figure 8 also shows that tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase were separate enzymes. The isoelectric point (pI) of tiglyl-CoA hydrase was pH 5.02. The optimal pH for enzyme activity was 9.9. Citraconyl-CoA and 3-methyl-crotonyl-CoA were inactive in the coupled assay. Due to the nature of the assay, it was impossible to determine if this inactivity was due to the specificity of tiglyl-CoA hydrase or 2-methyl-3-hydroxybutyryl-CoA dehydrogenase.

TABLE 8

INDUCTION OF 2-METHYL-3-HYDROXYBUTYRYL-CoA DEHYDROGENASE AND TIGLYL-CoA HYDRASE IN P. PUTIDA

Growth Substrates (0.3%, W/V)	Specific Activity[a] (nmoles/min/mg protein)	
	Enzyme Substrate	
	Tiglyl-CoA	2-Methyl-3-Hydroxybutyryl-CoA
DL-Isoleucine	198	166
DL-Valine	46	71
DL-Leucine	41	67
D-Glucose	0	22
Glutamate	30	57
Succinate	9	81

[a] Specific activity based on oxidation of 2-methyl-3-hydroxybutyryl-CoA by 2-methyl-3-hydroxybutyryl-CoA dehydrogenase. Specific activity of tiglyl-CoA hydrase rate limited by amount of dehydrogenase in extract.

TABLE 9

INDUCTION OF TIGLYL-CoA HYDRASE IN P. PUTIDA

Growth Substrate (0.3%, W/V)	Specific Activity[a] (nmoles/min/mg protein)
Tiglate	3,420
DL-Isoleucine	2,920
2-Keto-3-Methyl Valerate	630
DL-Valine	645
DL-Leucine	368
Acetate	157
Crotonate	154
3-Hydroxybutyrate	146
L-Glutamate	120

[a] .21 Unit of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase added per ml.

TABLE 10

PURIFICATION OF TIGLYL-CoA HYDRASE OF P. PUTIDA

Fraction	Total Protein (mgs)	Total Units	Specific Activity[a]	Purification (fold)
Crude Extract	5,472	1,390	.253	1
Ammonium Sulfate	705	313	.444	2
DEAE Cellulose Pool	66	105	1.640	7
Isoelectric Focusing Pool	10.5	189	17.970[b]	71

[a] Specific activity = μmoles/min/mg protein.

[b] Assayed by addition of purified 2-methyl-3-hydroxybutyryl-CoA dehydrogenase.

2-Methylacetoacetyl-CoA Cleavage Enzyme

Robinson and Coon postulated that a thiolase-like enzyme present in rat liver catalyzed the cleavage of 2-methylacetoacetyl-CoA to acetyl-CoA and propionyl-CoA (29). The role of thiolase in β-oxidation has been thoroughly studied in mammalian tissues. However, 2-methylacetoacetyl-CoA cleavage enzyme has not been previously characterized in bacteria. The catabolism of branched-chain amino acids is accomplished by several common enzymes (20, 21). With this observation in mind, the induction of 2-methylacetoacetyl-CoA cleavage enzyme and 3-hydroxy-3-methylglutaryl-CoA cleavage enzyme was compared in isoleucine, valine, and leucine grown cells (Table 11). 2-Methylacetoacetyl-CoA cleavage enzyme was induced only by growth on isoleucine and 3-hydroxy-3-methyl-glutaryl-CoA cleavage enzyme was induced by growth on each of the branched-chain amino acids. The inductive process for each enzyme appeared to be separate phenomena. The proportionality between NADH production and protein concentration was linear only with less than 0.30 mg protein per ml. Acetyl-CoA formation from 2-methylacetoacetyl-CoA in a complete reaction mixture was compared with assays in which one major component had been omitted (Table 12). In contrast to mammalian thiolase, magnesium did not appear to be essential for acetyl-CoA production. Omission of coenzyme A did not result in a drop in activity. L-malate appeared to be required for the measurement of enzyme activity.

