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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

REGULATION OF BRANCHED CHAIN AMINO ACID CATABOLISM IN *PSEUDOMONAS PUTIDA*

A DISSERTATION

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degree of

DOCTOR OF PHILOSOPHY

BY

VINCENT dePAUL MARSHALL

Oklahoma City, Oklahoma

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REGULATION OF BRANCHED CHAIN AMINO ACID

CATABOLISM IN *PSEUDOMONAS PUTIDA*

APPROVED BY

DISSERTATION COMMITTEE

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LIST OF ABBREVIATIONS

ATP	- Adenosine triphosphate
bp	- boiling point
C	- Centrigrade
CoA, CoASH	- Coenzyme A
EC	- Enzyme Commission
EDTA	- Ethylenedinitrilotetraacetic acid
2,6-DCPIP	- 2,6-Dichlorophenolindophenol
g	- gram or gravity
kc	- kilocycle
μeq	- microequivalent
μg	- microgram
mg	- milligram
ml	- milliliter
mμ	- millimicron
min	- minute
NAD	- Nicotinamide adenine dinucleotide (oxidized)
NADH	- Nicotinamide adenine dinucleotide (reduced)
Tris	- Tris(hydroxymethyl)aminomethane
WT	- wild type

REGULATION OF BRANCHED CHAIN AMINO ACID CATABOLISM IN *PSEUDOMONAS PUTIDA*

CHAPTER I

INTRODUCTION

Valine Catabolism in Bacteria

The valine catabolic pathway is shown in Fig. 1. The following experimental findings document the occurrence of this pathway in bacteria. Norton and Sokatch (26) reported transamination between L-valine and 2-oxoglutarate (Fig. 1, reaction 1') in *Pseudomonas aeruginosa* as a means of deamination of L-valine. The transaminase was later obtained in a purified form (27), and was found to be similar to transaminase B (33). The oxidative deamination of D-valine (Fig. 1, reaction 1') was also reported to occur in *Pseudomonas* by Norton, Bulmer, and Sokatch (25). The particulate D-amino acid dehydrogenase which catalyzed this reaction was partially purified from *P. aeruginosa*, and was found to be induced by growth of the organism on several L- and D-amino acids (19). Prior to the present study the oxidation of 2-oxoisovalerate (Fig. 1, reaction 2) was never found to occur in any organism. Coincident with the demonstration of this reaction in *Pseudomonas putida* other workers reported a reaction of identical stoichiometry to occur in *Bacillus subtilis* (24). Previously, reactions 3, 4, and 5 (Fig. 1) had never been studied in

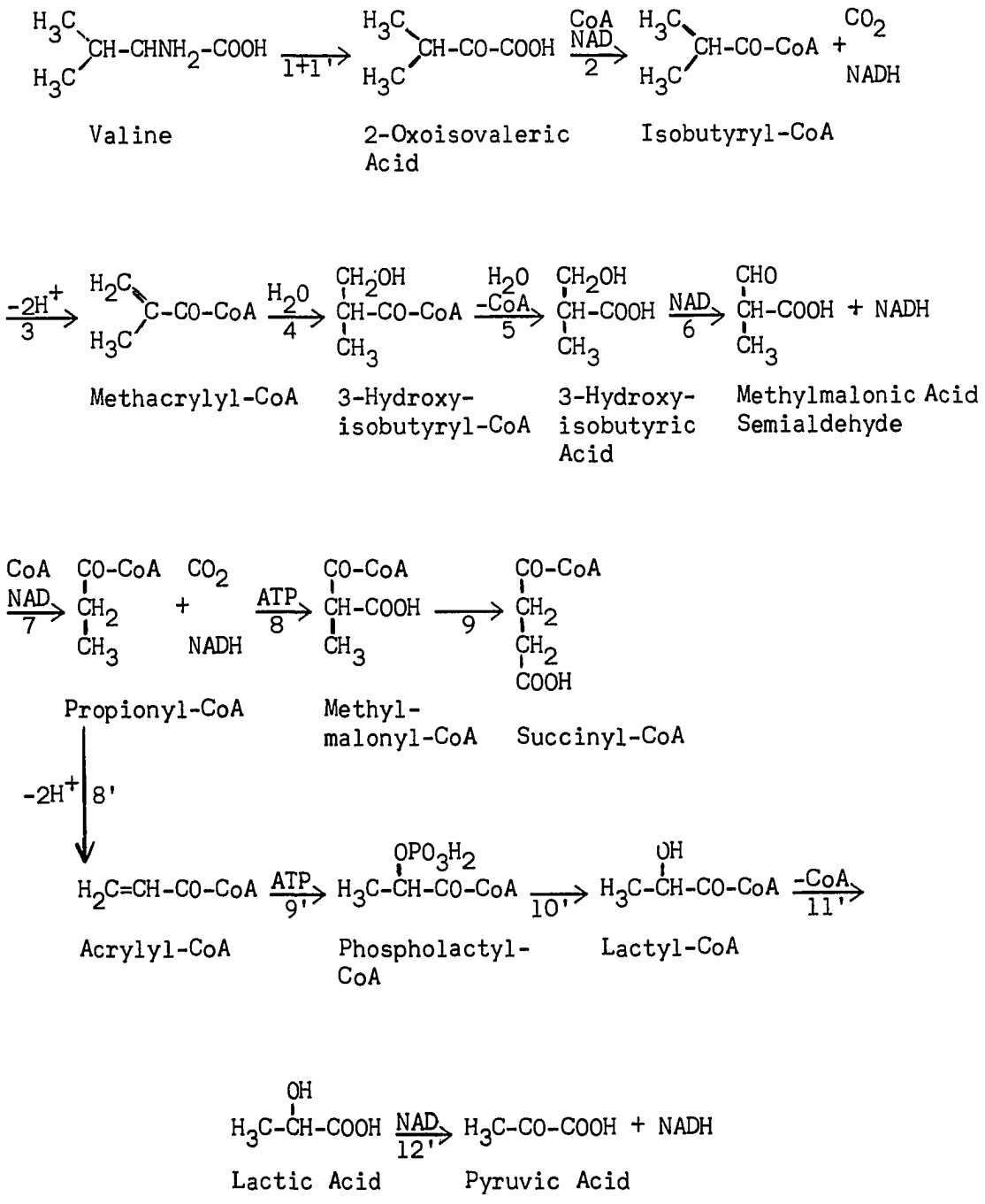

Figure 1. Valine catabolism in Pseudomonas.

bacteria, but were studied in animal tissue by Kinnory, Takeda and Greenberg (13), and by Robinson et al. (30). To propose a model for valine catabolism in bacteria (2), these three reactions were incorporated into a tentative catabolic sequence. In this study I have demonstrated that reactions 3, 4, and 5 (Fig. 1) also occur in Pseudomonas. Therefore all of the reactions in Fig. 1 are now known to occur in bacteria. 3-Hydroxyisobutyrate dehydrogenase, the enzyme which catalyzes reaction 6 (Fig. 1), was found in Pseudomonas, and was purified (44). In addition, the conversion of methylmalonate semialdehyde and CoA to propionyl-CoA and CO_2 (reaction 7, Fig. 1) was discovered in this organism (42). Recently this enzyme, methylmalonate semialdehyde dehydrogenase, was purified (2). In the past it was accepted without substantial evidence that methylmalonate semialdehyde reacted directly with CoA to form methylmalonyl-CoA. The studies by Bannerjee, Sanders and Sokatch (2), and by Sokatch, Sanders and Marshall (42) provided evidence that propionyl-CoA was a definite and free intermediate in valine catabolism. Propionyl-CoA carboxylase, the catalyst for reaction 8 (Fig. 1), was reported to occur in Pseudomonas (42), Micrococcus (40), and Rhodospirillum (14). The isomerization of methylmalonyl-CoA to succinyl-CoA (reaction 9, Fig. 1) was found to occur in Micrococcus (40), and in propionibacteria (48).

Evidence supporting the occurrence of an acrylate pathway (Fig. 1, reactions 8' through 12') as an alternate means of propionate metabolism in Pseudomonas was based partially on the results of an investigation by Sokatch (41). Pseudomonas grown on valine-4,4'-^{14}C synthesized alanine-1,3-^{14}C, which favored the theory of alanine's biosynthesis

via the acrylate pathway (41). The existence of an acrylate pathway of propionate oxidation was proposed earlier by Vagelos, Earl, and Stadtman (51). Sokatch, Sanders, and Marshall (42) assumed that the acrylate pathway in Pseudomonas functioned as a biosynthetic sequence, and was not a major energy yielding mechanism. An acyl-CoA dehydrogenase which catalyzes reaction 8' (Fig. 1) was found in Peptostreptococcus elsdinii (1). Recently this enzyme was purified (4). Acyl-CoA dehydrogenase activity was detected in crude extracts of P. putida during the course of the present study. Schneider and Wood (38) isolated the product of reaction 9' (Fig. 1), α-phospholactyl-CoA, which supported the occurrence of this reaction in P. elsdenii. Lactyl-CoA dehydrase was reported in P. elsdenii (1), P. aeruginosa (42), and a soil pseudomonad (51) which provided evidence for the occurrence of reaction 10' (Fig. 1) in the acrylate pathway. Lactyl-CoA is then deacylated and dehydrogenated (Fig. 1, reaction 11' and 12') to pyruvate.

Leucine and Isoleucine Catabolism in Bacteria

Figs. 2 and 3 show the proposed catabolic pathways for the metabolism of leucine and isoleucine in bacteria. Unlike the valine catabolic pathway, which is well documented to occur in bacteria, the pathways for the catabolism of leucine and isoleucine are based largely on reactions known only to occur in animal tissue. However, the first two reactions of each pathway are known to occur in bacteria. Norton and Sokatch (26, 27) found that all of the L-isomers of the branched chain amino acids served as substrates for transamination with 2-oxoglutarate in P. aeruginosa. In addition, the D-isomers of these amino acids were active as substrates with D-amino acid dehydrogenase in Pseudomonas

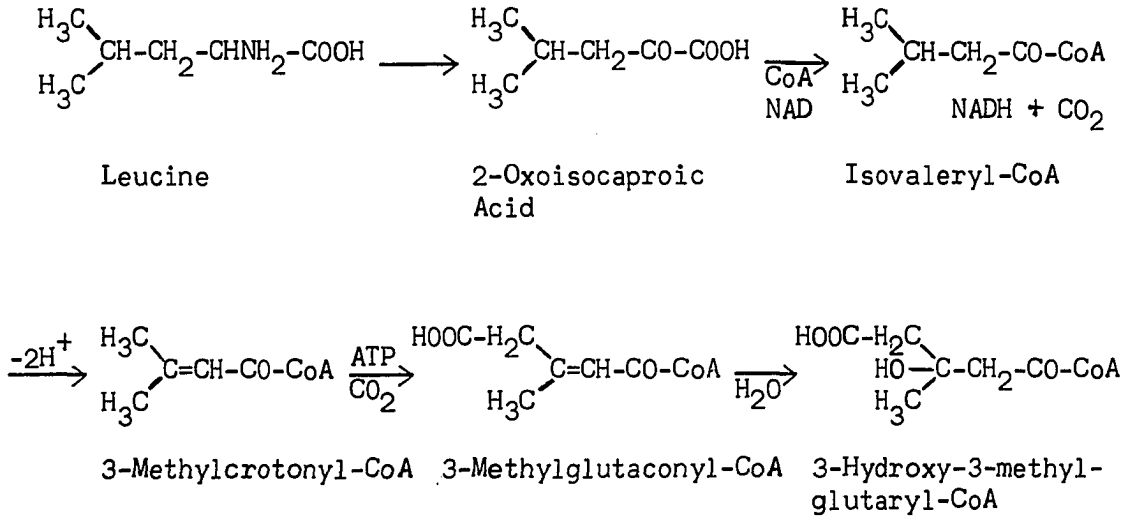

Figure 2. Proposed pathway for leucine catabolism in Pseudomonas.

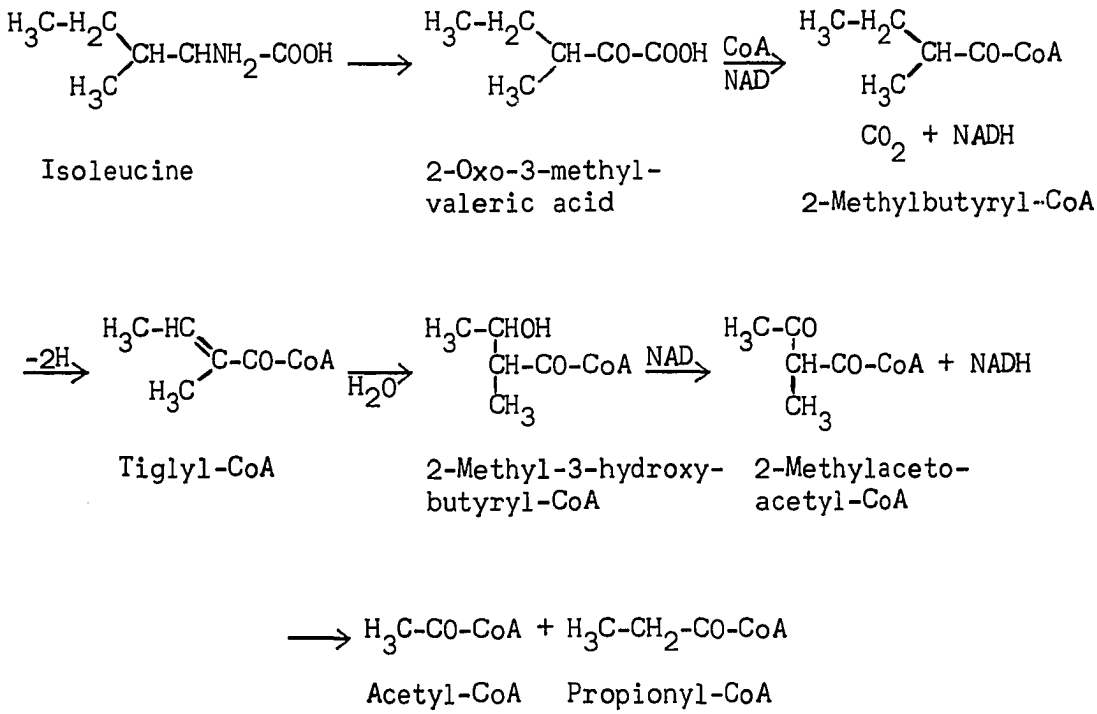

Figure 3. Proposed pathway for isoleucine catabolism in Pseudomonas.