Induction of Isoleucine Catabolic Enzymes in P. putida

The activity of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase

TABLE 11

INDUCTION OF CLEAVAGE ENZYMES IN P. PUTIDA

Enzyme	Specific Activity[a] When Carbon Source For Growth Was:			
	0.3% DL-Isoleucine	0.3% DL-Leucine	0.3% DL-Valine	0.3% D-Glucose
3-Hydroxy-3-Methyl-Glutaryl-CoA Cleavage Enzyme	91	108	56	0
2-Methylacetoacetyl-CoA Cleavage Enzyme	32	0	0	0

[a] nmoles/min/mg protein.

TABLE 12

ACETYL-CoA FORMATION FROM 2-METHYLACETOACETYL-CoA

Test System	Specific Activity (nmoles/min/mg protein)
Complete	38
Omit $MgCl_2$	48
Omit Coenzyme A	42
Omit Citrate Synthetase	20
Omit L-Malate	1

with 3-hydroxybutyryl-CoA raised the possibility that 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and 3-hydroxybutyryl-CoA dehydrogenase were the same enzyme (Table 6). Data in Table 13 compares the induction of these enzymes in cells grown on isoleucine, 3-hydroxybutyrate, and glutamate. Tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase were induced by growth on isoleucine but remained at basal levels in 3-hydroxybutyrate grown cells. 3-Hydroxybutyryl-CoA dehydrogenase appeared to be induced by growth on isoleucine and 3-hydroxybutyrate. These results were interpreted to mean that 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and 3-hydroxybutyryl-CoA dehydrogenase were individually induced enzymes. In this case, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase was equally capable of catalyzing the oxidation of 2-methyl-3-hydroxybutyryl-CoA and 3-hydroxybutyryl-CoA. However, 3-hydroxybutyryl-CoA dehydrogenase did not oxidize 2-methyl-3-hydroxybutyryl-CoA.

Table 14 shows a comparison of isoleucine catabolic enzymes in P. putida grown with branched-chain amino acids and neutral carbon sources. D-amino acid dehydrogenase and branched-chain keto acid dehydrogenase were induced by growth on isoleucine, valine, or leucine. Tiglyl-CoA hydrase, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, and 2-methylacetoacetyl-CoA cleavage enzyme were induced by growth on isoleucine and slightly by growth on valine.

A comparison of inducible isoleucine catabolic enzymes in P. putida grown on proposed intermediates is given in Table 15. D-amino acid dehydrogenase was induced by DL-isoleucine. Branched-chain keto acid dehydrogenase was induced by DL-isoleucine and 2-keto-3-methyl

TABLE 13

INDUCTION OF ISOLEUCINE CATABOLIC ENZYMES IN *P. PUTIDA*

Enzyme	Specific Activity[a] When Carbon Source For Growth Was:		
	0.3% Tiglate	0.3% 3-Hydroxy-butyrate	0.3% L-Glutamate
Tiglyl-CoA Hydrase	473	40	55
2-Methyl-3-Hydroxy-butyryl-CoA Dehydrogenase	526	62	68
3-Hydroxybutyryl-CoA Dehydrogenase	576	270	72
2-Methylacetoacetyl-CoA Cleavage Enzyme	198	23	0

[a] nmole/min/mg protein.

TABLE 14

COMPARISON OF ISOLEUCINE CATABOLIC ENZYMES IN P. PUTIDA GROWN ON DIFFERENT CARBON SOURCES

Enzyme	Specific Activity (nmoles/min/mg protein) When Carbon Source For Growth (0.3%, W/V) Was:					
	DL-Isoleucine	DL-Valine	DL-Leucine	Succinate	D-Glucose	L-Glutamate
D-Amino Acid Dehydrogenase	16	6	2	0	0	0
Branched-Chain Keto Acid Dehydrogenase	8	3	10	0	0	0
Tiglyl-CoA Hydrase	196	29	5	10	4	3
2-Methyl-3-Hydroxybutyryl-CoA Dehydrogenase	183	40	22	24	1	10
2-Methylacetoacetyl-CoA Cleavage Enzyme	133	41	0	0	-	-

TABLE 15

COMPARISON OF INDUCIBLE ISOLEUCINE CATABOLIC ENZYMES IN P. PUTIDA GROWN ON PROPOSED INTERMEDIATES

Enzyme	Specific Activity (nmoles/min/mg protein) When Growth Substrate (0.3%, W/V) Was:					
	DL-Isoleucine	2-Keto-Methyl Valerate	2-Methylbutyrate	Tiglate	L-Glutamate	D-Glucose
D-Amino Acid Dehydrogenase	4	0	0	0	0	0
Branched-Chain Keto Acid Dehydrogenase	26	10	0	0	0	0
Tiglyl-CoA Hydrase	239	113	170	221	4	5
2-Methyl-3-Hydroxybutyryl-CoA Dehydrogenase	132	149	147	334	28	11
2-Methylacetoacetyl-CoA Cleavage Enzyme	51	123	82	37	0	-

valerate. Tiglyl-CoA hydrase, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, and 2-methylacetoacetyl-CoA cleavage enzyme were induced by DL-isoleucine, 2-keto-3-methyl-valerate, 2-methylbutyrate, and tiglate. P. putida grew very scantily on 2-methyl-3-hydroxybutyrate. This was disappointing since tiglyl-CoA hydrase was induced by growth on tiglate and it would have been of interest to see if 2-methyl-3-hydroxybutyryl-CoA dehydrogenase could be induced without the concurrent induction of tiglyl-CoA hydrase. Based on these data, tiglate may be the inducer of tiglyl-CoA hydrase. The inducer(s) of dehydrogenase and cleavage enzyme were not determined.

L-alloisoleucine was not available for assay by branched-chain amino acid transaminase. Branched-chain amino acid transaminase was inactive with both isomers of D-isoleucine, but transaminated the L-isomers of all three branched-chain amino acids. The rate of enzyme synthesis appeared to be constitutive under all conditions used during the course of this investigation.

The enzyme catalyzing the oxidation of 2-methylbutyryl-CoA also appeared to be synthesized constitutively.

It was found during this investigation that D-isoleucine induced D-amino acid dehydrogenase which was active with D-isoleucine (specific activity of 8.8) and D-alloisoleucine (specific activity of 6.6) as well as a number of other D-amino acids.

CHAPTER IV

DISCUSSION

The purpose of this investigation was to elucidate the pathway used by P. putida to catabolize isoleucine. This objective was achieved by demonstrating that P. putida catabolized 2-methylbutyryl-CoA to acetyl-CoA and propionyl-CoA via a form of beta oxidation. It had been previously demonstrated that P. putida catabolized isoleucine to 2-methylbutyryl-CoA by enzymes common also to the catabolism of valine and leucine (21).

The proposed pathway for isoleucine catabolism (Fig. 1) was based in part on studies by Coon and his colleagues (6, 7, 29). The results of this investigation indicated that P. putida and mammalian tissues use essentially the same pathway to catabolize isoleucine. The evidence that tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase are unique to the catabolism of isoleucine is as follows: common catabolic enzymes, such as D-amino acid dehydrogenase and branched-chain keto acid dehydrogenase have comparable magnitudes of induction when grown on any of the branched chain amino acids. Therefore, if the same enzyme catalyzed the hydration of all three branched-chain enoyl-CoA derivatives, tiglyl-CoA hydrase should be induced by isoleucine, valine, and leucine. However, tiglyl-CoA hydrase was

significantly induced only by isoleucine (Table 9) implying that the hydration of methylacrylyl-CoA and 3-methylglutaconyl-CoA was accomplished by other enzyme(s). Considerable effort was expended to determine if tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase were synonymous terms for crotonase and β-hydroxyacyl dehydrogenase, both of which have been well studied in beta-oxidation. Crotonate and 3-hydroxybutyrate did not induce tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, although both enzymes are induced by tiglate (Tables 8, 9). 3-Hydroxybutyrate did induce 3-hydroxybutyryl-CoA dehydrogenase (Table 13). The apparent induction of 3-hydroxybutyryl-CoA dehydrogenase by tiglate may be explained by the substrate specificity of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase which is active with 3-hydroxybutyryl-CoA (Table 6). On the other hand, the substrate specificity of 3-hydroxybutyryl-CoA dehydrogenase seemingly precludes the reduction of 2-methyl-3-hydroxybutyryl-CoA. The most logical explanation is that the metabolism of tiglate and crotonate in P. putida proceeds through similar but separate pathways and are catalyzed by different enzymes. The enzymic characteristics of 2-methylacetoacetyl-CoA cleavage enzyme differentiated it from mammalian thiolase and 3-hydroxy-3-methylglutaryl-CoA cleavage enzyme. However, 2-methylacetoacetyl-CoA cleavage enzyme was not sufficiently characterized to the point that it can be stated that it is unique to the catabolism of isoleucine.