(19, 26). Sanwall and Zink (34) purified an NAD linked dehydrogenase from Bacillus cereus which was active with the L-isomers of valine, leucine, and isoleucine. During the present study I have shown that the branched chain oxo acids may be oxidized by a single enzyme or a single enzyme complex in Pseudomonas. The other catabolic reactions for the degradation of leucine and isoleucine have been described (22, 43). It is interesting that one of the endproducts of isoleucine catabolism is propionyl-CoA. Propionyl-CoA is also an intermediate in the catabolism of valine.

Regulation of Amino Acid Catabolism

Regulation of Histidine Catabolism

The regulation of histidine catabolism has been studied in several microorganisms. Hartwell and Magasanik (9) found that the synthesis of histidine ammonia lyase (histidase) was induced in B. subtilis grown on glutamate by the addition of histidine to the growth medium. The synthesis of histidase was reported to be sensitive to catabolite repression by glucose, glycerol, and sucrose. Other studies with B. subtilis (5) indicated that histidine served as an inducer for the synthesis of urocanase and formiminoglutamate hydrolase, as well as histidase. The synthesis of these three enzymes was sensitive to catabolite repression by glycerol and glucose.

Histidine catabolism was extensively investigated by Magasanik and coworkers in Aerobacter aerogenes. Magasanik et al. (18) observed that urocanate, as well as histidine induced the synthesis of the histidine catabolic enzymes in A. aerogenes. The synthesis of urocanase

was found to be induced coordinately with that of histidase. Although formiminoglutamate hydrolase was induced by histidine and urocanate, its synthesis was not coordinate with that of histidase and urocanase. The observation that urocanate served as an inducer for histidase, urocanase, and formiminoglutamate hydrolase suggested that histidine was an inducer only because of its conversion to urocanate. The synthesis of these three enzymes was repressed by glucose and glycerol.

Imidazolepropionate, a nonmetabolizable analog of urocanate, induced the synthesis of the histidine catabolic enzymes, as well as a urocanate permease (35). This experiment indicated that the synthesis of histidase, urocanase, and formiminoglutamate hydrolase responded to a single inducer, and therefore eliminated a sequential induction mechanism.

Schlesinger, Scotto, and Magasanik (36) presented evidence that urocanate, not histidine, was responsible for the induction of the enzymes of histidine catabolism in A. aerogenes. This conclusion was based on the finding that mutants deficient in histidase responded more weakly to induction by histidine than the parent wild type strain. Both strains responded equally well to urocanate as the inducer. It is of interest that urocanate was not reported as an inducer of the histidine catabolic enzymes in B. subtilis (9).

The genetic loci for histidase and urocanase in Salmonella typhimurium appear to be closely linked (3, 21). Histidine coordinately induced histidase and urocanase to various degrees in different strains of S. typhimurium (3, 21). Urocanate could not be used as an inducer for histidase and urocanase, since there is a permeability barrier for uro-

canate in S. typhimurium. However since imidazolepropionate, an analog of urocanate, can induce histidase and urocanase in S. typhimurium, it is possible that urocanate may have exerted an inductive effect similar to that in A. aerogenes. The coordinate control over the synthesis of histidase and urocanase, as well as the close linkage of the genes responsible for the formation of these enzymes suggested that they belonged to a single operon.

Lessie and Neidhardt (16) reported that in P. aeruginosa, histidase, urocanase, and formiminoglutamate hydrolase were induced by growth of the organism on histidine and urocanate, or growth in the presence of dihydrourocanate, a gratuitous inducer. The enzymes of this pathway were shown to be sensitive to catabolite repression.

Succinate was shown to control the histidine catabolic pathway in P. putida by negative feedback inhibition (11). Succinate inhibited urocanase, which caused the accumulation of urocanate. Urocanate was shown to inhibit histidase competitively.

Regulation of Tryptophan Catabolism

The regulatory mechanisms governing the synthesis of the tryptophan catabolic enzymes in Pseudomonas flourescens were investigated by Palleroni and Stanier (28). The synthesis of tryptophan pyrrolase and formylkynurenine formamidase were found to be induced coordinately by L-kynurenine. The other enzymes of the pathway studied were induced sequentially by their respective substrates.

Regulation of Branched Chain Amino Acid Catabolism

A preliminary regulatory study of the isobutyrate portion of the

valine catabolic pathway in P. putida was reported by Schmidt (37). 3-Hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase were found to be induced by isobutyrate, and repressed by succinate.

Although Schmidt made these observations concerning the regulation of a section of the valine catabolic pathway, the nature of the regulation of branched chain amino acid catabolism for the most part is unknown. Two of the primary obstacles which have previously blocked a study concerning the regulation of branched chain amino acid catabolism were the lack of mutants blocked in the catabolism of these amino acids, and the inability to assay several early catabolic reactions. The early part of the present research was devoted to the development of several new assays which could be used to measure these reactions. In another study, Martin (20) isolated several presumptive valine catabolic mutants of P. putida.

Statement of Project

The present study is directed toward the elucidation of the nature of the regulation of branched chain amino acid catabolism in P. putida. In addition to the regulation of the synthesis of D-amino acid dehydrogenase, branched chain amino acid transaminase, branched chain oxo acid dehydrogenase, isobutyryl-CoA dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, and methylmalonate semialdehyde dehydrogenase, the allosteric regulation of branched chain oxo acid dehydrogenase was investigated.

It may be of interest that P. putida was chosen as the experimental organism, since previous work from this laboratory concerned

valine catabolism in P. aeruginosa. P. putida was primarily selected because the genetics of this organism have been reasonably well studied (10). However it was also advantageous to use P. putida, since mutants blocked in valine catabolism have previously been isolated from this organism (20). In the future when linkage studies are made of the branched chain amino acid catabolic markers, it will be valuable to have made these preliminary studies in an organism with a workable transduction system.

It is of interest that prior to the present study so little was known concerning the regulation of branched chain amino acid catabolism, despite the occurrence of a hereditary syndrome, Maple Syrup disease, which involves a metabolic block in the catabolism of valine, leucine, and isoleucine (22, 23). Maple Syrup disease usually results in infant death, but older mentally retarded children have been observed with this condition (6). Investigations into the regulation of branched chain amino acid catabolism may yield information directly pertinent to medical knowledge, as well as that important to the understanding of normal cellular metabolism.

CHAPTER II

METHODS

Culture Methods

Organisms

P. putida, C1S-WT from the laboratory of Dr. I. C. Gunsalus was the organism used in the major portion of this investigation; additionally, valine catabolic mutants of this organism, MUS 5, a branched chain amino acid transaminase mutant, and MUS 17, a branched chain oxo acid dehydrogenase mutant were supplied by R. R. Martin from the laboratory of Dr. L. Unger. These mutants were isolated and tentatively characterized by growth on several valine catabolic intermediates (20). MUS 5 was demonstrated in this study to be a branched chain amino acid transaminase mutant by the assay of Taylor and Jenkins (49), and MUS 17 shown to be a branched chain oxo acid dehydrogenase mutant by the branched chain oxo acid dehydrogenase assay developed in this study. The MUS nomenclature was developed by Martin (20). In addition, *P. aeruginosa* from the stock cultures of Dr. J. R. Sokatch was used. This was the same strain of *P. aeruginosa* used in all previous studies reported from this laboratory.

P. putida was grown at 30 C on an inorganic salts medium described in the next section with the carbon source added at the appropri-

ate concentration. It was necessary to supplement valine media with 0.005% L-isoleucine (12) to overcome valine toxicity (50) in P. putida. P. aeruginosa was grown on the same medium at 37 C.

Stock cultures of P. putida were maintained on 2% nutrient agar slants at 4 C. P. aeruginosa was kept at 4 C on the inorganic salts medium with 0.3% DL-valine as the carbon source with 2% agar. Both P. putida and P. aeruginosa were subcultured at monthly intervals.

Media

The basal medium used in this study was previously described by Jacobson (12) and Martin (20). The following are the ingredients of the basal medium per liter: K_2HPO_4, 43.5 g; KH_2PO_4, 17 g; and NH_4Cl, 21.4 g. The basal medium was diluted ten-fold with water, and was sterilized by steam under pressure at 121 C for 15 min.

Salts S described by Jacobson (12) and Martin (20) was used to provide the necessary inorganic materials not supplied in the basal medium. The following are the ingredients of Salts S per liter of aqueous 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; $Na_2MoO_4 \cdot 2\ H_2O$, 0.48 g; $CaCl_2$, 0.33 g; and NaCl, 0.12 g. This solution was sterilized through a 250 mμ sintered glass filter.

The complete medium was made by combining the presterilized subfractions. Per liter this medium contains: basal medium, 100 ml; Salts S, 10 ml; carbon source, appropriate amount; and enough water to bring the final volume to 1 liter.

Mass Culture

Loopfulls of cells, transferred from a stock culture of P. putida

or P. aeruginosa, were aseptically inoculated into 5.0 ml amounts of complete medium, and were shaken overnight at 30 or 37 C. After this period the 5 ml cultures were introduced into 1 liter batches of complete medium, which were incubated at the same temperature for the appropriate period of time with aeration provided by a bubbler. These cells were either harvested directly by centrifugation when used as a source of enzyme, or they were inoculated into 14 liters of complete medium in a fermenter produced by Fermentation Design, Inc. In the fermenter the organism was grown with aeration and agitation at 30 or 37 C to the end of log phase growth. Growth was followed by diluting aliquots of the culture five-fold with water, and reading at 660 mμ with a Bausch and Lomb Spectronic 20 spectrophotometer. Optical densities are reported as optical density units per ml. When the culture approached stationary phase the cells were harvested at 0 C with the continuous flow attachment to the Servall RC-2 refrigerated centrifuge. The cells were frozen and kept at -15 C.

Synthesis of Substrates

Isobutyryl-CoA, methacrylyl-CoA, and 3-hydroxyisobutyryl-CoA were synthesized by methods described by Stadtman (45) which were based on the method of Simon and Shemin (39). 3-Hydroxyisobutyrate was synthesized according to the method of Robinson and Coon (32), and methylmalonate semialdehyde was prepared by the technique of Kupiecki and Coon (15).

Enzyme Preparation

For the preparation of the enzyme, 1 to 5 g of the organism (wet weight) were suspended in 100 ml of 0.05 M potassium phosphate buf-

fer, pH 7.5, and were centrifuged for 15 min at 10^4 g at 5 C. The pellet was resuspended in 15 ml of the same buffer, and was subjected to sonic distruption with a Raytheon 10 kc sonic oscillator for 10 min, or a Branson Model S 75 sonifier from 5 to 10 min depending on the preparation desired. The resulting material was centrifuged as previously mentioned, and the supernatant fluid was used as the source of enzyme.

To eliminate reactions which interfered with the 3-hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase it was necessary to centrifuge the enzyme preparation at 3×10^4 g with the Beckman Model L-2 ultracentrifuge for 30 min. The supernatant fluid was then used for 3-hydroxyisobutyrate and methylmalonate semialdehyde dehydrogenase assays.

Protein Determination

Protein concentrations were determined by the method of Warburg and Christian (52). The enzyme was diluted with 0.05 M phosphate buffer, pH 7.5, and read against a blank containing the same buffer at 260 and 280 mμ with the Beckman model DU spectrophotometer.

Assay of Enzymic Activity

D-Amino Acid Dehydrogenase

D-Amino acid dehydrogenase was assayed by the method of Norton, Bulmer, and Sokatch (25). The enzymic activity was assayed by following the reduction of 2,6-dichlorophenolindophenol at 600 mμ with the Beckman model DU spectrophotometer equipped with a Gilford model 2000 multiple absorbence recorder. The spectrophotometer was equipped with thermospacers through which water at 37 C was allowed to flow to keep the tem-

perature constant. The standard assay was performed with 2 cuvettes, and was read with the spectrophotometer set with a blank which contained all reagents except substrate. The first cuvette contained 50 μmoles of amino acid, enzyme, 300 μmoles of tris buffer, pH 7.5, 20 μmoles of NaCN, 40 μg of 2,6-DCPIP, and was brought to 3 ml with water. The second cuvette was used as a blank and a control. It contained all reagents except amino acid. The extinction coefficient of 21 was used to calculate the amount of dye reduced in μmoles (47). One unit of enzyme activity was that amount which caused the reduction of 1 mμmole 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 (49). L-valine and 2-oxoglutarate, 20 μmoles each, and 100 μmoles of tris buffer, pH 7.0, were placed in test tubes. Water was added to take the final volume to 3 ml after the addition of enzyme. The mixture was allowed to equilibrate for 5 min at 37 C. Reactions were then initiated by the addition of 1 to 5 mg of enzyme, and were allowed to incubate for 10 min at 37 C. The reactions were terminated by the addition of 1.0 ml fo 2,4-dinitrophenylhydrazine reagent made by the method described by Taylor and Jenkins (49). 2,4-Dinitrophenylhydrazone formation was allowed to proceed for 10 min at room temperature before the 2,4-dinitrophenylhydrazone of 2-oxoisovalerate was selectively extracted with ethyl acetate. The 2,4-dinitrophenylhydrazone of 2-oxoisovalerate was then extracted with 5 ml of ethyl acetate, and centrifuged for 1 min; 3.5 ml of the upper phase (organic) was transferred to another tube and

was extracted with 2 ml of carbonate reagent made by adding 5.3 g of anhydrous Na_2CO_3, 0.83 g of $NaHCO_3$, and 200 g of Na_2SO_4 per liter of water. The tube was centrifuged for 1 min, and 3.0 ml of the organic phase was placed in another centrifuge tube. Five ml of petroleum ether (b.p. range 30 to 60 C) and 1.5 ml of 0.1 N Na_2CO_3-0.01 N $NaHCO_3$ were added in succession. After having been shaken well and centrifuged for 1 min, 1 ml of the lower phase (aqueous phase) was removed and was treated with 2 ml of 1 N NaOH. The color change was read at 440 mμ with the Beckman model DU spectrophotometer. By a standard curve it was demonstrated that 0.95 optical density units equals 1 μmole of 2-oxoisovalerate. One unit of transaminase activity was set equal to 1 mμmole of 2-oxoisovalerate produced per min.