Sokatch and his associates demonstrated that at least three enzymes in Pseudomonas were common to the catabolism of isoleucine, valine, and leucine (Fig. 9) (19, 20, 21, 25). The D-amino acid

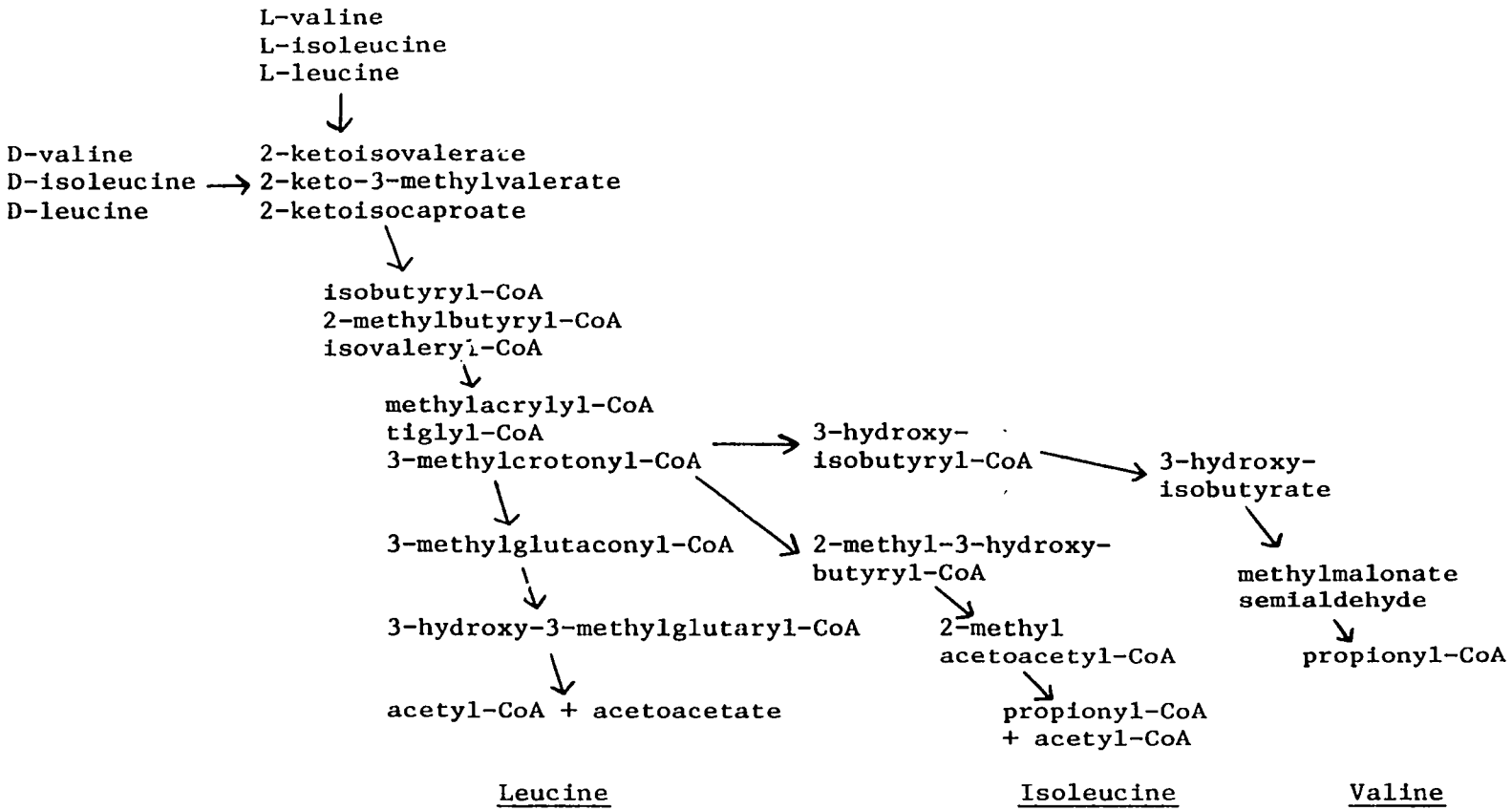

Figure 9. Catabolism of isoleucine, valine, and leucine.

dehydrogenase of P. aeruginosa was partially purified by Marshall (19). It was induced by a number of amino acids and displayed a broad substrate specificity, which included the D-isomers of all three branched-chain amino acids (20). Norton and Sokatch found that branched-chain amino acid transaminase was active with the L-isomers of isoleucine, valine, and leucine (24). Branched-chain amino acid transaminase appeared to be synthesized constitutively in both P. putida (20) and P. aeruginosa (24). Marshall showed that the third common catabolic enzyme, branched-chain keto acid dehydrogenase, was induced by growth on any of the branched-chain keto acid derivatives. Regardless of inducer source, the enzyme oxidatively decarboxylated the keto acid of isoleucine, valine, and leucine (21).

It was not determined if the same enzyme catalyzed the dehydrogenation of 2-methylbutyryl-CoA (isoleucine), isobutyryl-CoA (valine) and isovaleryl-CoA (leucine). There is no irrefutable evidence that 2-methylbutyryl-CoA dehydrogenase is constitutive, even though induction was not observed in this investigation. Earlier workers have found that similar assays based on the reduction of 2,6-DCPIP can be misleading due to the presence of bacterial deacylases (37). Under these circumstances, "specific activity" would be a function of the concentration of coenzyme A liberated by deacylase, rather than true dehydrogenation.

CHAPTER V

SUMMARY