Branched Chain Oxo Acid Dehydrogenase

The branched chain oxo acid dehydrogenase assay was developed during the course of the present study. The oxidation of 2-oxoisovalerate and the other branched chain oxo acids was followed by measuring the production of NADH spectrophotometrically at 340 mμ. The spectrophotometer and the recorder system were described earlier in this chapter. The standard assay was performed with 2 cuvettes, and was read with the spectrophotometer set with a blank which contained all reagents except substrate. The first cuvette contained 10 μmoles of 2-oxoisovalerate, enzyme, 100 μmoles of phosphate buffer, pH 8.0, 10 μmoles of NAD, 1 μmole of CoA, and enough water to take the total volume to 1 ml. The second cuvette contained all reagents except substrate, and was used as a blank and as control. The μmolar extinction coefficient of 6.22 was used to calculate the amount of NADH produced. One unit of enzyme activity was

defined as 1 mμmole of NADH produced per min.

Isobutyryl-CoA Dehydrogenase (EC 1.3.99.2)

The oxidation of isobutyryl-CoA was assayed by the method developed by Green et al. (7) for butyryl-CoA dehydrogenase. Into 10 ml test tubes were placed 1.5 μmoles of isobutyryl-CoA, enzyme, 30 μmoles of tris buffer, pH 8.0, 160 μg of pyocyanine, 2.0 mg of 2,3,5-triphenyltetrazolium, 300 μg of serum albumin, and sufficient water to bring the final volume to 0.5 ml. Two blanks, one containing no enzyme and one without substrate, were set up at the same time. The tubes were placed inside of a vacuum desiccator for 1 hr. Air was admitted and 1 drop of 1 N HCl, 1.5 ml of acetone, and 4.5 ml of carbon tetrachloride were added to each tube. The red formazan was extracted into the organic phase, and the layers were separated by centrifugation. The optical density at 485 mμ was then determined with the Beckman model DU spectrophotometer. To determine the number of μmoles of formazan formed the optical density was multiplied by 0.5 (7). One unit of enzymic activity was defined as 1 mμmole of formazan produced per min.

Crotonase (EC 4.2.1.17)

The assay for the hydration of methacrylyl-CoA was based on a technique developed by Stern, del Campillo, and Raw (46). To the experimental cuvette were added 0.2 ml of assay mixture, 100 μmoles of methacrylyl-CoA, enzyme, and sufficient water to bring the volume to 3 ml. The assay mixture contained 1 mmole of tris buffer, pH 7.5, 1 mg of serum albumin, 15 μmoles of potassium EDTA, pH 7.4, and enough water to take the final volume to 3 ml. The blank contained all reagents except sub-

strate. The disappearance of the double bond was followed spectrophotometrically at 263 mμ with the recorder and spectrophotometer system described earlier in this chapter. The molar extinction coefficient for this reaction was reported to be 6700 (46). One unit of crotonase activity was defined as the hydration of 1 mμmole of methacrylyl-CoA per min.

3-Hydroxyisobutyryl-CoA Deacylase (EC 3.1.2.4)

The deacylation of 3-hydroxyisobutyryl-CoA was measured by the nitroprusside assay of Grunert and Phillips (8) as modified by Stadtman (45). To the reaction vessel were added 0.5 μmoles of substrate, enzyme, 80 μmoles of tris buffer, pH 8.0, and sufficient water to bring the final volume to 0.4 ml. This mixture was allowed to incubate aerobically for 3 min at 37 C. A control was used which contained all reagents except substrate. After incubation the contents of these tubes were allowed to react with 0.4 ml of saturated ammonium sulfate, 0.2 ml of 2% nitroprusside, and 0.2 ml of cyanide reagent. The cyanide reagent was made by the addition of 20.7 g of K_2SO_3 and 0.33 g of NaCN to 100 ml of water. A standard curve made with glutathione demonstrated that 1 optical density unit at 540 mμ is equivalent to 1.5 μeq of sulfhydryl group. One unit of enzyme activity was defined as 1 mμmole of CoA liberated per min.

3-Hydroxyisobutyrate Dehydrogenase (EC 1.1.1.31)

The assay used to measure the oxidation of 3-hydroxyisobutyrate to methylmalonate semialdehyde was based on the method of Robinson and Coon (31). 3-Hydroxyisobutyrate dehydrogenase was assayed spectrophotometrically by determination of the rate of NADH production at 340 mμ with

the same spectrophotometer and recorder combination described in the section on D-amino acid dehydrogenase. The assay system contained per ml: 100 μmoles of tris buffer, pH 9.2, 10 μmoles of 3-hydroxyisobutyrate, 1 μmole of NAD, enzyme, and water. The reaction was initiated by the addition of substrate, and the increase of optical density was recorded. The extinction coefficient of 6.22 was used to calculate activity in terms of μmoles. One unit of enzyme activity was defined as 1 mμmole of NADH formed per min.

Methylmalonate Semialdehyde Dehydrogenase

The standard assay for the oxidation of methylmalonate semialdehyde was the same assay developed by Sokatch, Sanders and Marshall (42). The assay system contained 100 μmoles of tris buffer, pH 9.2, 200 μmoles of β-mercaptoethanol, 1 μmole of NAD, 5 μmoles of methylmalonate semialdehyde, enzyme, and enough water to bring the final volume to 1 ml. The reaction was initiated by addition of substrate, and was followed at 340 mμ by the spectrophotometer and recorder combination described earlier in this chapter. The extinction coefficient for this assay was the same as used in the previous assay. One unit of enzyme activity was described as 1 mμmole of NADH formed per min.

CHAPTER III

RESULTS

Development of Enzyme Assays

D-Amino Acid Dehydrogenase

D-Amino acid dehydrogenase of P. putida was assayed by following the reduction of 2,6-dichlorophenolindophenol spectrophotometrically. This same assay was used in previous studies (19, 25) with P. aeruginosa. The D-amino acid dehydrogenase catalyzes reaction 1', Fig. 1. Fig. 4 shows enzyme activity plotted as a function of protein concentration. Activity was proportional to enzyme concentration up to 6 mg of protein per 3 ml of reaction mixture.

Branched Chain Amino Acid Transaminase

Transamination between L-valine and 2-oxoglutarate was previously studied in P. aeruginosa (26, 27). This reaction is shown in Fig. 1 as reaction 1. The activity of the transaminase in P. putida was measured spectrophotometrically by quantitation of the 2,4-dinitrophenylhydrazones of 2-oxoisovalerate formed per unit time. This assay was employed instead of the chromatographic assay of Norton and Sokatch (26), because it was faster, although not as flexible. The activity of the enzyme was linearly proportional with protein concentration up to 15 mg of protein

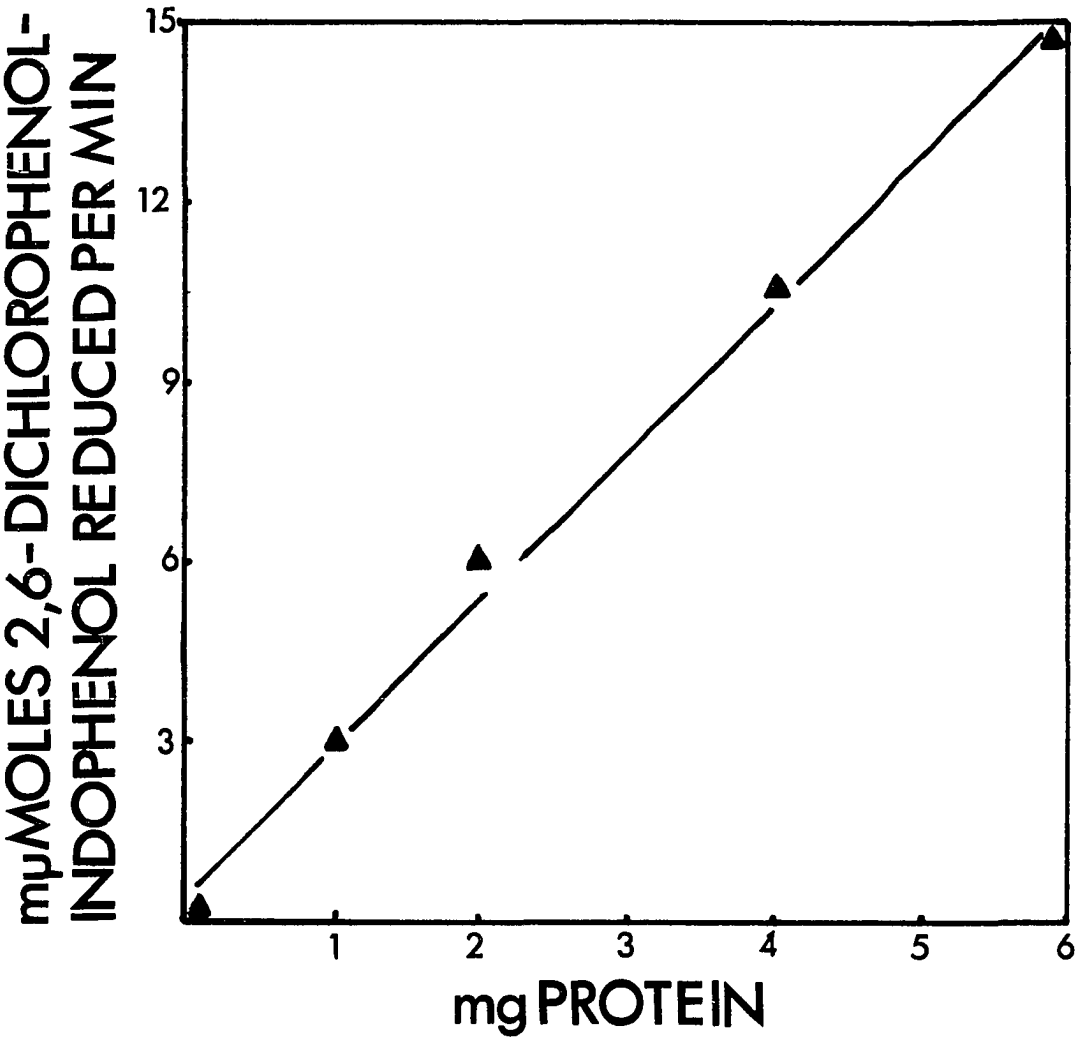

Figure 4. Activity of D-amino acid dehydrogenase as a function of protein concentration.

per ml (Fig. 5). The buffer pH optimum of this reaction was determined for P. putida (Table 1), and was found to be pH 7.0.

Branched Chain Oxo Acid Dehydrogenase

The activity of the branched chain oxo acid dehydrogenase was measured by following the production of NADH spectrophotometrically at 340 mμ, reaction 2, Fig. 1. The rate of NADH production was linearly proportional to enzyme concentration up to 0.65 mg of protein per ml (Fig. 6). The optimum pH of the buffer used in the assay was pH 8.0 (Table 2). The level of substrate necessary to saturate the enzyme was determined by varying the level of substrate in the standard assay from 0 to 10 μmoles of 2-oxoisovalerate per ml. Maximal activity was achieved at a substrate concentration of 0.3 μmoles per ml (Fig. 7); there was no substrate inhibition at 10 μmoles of 2-oxoisovalerate per ml. Branched chain oxo acid dehydrogenase was active with 2-oxoisovalerate, 2-oxoisocaproate, 2-oxo-3-methylvalerate, and 2-oxoglutarate, but not with pyruvate (Table 3). The product of reaction 2, Fig. 1 was treated with hydroxylamine, and the derivative was identified by the procedure of Yamada and Jacoby (54) as isobutyryl hydroxamate. This strongly indicated that isobutyryl-CoA was a product of the oxidation of 2-oxoisovalerate.

Isobutyryl-CoA Dehydrogenase

The reaction catalyzed by isobutyryl-CoA dehydrogenase is reaction 3, Fig. 1. The activity of isobutyryl-CoA dehydrogenase was assayed by the measurement of formazan produced in the procedure described in the Methods section. The rate of formazan production was linearly proportional to enzyme concentration from 1 to 4 mg of protein per ml (Fig. 8).

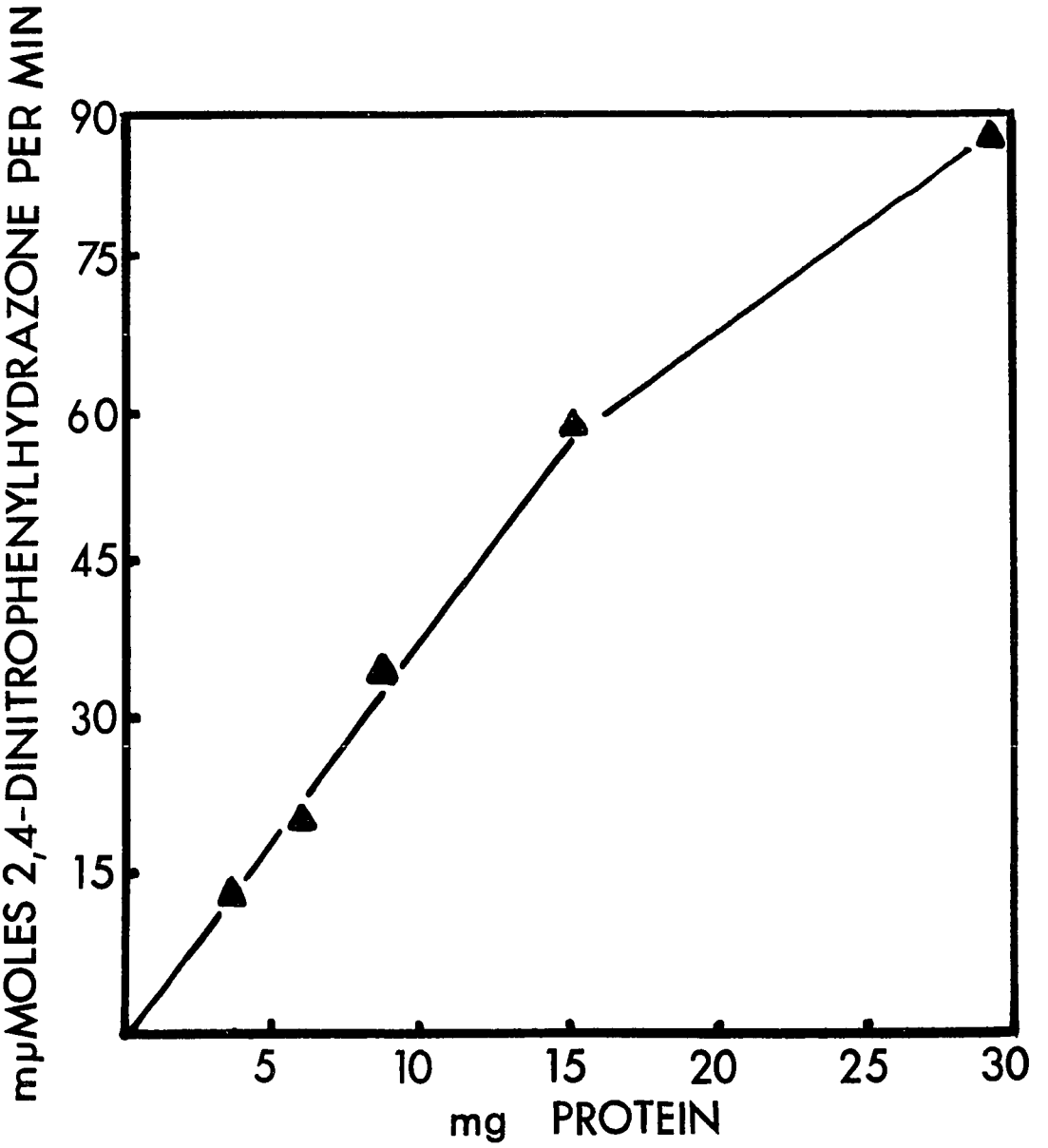

Figure 5. Activity of branched chain amino acid transaminase as a function of protein concentration.

TABLE 1

pH OPTIMUM OF BRANCHED CHAIN AMINO ACID TRANSAMINASE OF *P. putida*

Buffer pH	Specific Activity (mμmoles branched chain oxo acid per min per mg protein)
6.5	17.0
7.0	19.0
7.5	16.0
8.0	1.3
8.5	2.2
9.0	1.9

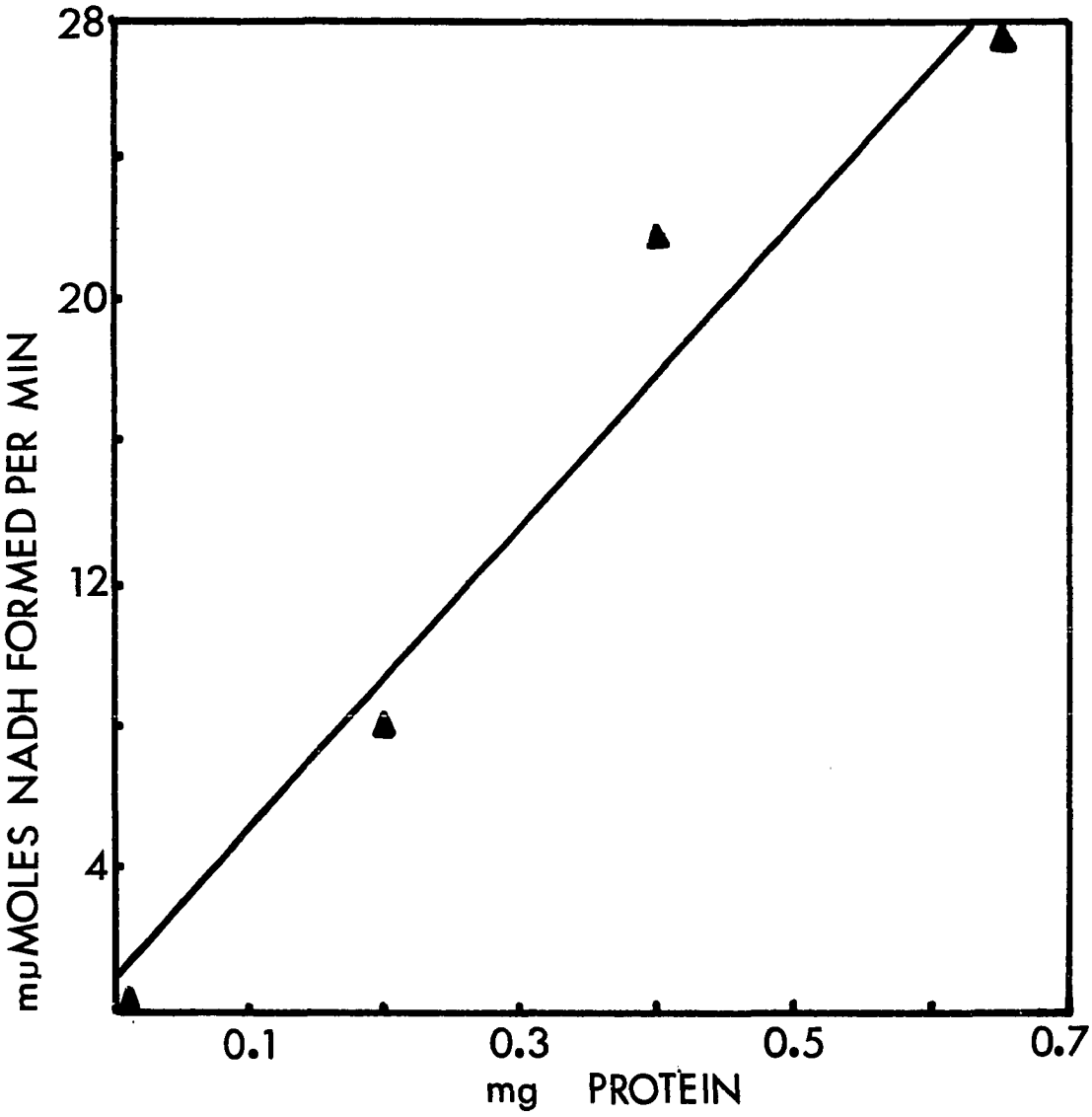

Figure 6. Activity of branched chain oxo acid dehydrogenase as a function of protein concentration.

TABLE 2

pH OPTIMUM OF BRANCHED CHAIN OXO ACID DEHYDROGENASE

Buffer pH	Specific Activity (mμmoles NADH per min per mg protein)
6.5	8
7.0	15
7.5	25
8.0	30
8.5	26
9.0	26

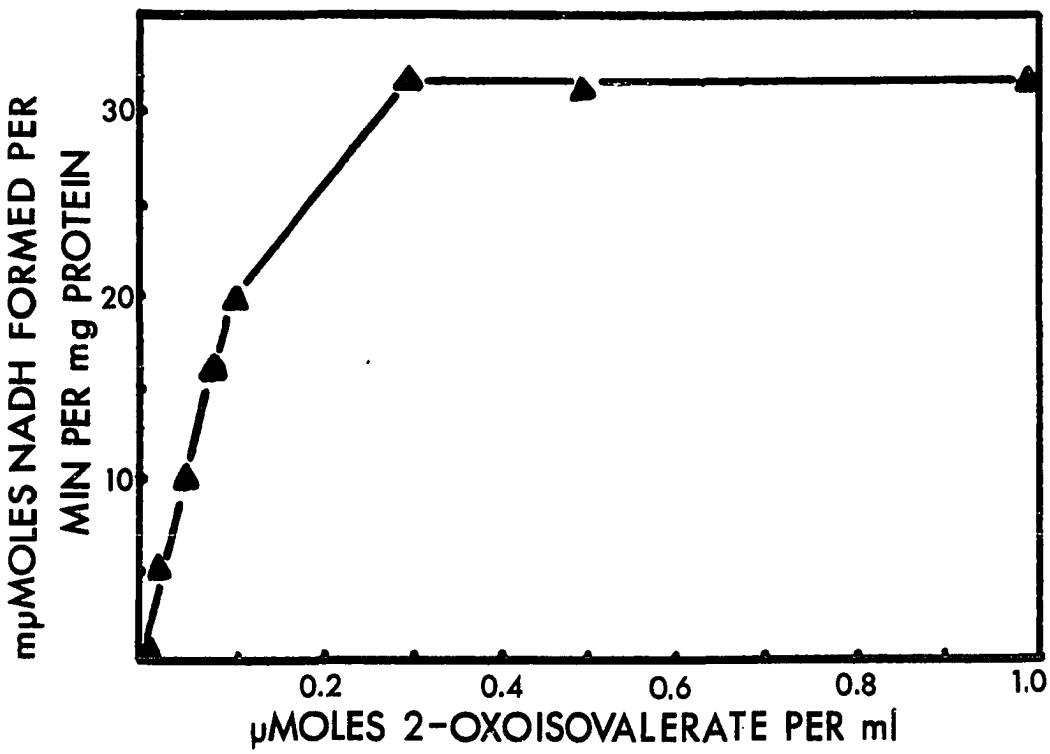

Figure 7. NADH production as a function of 2-oxoisovalerate concentration in the assay for branched chain oxo acid dehydrogenase.

TABLE 3

SUBSTRATE SPECIFICITY OF BRANCHED CHAIN OXO ACID DEHYDROGENASE

Substrate	Specific Activity (mμmoles NADH per min per mg protein)
2-Oxoisocaproate	50
2-Oxoisovalerate	20
2-Oxo-3-methylvalerate	14
2-Oxoglutarate	7
Pyruvate	0

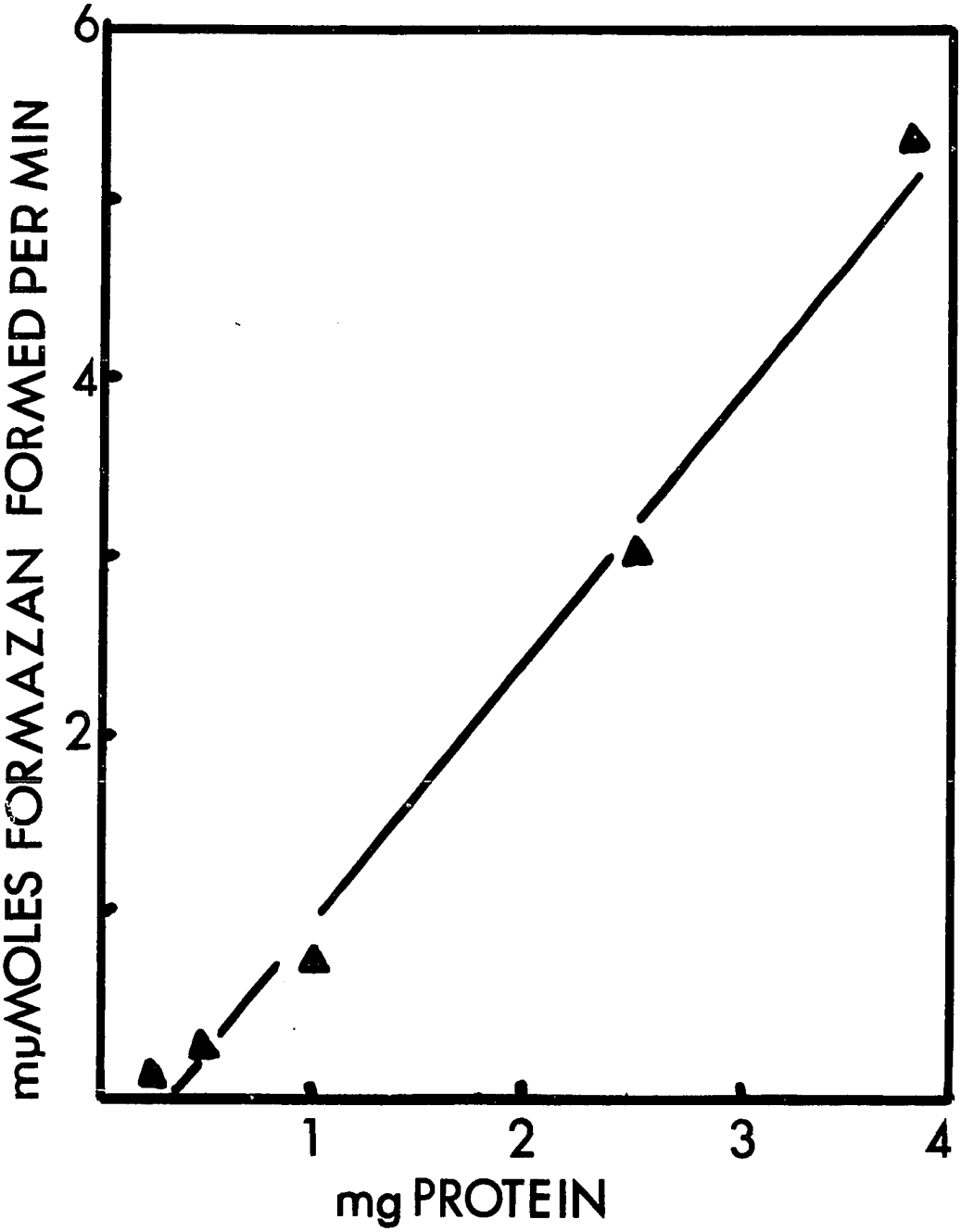

Figure 8. Activity of isobutyryl-CoA dehydrogenase as a function of protein concentration.