The objective of this investigation was the elucidation of the pathway for the catabolism of isoleucine in Pseudomonas putida. Growth rates and cell yeilds were calculated for stereoisomers of branched-chain amino acids. Assays were developed for the dehydrogenation of 2-methylbutyryl-CoA, the hydration of tiglyl-CoA, the dehydrogenation of 2-methyl-3-hydroxybutyryl-CoA and the cleavage of 2-methylacetoacetyl-CoA. Previously described assays for D-amino acid dehydrogenase, branched-chain amino acid transaminase and branched-chain keto acid dehydrogenase were modified for use with P. putida.

D-amino acid dehydrogenase was induced by growth in the presence of D-isoleucine. Branched-chain keto acid dehydrogenase was induced by growth in the presence of 2-keto-3-methyl valeric acid. Growth on isoleucine, 2-keto-3-methyl valeric acid, 2-methylbutyrate and tiglic acid induced tiglyl-CoA hydrase, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, and 2-methylacetoacetyl-CoA cleavage enzyme. Branched-chain amino acid transaminase and 2-methylbutyryl-CoA dehydrogenase appeared to be synthesized constitutively.

2-Methyl-3-hydroxybutyryl-CoA dehydrogenase was enriched 106 fold by ammonium sulfate precipitation, ion exchangers and

isoelectric focusing. Molecular sieving of this fraction gave one band upon disc gel electrophoresis. An end product determination proved that the enzyme catalyzes the oxidation of 2-methyl-3-hydroxybutyryl-CoA to 2-methylacetoacetyl-CoA. Tiglyl-CoA hydrase was enriched 76 fold by the same procedure. Two bands of protein were present in disc gel electrophoresis. Tiglyl-CoA hydrase was inseparable from 2-methyl-3-hydroxybutyryl-CoA dehydrogenase until the isoelectric focusing step. Isoelectric points, optimal pHs, and substrate specificities were determined for both enzymes. The catabolic pathway for isoleucine in P. putida appears to be essentially the same as the pathway previously demonstrated in mammalian tissues. Three and possibly four enzymes are common to valine and leucine catabolism.

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