The buffer pH optimum of isobutyryl-CoA dehydrogenase was pH 8.0. Table 4 shows the activity of isobutyryl-CoA dehydrogenase at several buffer pH's. The amount of substrate necessary to saturate the enzyme was determined by varying the level of substrate from 0 to 3.0 μmoles of isobutyryl-CoA per ml. Maximal activity was achieved at about 2.0 μmoles per ml. These data are shown in Fig. 9. The crude enzyme preparations were active with isobutyryl-CoA, butyryl-CoA, and propionyl-CoA (Table 5). The product of reaction 3, Fig. 1 was treated with hydroxylamine, and the derivative was identified by the procedure of Yamada and Jacoby (54) as 3-hydroxyisobutyryl hydroxamate. Methacrylyl-CoA is known to be spontaneously hydrated to 3-hydroxyisobutyryl-CoA (30) which may account for the isolation and identification of 3-hydroxyisobutyryl hydroxamate instead of methacryl hydroxamate. These data indicated that methacrylyl-CoA was the product of the dehydrogenation of isobutyryl-CoA. The oxidation of isobutyryl-CoA was linear with time from 0 to 90 min (Fig. 10).

Crotonase

The hydration of methacrylyl-CoA (Fig. 1, reaction 4), measured spectrophotometrically at 263 mμ (46), was linear with protein concentration up to 0.6 mg of protein per 3 ml (Fig. 11).

3-Hydroxyisobutyryl-CoA Deacylase

The deacylation of 3-hydroxyisobutyryl-CoA (Fig. 1, reaction 5) was measured by the nitroprusside assay presented in the Methods section (8, 45). The production of CoASH was linear with increasing enzyme concentration from about 0.25 to 1.50 mg of protein per ml (Fig. 12).

TABLE 4

pH OPTIMUM OF ISOBUTYRYL-CoA DEHYDROGENASE

pH	Specific Activity (mμmoles formazan per min per mg protein)
6.5	0.38
7.0	0.33
7.5	0.30
8.0	0.80
8.5	0.53
9.0	0.38

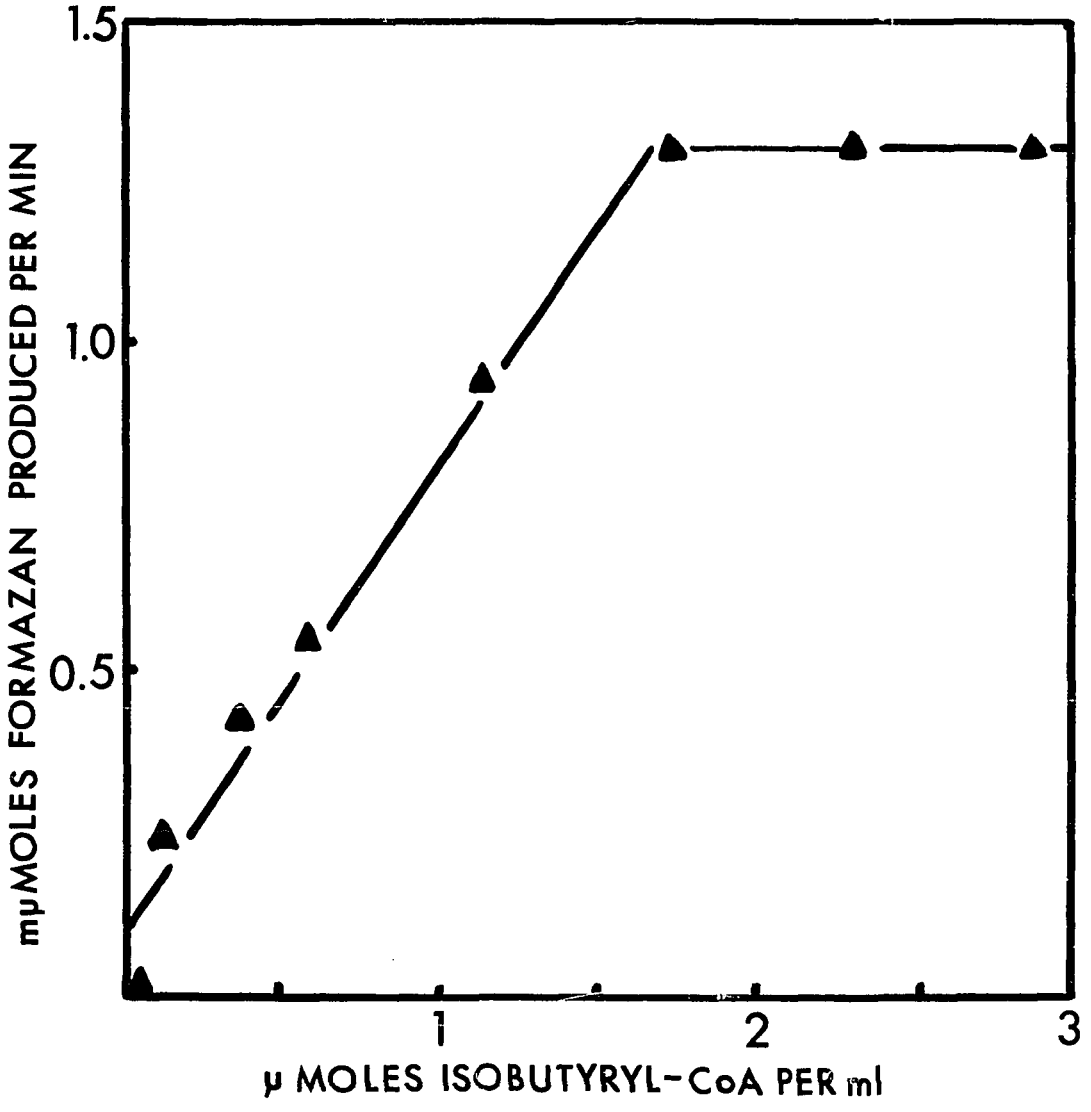

Figure 9. Formazan production as a function of isobutyryl-CoA concentration in the assay for isobutyryl-CoA dehydrogenase.

TABLE 5

SUBSTRATE SPECIFICITY OF ISOBUTYRYL-CoA DEHYDROGENASE

Substrate	Specific Activity (mμmoles of formazan per min per mg protein)
Butyryl-CoA	1.8
Isobutyryl-CoA	1.3
Propionyl-CoA	0.5

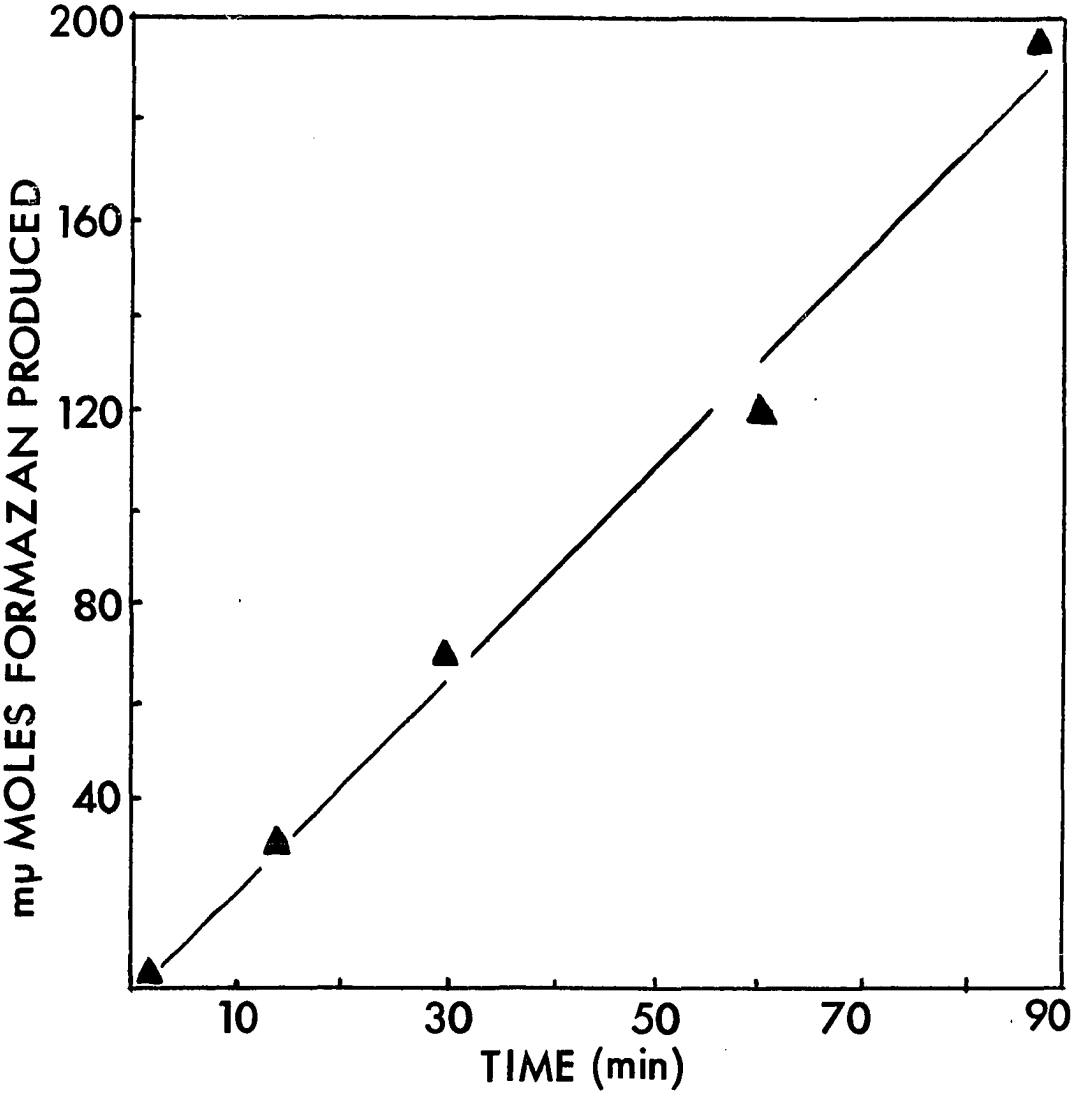

Figure 10. Proportionality of isobutyryl-CoA dehydrogenase activity to time.

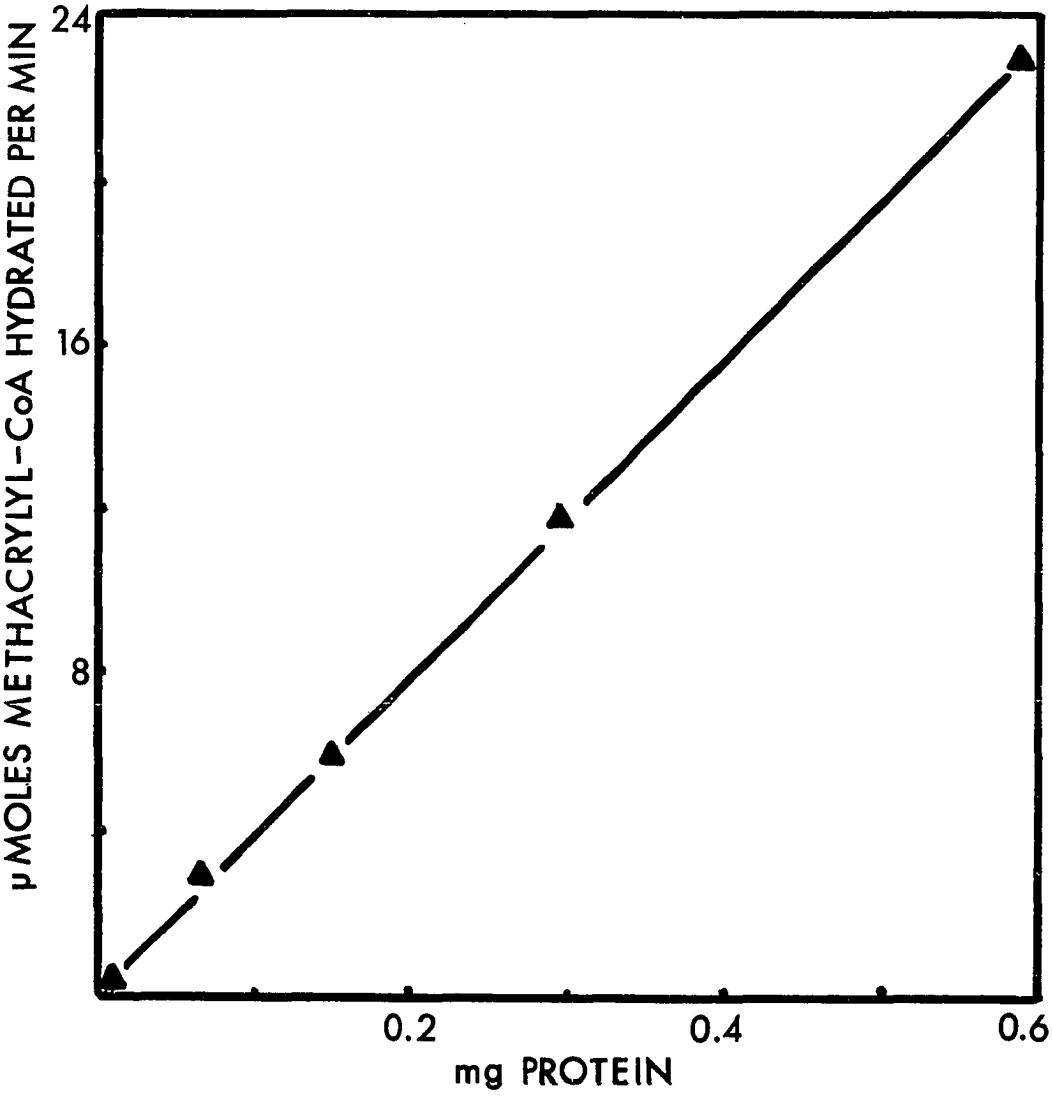

Figure 11. Activity of crotonase as a function of protein concentration.

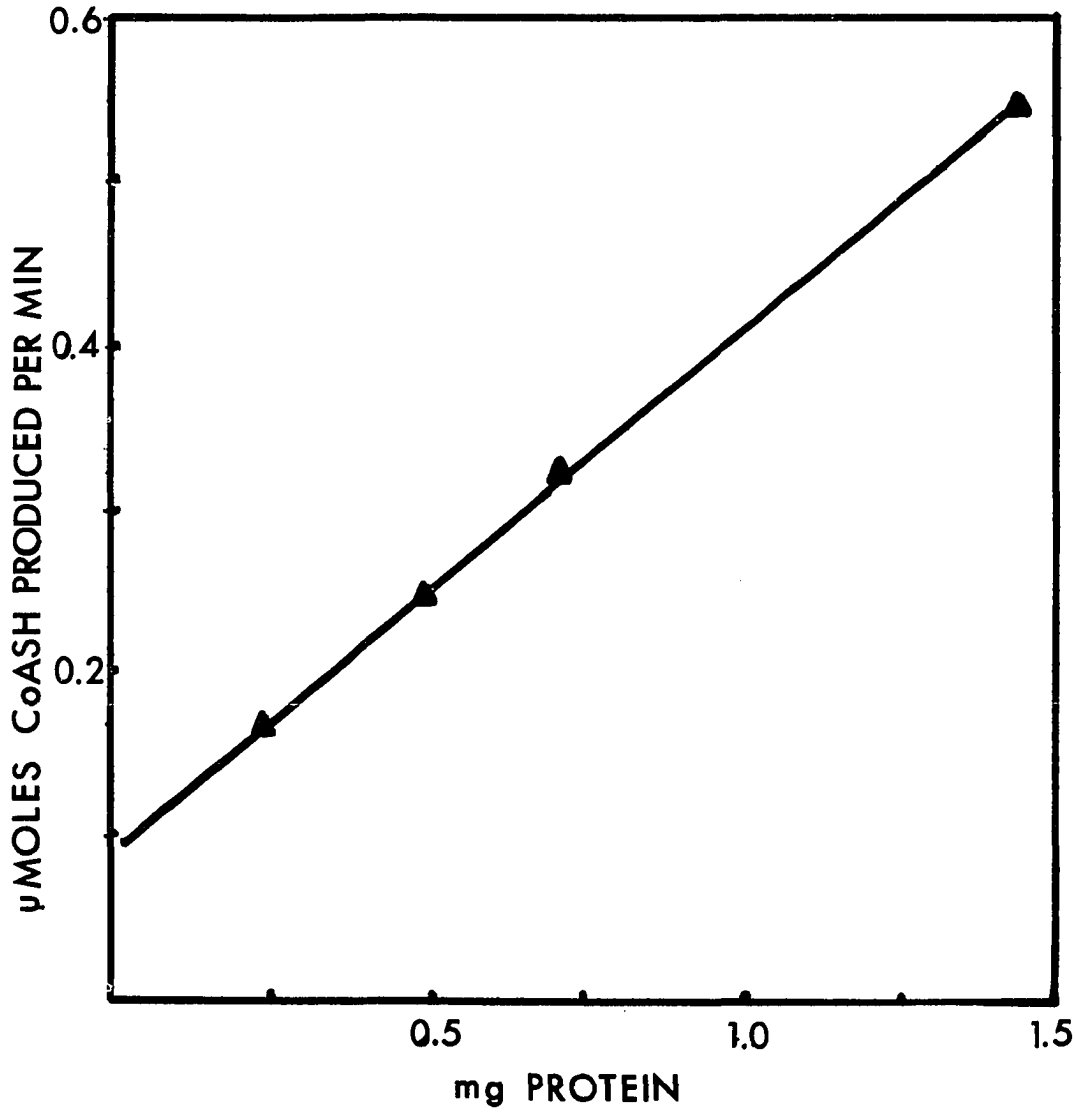

Figure 12. Activity of 3-hydroxyisobutyryl-CoA deacylase as a function of protein concentration.

3-Hydroxyisobutyrate Dehydrogenase

The activity of 3-hydroxyisobutyrate dehydrogenase was followed by measuring the production of NADH spectrophotometrically (31). The same assay was used to measure this reaction in P. aeruginosa (44). 3-Hydroxyisobutyrate dehydrogenase catalyzes reaction 6, Fig. 1. Enzyme activity was linear with protein concentration up to approximately 0.50 mg of protein per ml. These data appear in Fig. 13.

Methylmalonate Semialdehyde Dehydrogenase

The activity of methylmalonate semialdehyde dehydrogenase was measured spectrophotometrically by quantitation of NADH produced (Fig. 1, reaction 7). This assay was developed to measure the dehydrogenation of methylmalonate semialdehyde in P. aeruginosa (42). Enzyme activity was linear with protein concentration up to 0.5 mg of protein per ml (Fig. 14).

Regulation of the Synthesis of Valine Catabolic Enzymes

Induction by Valine of Enzymes in Wild Type Cells

The inducibility of the valine catabolic enzymes in P. putida, C1S-WT was determined by growth of the organism in individual cultures with 0.3% L-valine-0.005% L-isoleucine, 0.3% L-glutamate, 0.3% glucose, and 0.3% succinate as the sole carbon sources. Succinate and glucose are known to repress the formation of inducible catabolic enzymes by catabolite repression (17); therefore, the inducible enzymes formed in cells grown on these carbon sources are formed at a basal level. If the valine catabolic enzymes are inducible they should be formed at elevated

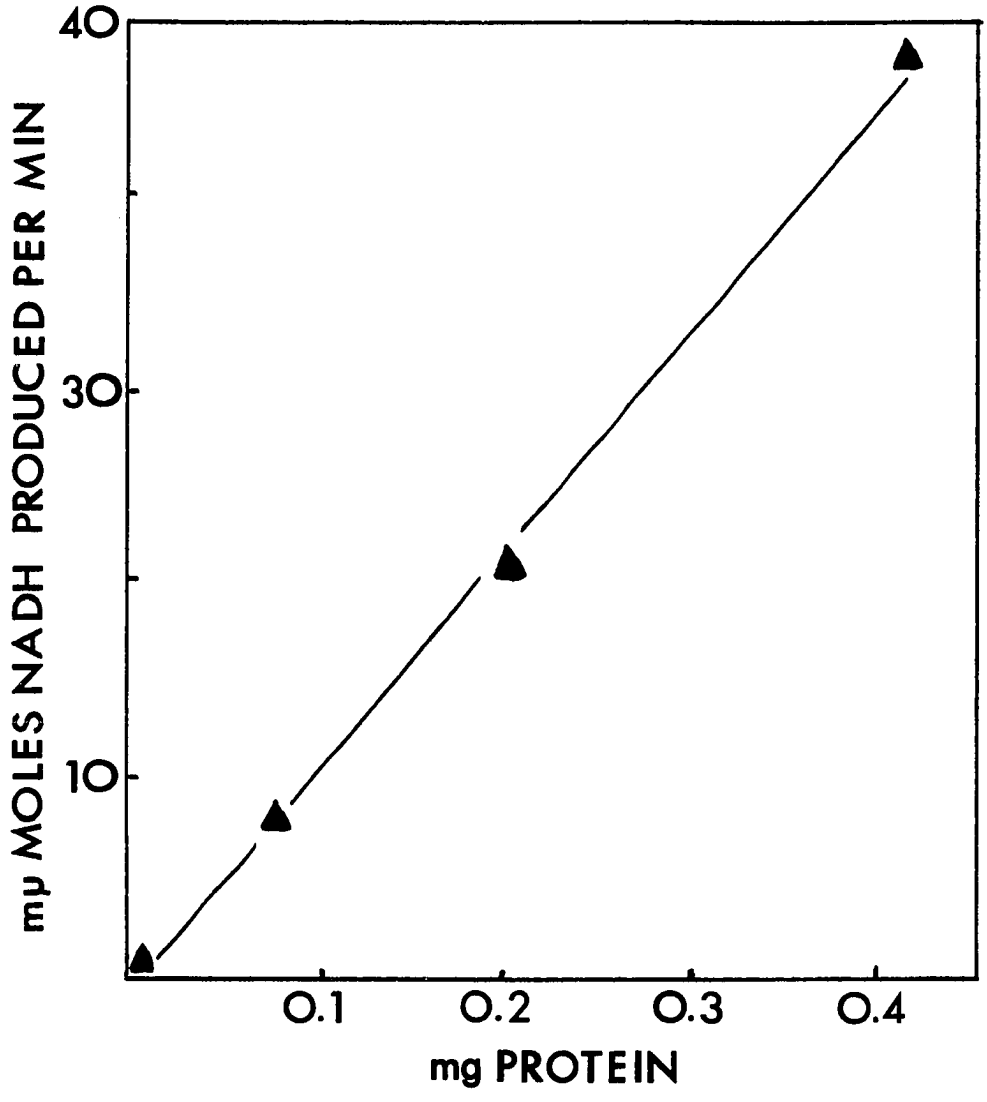

Figure 13. Activity of 3-hydroxyisobutyrate dehydrogenase as a function of protein concentration.

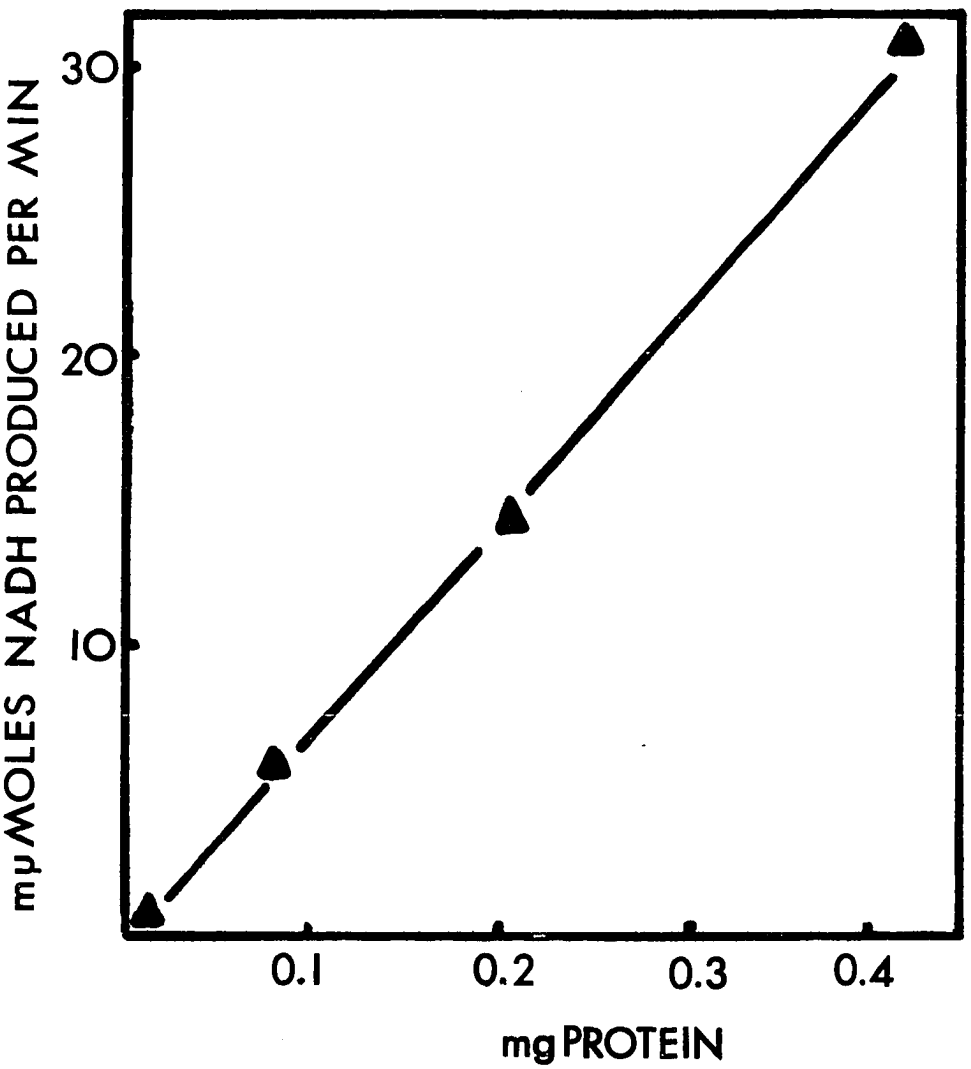

Figure 14. Activity of methylmalonate semialdehyde dehydrogenase as a function of protein concentration.

levels when the organism is grown on valine-isoleucine. Glutamate does not act as a catabolite repressor (9), and should not induce the enzymes of the valine catabolic pathway.

When the cultures were harvested the turbidities expressed in optical density units per ml at 660 mμ were: valine-isoleucine, 1.20; glutamate, 0.90; succinate, 0.75; and glucose, 1.50. The optical densities were determined and the cells were harvested by the procedures listed in the Methods section. Crude enzyme preparations were made and assayed for D-amino acid dehydrogenase, branched chain amino acid transaminase, branched chain oxo acid dehydrogenase, isobutyryl-CoA dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, and methylmalonate semialdehyde dehydrogenase. The results of this study are presented in Table 6. Branched chain amino acid transaminase and isobutyryl-CoA dehydrogenase activities were present in cells grown on succinate, glucose, and glutamate at the same levels as found in cells grown on valine-isoleucine; therefore, the synthesis of these enzymes was constitutive. D-Amino acid dehydrogenase, and branched chain oxo acid dehydrogenase were inducible, since enzyme activity was present only in cells grown on valine-isoleucine. In addition, 3-hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase were inducible, since elevated activities were found only in cells grown on valine-isoleucine.

To study the kinetics of induction of the valine catabolic enzymes, P. putida, C1S-WT was grown on a medium containing 0.3% glucose as the sole source of carbon to the middle of the logarithmic growth phase in the 14 liter fermenter previously described. At this time enough DL-valine and DL-isoleucine were added to the growing cells to bring their

TABLE 6

INDUCTION BY VALINE OF SEVERAL VALINE CATABOLIC ENZYMES IN *P. putida*, C1S-WT

Enzyme	Growth Substrates			
	0.3% L-Valine 0.005% L-Isoleucine	0.3% L-Glutamate	0.3% Succinate	0.3% Glucose
	mμmoles per min per mg protein			
D-Amino Acid Dehydrogenase	4	0	0	0
Branched Chain Amino Acid Transaminase	27	27	27	28
Branched Chain Oxo Acid Dehydrogenase	29	0	0	0
Isobutyryl-CoA Dehydrogenase	1	1	1	1
3-Hydroxyiso-butyrate Dehydrogenase	170	20	10	10
Methylmalonate Semialdehyde Dehydrogenase	48	12	7	7

respective concentrations to 0.1% and 0.005%. At various times thereafter 50 ml aliquots of growth were taken as sources of enzyme. Samples were taken before induction at initial log phase and near middle log phase at an interval of 90 min. After induction, samples were taken at 30 min intervals until the culture approached stationary phase. All samples were immediately frozen in an acetone-dry ice bath. The activities of D-amino acid dehydrogenase, branched chain oxo acid dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, and methylmalonate semialdehyde dehydrogenase increased linearly with increase in total protein, indicating that there was induction of these enzymes by growth in the presence of valine (29). These results are shown in Fig. 15.

Induction by Branched Chain Amino Acids of Enzymes in Wild Type Cells

Cultures of P. putida, C1S-WT were grown individually in the presence of the following carbon sources: 0.3% L-valine-0.005% L-isoleucine, 0.3% L-isoleucine, 0.3% L-leucine, and 0.3% glucose. The object of this experiment was to determine if isoleucine and leucine exerted a regulatory influence on the synthesis of the valine catabolic enzymes. When harvested the optical densities per ml were: valine-isoleucine, 1.40; isoleucine, 1.60; leucine, 1.45; and glucose, 1.55. Enzyme preparations were made from the harvested cells, and the activities of the inducible valine catabolic enzymes were measured (Table 7). The D-amino acid dehydrogenase was induced by growth of the organism on all of the branched chain amino acids. The branched chain oxo acid dehydrogenase was induced by growth of the organism on all of the branched chain amino acids, but to the greatest extent by growth on isoleucine. 3-Hydroxyiso-

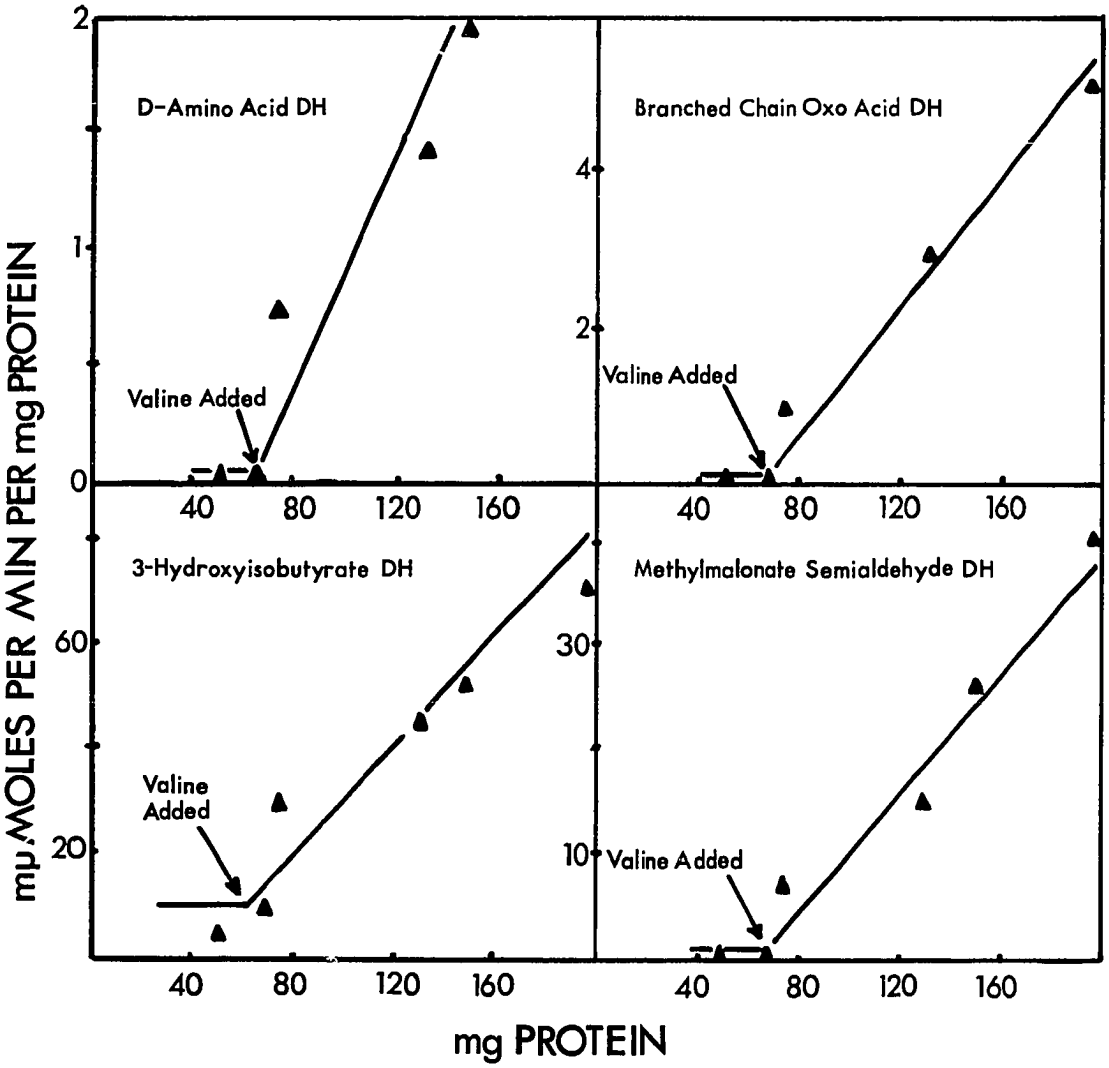

Figure 15. Induction of valine catabolic enzymes.

TABLE 7

INDUCTION BY BRANCHED CHAIN AMINO ACIDS OF VALINE CATABOLIC ENZYMES IN *P. putida*, C1S-WT

Enzyme	Growth Substrates			
	0.3% L-Valine 0.005% L-Isoleucine	0.3% L-Isoleucine	0.3% L-Leucine	0.3% Glucose
	mμmoles per min per mg protein			
D-Amino Acid Dehydrogenase	1	1	1	0
Branched Chain Oxo Acid Dehydrogenase	80	200	40	0
3-Hydroxyisobutyrate Dehydrogenase	160	66	22	15
Methylmalonate Semialdehyde Dehydrogenase	50	27	12	8

butyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase catalyze reactions having no counterparts in the catabolism of leucine and isoleucine; therefore, one would expect only valine to induce these enzymes. However these enzymes were induced by growth on isoleucine at a level about four-fold above the repressed level observed when the cells were grown on glucose. In addition, leucine appeared to have a slight inductive effect.

Cultures of P. putida, C1S-WT were grown separately on various combinations of the branched chain amino acids to determine if these amino acids exerted a multivalent regulatory effect on the synthesis of the valine catabolic enzymes. These media contained 0.3% L-valine-0.005% L-isoleucine, 0.3% L-valine with 0.1% isoleucine, 0.3% L-valine-0.005% L-isoleucine and 0.1% L-leucine, and 0.3% L-valine with 0.1% L-isoleucine and 0.1% L-leucine. Cells were harvested after overnight growth as previously described. At harvest optical densities were per ml: valine-isoleucine, 1.60; valine and isoleucine, 1.70; valine-isoleucine with leucine, 1.00; and valine with leucine and isoleucine, 1.25. The data concerning this study are presented in Table 8. Combinations of the branched chain amino acids did not induce D-amino acid dehydrogenase to higher levels than did valine-isoleucine. 0.1% Isoleucine in combination with valine served as the best inducer for branched chain oxo acid dehydrogenase; however, leucine in combination with valine-isoleucine served to induce branched chain oxo acid dehydrogenase better than did valine-isoleucine. Although leucine induced branched chain oxo acid dehydrogenase (Table 7), the addition of leucine to valine and 0.1% isoleucine did not increase the rate of synthesis above that achieved by valine and 0.1%

TABLE 8

INDUCTION BY COMBINATIONS OF THE BRANCHED CHAIN AMINO ACIDS OF SEVERAL VALINE CATABOLIC ENZYMES IN *P. putida*, C1S-WT

Enzyme	Growth Substrates			
	0.3% L-Valine 0.005% L-Isoleucine	0.3% L-Valine 0.1% L-Isoleucine	0.3% L-Valine 0.005% L-Isoleucine 0.1% L-Leucine	0.3% L-Valine 0.1% L-Isoleucine 0.1% L-Leucine
	mμmoles per min per mg protein			
D-Amino Acid Dehydrogenase	4	5	3	3
Branched Chain Amino Acid Transaminase	26	24	26	26
Branched Chain Oxo Acid Dehydrogenase	33	170	60	160
Isobutyryl-CoA Dehydrogenase	1	1	1	1
3-Hydroxyisobutyrate Dehydrogenase	150	200	180	200
Methylmalonate Semialdehyde Dehydrogenase	66	90	70	80

isoleucine. 3-Hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase were also induced to the greatest extent in cells grown on valine and 0.1% isoleucine.

Induction by Isobutyrate and Propionate of Enzymes in Wild Type Cells

Cultures of P. putida, C1S-WT were grown overnight individually in these media: 0.3% isobutyrate, 0.3% propionate, 0.3% L-valine-0.005% L-isoleucine, and 0.3% glucose to determine the role of isobutyrate and propionate in the induction of the valine catabolic enzymes. The optical densities per ml at harvest were 1.55, 1.35, 1.50, and 1.50, respectively. The cells were harvested and the enzyme preparations were made as described earlier. Each preparation was assayed for D-amino acid dehydrogenase, branched chain oxo acid dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, and methylmalonate semialdehyde dehydrogenase (Table 9). Neither D-amino acid dehydrogenase, nor branched chain oxo acid dehydrogenase were induced by growth on isobutyrate or propionate; however, 3-hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase were induced by isobutyrate better than by valine-isoleucine, and to some extent by propionate.

Comparative Study of Enzyme Induction in P. putida, WT and P. aeruginosa, WT

P. putida, C1S-WT and P. aeruginosa, WT were grown overnight on media containing 0.3% L-valine-0.005% L-isoleucine, and on 0.3% glucose. P. putida grown on valine-isoleucine was harvested at an optical density per ml of 1.25, and 1.50 when grown on glucose. P. aeruginosa grown on valine-isoleucine was harvested at an optical density per ml of 1.60, and

TABLE 9

INDUCTION BY GROWTH OF P. putida, C1S-WT ON ISOBUTYRATE OF SEVERAL VALINE CATABOLIC ENZYMES

Enzyme	Growth Substrates			
	0.3% L-Valine 0.005% L-Isoleucine	0.3% Isobutyrate	0.3% Propionate	0.3% Glucose
	mμmoles per min per mg protein			
D-Amino Acid Dehydrogenase	1	0	0	0
Branched Chain Oxo Acid Dehydrogenase	50	0	0	0
3-Hydroxyisobutyrate Dehydrogenase	200	330	60	23
Methylmalonate Semialdehyde Dehydrogenase	70	140	50	17

1.55 when grown on glucose. The enzyme preparations were assayed for all six of the valine catabolic enzymes studied (Table 10). Branched chain amino acid transaminase, and isobutyryl-CoA dehydrogenase were formed in both organisms grown on glucose at the same levels found in cells grown on valine-isoleucine; therefore, these enzymes were constitutive. Branched chain oxo acid dehydrogenase was inducible in both organisms, but was induced to a higher level in P. putida. 3-Hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase were induced in both species; however, the level of enzyme activity was greater in P. putida. In addition, the synthesis of 3-hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase was not totally repressed by growth on glucose in P. putida as it was in P. aeruginosa; this could be due to a difference in the basal levels of these enzymes.

Induction by Branched Chain Amino Acids and Branched Chain Oxo Acids of Enzymes in P. putida, MUS 5

P. putida, MUS 5 was identified as a transaminase mutant (49), although the enzymic block was not complete (Table 11). Since growth of MUS 5 in the presence of valine does not result in the induction of branched chain oxo acid dehydrogenase, it appears that the block is tight enough to interfere with the induction of this enzyme by 2-oxoisovalerate (Table 11). P. putida, C1S-MUS 5 was grown overnight in individual cultures on these substrates: 0.1% 2-oxoisovalerate with 0.3% L-glutamate, 0.1% 2-oxoisocaproate with 0.3% L-glutamate, 0.1% 2-oxo-3-methylvalerate with 0.3% L-glutamate, 0.3% L-glutamate, and 0.1% L-valine-0.005% L-isoleucine with 0.3% L-glutamate. As a control, P. putida, C1S-WT was grown on a medium containing 0.1% L-valine-0.005% L-isoleucine with 0.3% L-

TABLE 10

COMPARISON OF VALINE CATABOLIC ENZYME INDUCTION IN *Pseudomonas*

Enzyme	Growth Substrates			
	0.3% L-Valine 0.005% L-Isoleucine	0.3% Glucose	0.3% L-Valine 0.005% L-Isoleucine	0.3% Glucose
	P. putida		*P. aeruginosa*	
	mμmoles per min per mg protein			
D-Amino Acid Dehydrogenase	2.5	0	2.5	0
Branched Chain Amino Acid Transaminase	30	33	30	29
Branched Chain Oxo Acid Dehydrogenase	40	0	7	0
Isobutyryl-CoA Dehydrogenase	1.5	1.7	1.5	1.7
3-Hydroxyisobutyrate Dehydrogenase	200	20	100	0
Methylmalonate Semialdehyde Dehydrogenase	100	10	62	0

TABLE 11

INDUCTION BY BRANCHED CHAIN OXO ACIDS OF VALINE CATABOLIC ENZYMES

Enzyme	0.3% L-Glutamate 0.1% L-Valine 0.005% L-Isoleucine	0.3% L-Glutamate 0.1% L-Valine 0.005% L-Isoleucine	Growth 0.3% L-Glutamate
	P. putida, WT	*P. putida*, MUS 5	*P. putida*, MUS 5
			mμmoles per
D-Amino Acid Dehydrogenase	1	1	0
Branched Chain Amino Acid Transaminase	52	28	33
Branched Chain Oxo Acid Dehydrogenase	45	0	0
Isobutyryl-CoA Dehydrogenase	1	1	1
3-Hydroxyiso-butyrate Dehydrogenase	150	100	28
Methylmalonate Semialdehyde Dehydrogenase	40	28	17

TABLE 11--Continued

Substrates		
0.3% L-Glutamate 0.1% 2-Oxoisovalerate	0.3% L-Glutamate 0.1% 2-Oxoisocaproate	0.3% L-Glutamate 0.1% 2-Oxo-3-methylvalerate
P. putida, MUS 5	*P. putida*, MUS 5	*P. putida*, MUS 5
min per mg protein		
0	0	0
33	33	31
15	10	15
1	1	1
50	31	33
25	16	27

glutamate. When the mutant was grown on these media the optical densities per ml at harvest were: 2-oxoisovalerate with glutamate, 1.50; 2-oxo-3-methylvalerate with glutamate, 1.55; 2-oxoisocaproate with glutamate, 1.50; glutamate, 1.65; and valine-isoleucine with glutamate, 1.60. When P. putida, WT was harvested its optical density per ml was 1.60. Enzyme preparations were assayed for D-amino acid dehydrogenase, branched chain amino acid transaminase, branched chain oxo acid dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, and methylmalonate semialdehyde dehydrogenase (Table 11). D-amino acid dehydrogenase was induced by growth on valine-isoleucine in the mutant and wild type, but not by oxo acids in the mutant or wild type. Since induction occurs in MUS 5 which is blocked in the transaminase, it appears that valine induces D-amino acid dehydrogenase. This eliminates the possibility of induction by a valine catabolic intermediate. Branched chain oxo acid dehydrogenase was apparently induced by the 2-oxo acids of the branched chain amino acids, since the transaminase mutant was not induced by valine, but only by the branched chain oxo acids. 3-Hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase were induced by valine in the transaminase mutant which indicated that valine, and not a valine catabolic intermediate, induced these enzymes, since valine is presumably not degraded in this organism. However, previous studies (Table 9) indicated that isobutyrate or valine were capable of inducing both of these enzymes.

In order to determine the inductive role of 2-oxoisovalerate in the synthesis of branched chain oxo acid dehydrogenase by P. putida, wild type cells and MUS 5 were grown in media containing 0.1% 2-oxoisovalerate with 0.3% L-glutamate. As a control, both organisms were grown

on 0.1% L-valine-0.005% L-isoleucine with 0.3% L-glutamate. These data are presented in Table 12. In the wild type organism, grown on valine-isoleucine with glutamate, the activity of branched chain oxo acid dehydrogenase was about two-fold greater than in the same organism grown on 2-oxoisovalerate with glutamate. This enzyme was not formed in the transaminase mutant grown on valine, since no 2-oxoisovalerate could be synthesized from L-valine at the appropriate level required for induction by this organism. However, branched chain oxo acid dehydrogenase was induced at low levels in the mutant by 2-oxoisovalerate added in the medium. These experiments indicated that branched chain oxo acid dehydrogenase was induced sequentially by 2-oxoisovalerate.

To determine the inductive role of several amino acids for D-amino acid dehydrogenase, MUS 5 was grown on these media: 0.1% L-valine-0.005% L-isoleucine with 0.3% L-glutamate, 0.1% L-isoleucine with 0.3% L-glutamate, 0.1% L-leucine with 0.3% L-glutamate, 0.1% L-alanine with 0.3% L-glutamate, and 0.3% L-glutamate. As a control, P. putida, C1S-WT was grown on 0.3% L-glutamate. The optical density per ml of the wild type cells at harvest was 1.24. The optical densities per ml for the mutant organism at harvest were: valine-isoleucine with glutamate, 1.40; isoleucine with glutamate, 1.70; leucine with glutamate, 1.35; alanine with glutamate, 1.55; and glutamate, 1.50. Enzyme preparations were made from each culture and assayed for D-amino acid dehydrogenase and branched chain amino acid transaminase. All of the amino acids tested, except glutamate induced the synthesis of D-amino acid dehydrogenase. These data are presented in Table 13.

Since P. putida, C1S-WT is known to have a D-amino acid dehydro-

TABLE 12

INDUCTION BY 2-OXOISOVALERATE OF BRANCHED CHAIN OXO ACID DEHYDROGENASE

Organism	Growth Substrate	Specific Activity (mμmoles NADH formed per min per mg of protein)
P. putida, C1S-WT	0.1% L-Valine with 0.005% L-Isoleucine and 0.3% L-Glutamate	18
	0.1% 2-Oxoisovalerate and 0.3% L-Glutamate	8
P. putida, C1S-MUS 5	0.1% L-Valine with 0.005% L-Isoleucine and 0.3% L-Glutamate	0
	0.1% 2-Oxoisovalerate and 0.3% L-Glutamate	7

TABLE 13

INDUCTION BY AMINO ACIDS OF D-AMINO ACID DEHYDROGENASE IN *P. putida*

Organism*	Growth Substrates	Specific Activity (mμmoles per min per mg protein)
P. putida, MUS 5	0.3% L-Glutamate	0
	0.1% L-Valine 0.005% L-Isoleucine 0.3% L-Glutamate	1.5
	0.1% L-Isoleucine 0.3% L-Glutamate	1.5
	0.1% L-Leucine 0.3% L-Glutamate	1.7
	0.1% L-Alanine 0.3% L-Glutamate	1.9

*As a control the transaminase was assayed in *P. putida*, WT and MUS 5 grown on L-glutamate; the activities, respectively, were 53 and 20 mμmoles per min per mg protein.

genase, it was necessary to determine if MUS 5 was able to circumvent the transaminase block by racemization of L-valine to D-valine. It is conceivable that P. putida could then oxidize D-valine to 2-oxoisovalerate where it would enter into the L-valine catabolic pathway, and possibly supply intermediates for the induction of other valine catabolic enzymes. Valine racemase was assayed by the method of Wood and Gunsalus (53) (Table 14), which demonstrated that P. putida was unable to racemize valine. As a control the assay was performed with extracts of P. aeruginosa which does not have a valine racemase (26). Alanine racemase was also determined, since P. aeruginosa is known to have this enzyme (26). Separate reaction mixtures with 20 μmoles each of D-valine and D-alanine were prepared in order to determine the accuracy of recovery of these amino acids. The data in Table 14 provide evidence that there is not a valine racemase in P. putida.

The ability of P. putida to use D-valine as a source of carbon was studied. P. putida, C1S-WT was inoculated into media containing the following carbon sources: 0.3% D-valine-0.005% L-isoleucine, 0.3% L-valine-0.005% L-isoleucine, 0.3% D-alanine, and 0.3% L-alanine. P. aeruginosa, WT which has the ability to use valine as a carbon source, was inoculated into the same media. All cultures were incubated aerobically at 34 C, and the growth was measured at 24, 36, and 48 hr by the method previously described. The results of these studies demonstrated that P. putida could not grow on D-valine as the sole carbon source (Table 15). This finding is also evidence that a valine racemase is not present in P. putida.

TABLE 14

AMINO ACID RACEMASE ACTIVITY IN *Pseudomonas*

Organism	Substrate	µmoles Substrate	µmoles D-Amino Acid Observed
P. putida, WT	L-Alanine	80	48
	L-Valine	80	3
P. aeruginosa, WT	L-Alanine	80	40
	L-Valine	80	2.6
Control	D-Alanine	20	15.8
	D-Valine	20	19.4

TABLE 15

GROWTH OF *Pseudomonas* ON AMINO ACIDS

Organism	Growth Medium	Optical Density at 660 mμ		
		24 hr	36 hr	48 hr
P. putida, WT	0.3% L-Valine 0.005% L-Isoleucine	0.65	2.00	2.20
	0.3% D-Valine 0.005% L-Isoleucine	0.10	0.20	0.25
	0.3% L-Alanine	0.70	1.65	1.75
	0.3% D-Alanine	0.70	1.75	1.75
P. aeruginosa, WT	0.3% L-Valine 0.005% L-Isoleucine	0.55	2.85	3.15
	0.3% D-Valine 0.005% L-Isoleucine	0.40	2.95	3.00
	0.3% L-Alanine	0.45	2.60	2.85
	0.3% D-Alanine	0.50	2.55	2.75

Induction by Valine of Enzymes in the Branched Chain Oxo Acid Dehydrogenase Mutant

P. putida, C1S-MUS 17 was found to be completely blocked in branched chain oxo acid dehydrogenase (Table 16). To support the finding with MUS 5 that valine induces 3-hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase over the transaminase block, P. putida, C1S-MUS 17 was grown overnight in media containing 0.1% L-valine-0.005% L-isoleucine with 0.3% L-glutamate, 0.3% L-glutamate, and 0.3% glucose. As a control, P. putida, C1S-WT was grown on 0.1% L-valine-0.005% L-isoleucine with 0.3% L-glutamate. The mutant cells were harvested at these optical densities per ml: valine-isoleucine with glutamate, 1.75; glutamate, 1.80; and glucose 1.80. The wild type control was harvested at an optical density per ml of 1.90. Enzyme preparations made from the cells in each culture were used as a source for D-amino acid dehydrogenase, branched chain oxo acid dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, and methylmalonate semialdehyde dehydrogenase. The results of these assays are presented in Table 16. Since MUS 17 is blocked at the branched chain oxo acid dehydrogenase, the induction of 3-hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase was due to either valine or 2-oxoisovalerate.

Specificity of Branched Chain Oxo Acid Dehydrogenase

To determine if catabolism of the branched chain oxo acids occurs by one enzyme or enzyme complex, MUS 17 (branched chain oxo acid dehydrogenase mutant) was grown on 0.1% L-valine and 0.1% L-isoleucine with 0.3% L-glutamate. As a control, the wild type organism was grown on the same medium. Enzyme preparations were made from both organisms, and

TABLE 16

INDUCTION OF VALINE CATABOLIC ENZYMES IN P. putida, C1S-MUS 17

Enzyme	Growth Substrates			
	0.1% L-Valine 0.005% L-Isoleucine	0.1% L-Valine 0.005% L-Isoleucine	0.3% L-Glutamate	0.3% Glucose
	P. putida, WT	P. putida, C1S-MUS 17		
	mμmoles per min per mg protein			
D-Amino Acid Dehydrogenase	1	1	0	0
Branched Chain Oxo Acid Dehydrogenase	20	0	0	0
3-Hydroxyiso-butyrate Dehydrogenase	100	65	23	16
Methylmalonate Semialdehyde Dehydrogenase	38	40	17	8

assayed with all of the branched chain oxo acids used as substrates (Table 17). Since a single mutation blocks the oxidation of all three oxo acid substrates, there was apparently only one enzyme or enzyme complex responsible for their oxidation.

Regulation of Branched Chain Oxo Acid Dehydrogenase Activity in P. putida C1S-WT by Leucine and Isoleucine

To determine if branched chain oxo acid dehydrogenase was allosterically regulated by branched chain amino acids, enzyme extracts were treated with leucine, isoleucine and valine to determine if these compounds altered the rate of catalysis.

P. putida, C1S-WT was grown overnight on 0.3% DL-valine-0.005% DL-isoleucine, 0.3% DL-valine and 0.1% DL-isoleucine, and 0.3% DL-isoleucine. The cells were harvested and crude enzyme preparations were made from each culture. The activity of branched chain oxo acid dehydrogenase was assayed in each of the enzyme preparations. In addition, the preparation from the cells grown on valine with minimal isoleucine was assayed in the presence of 3 mM DL-isoleucine, and 3 mM DL-leucine. Both of these effectors served to stimulate branched chain oxo acid dehydrogenase activity several fold (Table 18).

To determine the optimal effector concentration for branched chain oxo acid dehydrogenase, extracts were made from P. putida, C1S-WT grown on 0.3% L-valine-0.005% L-isoleucine, and were assayed in the presence of several concentrations of L-valine, L-isoleucine, and L-leucine (Table 19). L-isoleucine and L-leucine were demonstrated to be regulators that exerted their optimal effect at 1 mM. L-valine was found not to be a regulator. Low molecular weight effectors may have been present to alter enzymic activity.

TABLE 17

SPECIFICITY OF BRANCHED CHAIN OXO ACID DEHYDROGENASE

Assay Substrate	*P. putida*, C1S-WT	*P. putida*, C1S-MUS 17
	mμmoles per min per mg protein	
2-Oxoisovalerate	20	0
2-Oxo-3-methyl-valerate	12	0
2-Oxoisocaproate	40	0

TABLE 18

REGULATION BY ISOLEUCINE AND LEUCINE OF BRANCHED CHAIN OXO ACID DEHYDROGENASE ACTIVITY IN *P. putida*, C1S-WT

Addition to Assay	Growth Substrates		
	0.3% DL-Valine 0.005% DL-Isoleucine	0.3% DL-Isoleucine	0.3% DL-Valine 0.1% DL-Isoleucine
	mμmoles per min per mg protein		
None	16	75	117
3 mM DL-Isoleucine	62	-	-
3 mM DL-Leucine	42	-	-

TABLE 19

OPTIMAL EFFECTOR CONCENTRATIONS FOR REGULATION BY BRANCHED CHAIN AMINO ACIDS OF BRANCHED CHAIN OXO ACID DEHYDROGENASE IN P. putida, C1S-WT GROWN ON VALINE AND ISOLEUCINE

Effector	Effector Concentration				
	No Addition	0.1 mM	0.5 mM	1.0 mM	2.5 mM
	mμmoles per min per mg protein				
No Addition	150	-	-	-	-
L-Isoleucine	-	250	350	520	440
L-Leucine	-	350	480	566	566
L-Valine	-	160	160	160	160

CHAPTER IV

DISCUSSION

The aims of this study concerning the regulation of branched chain amino acid catabolism, and those studies on tryptophan (28) and histidine (3, 5, 9, 11, 16, 18, 21, 35, 36) were to determine the nature of the regulation of the catabolism of the branched chain amino acids, histidine, and tryptophan. Although the reactions of histidine, tryptophan, and valine catabolism are significantly different, some aspects of the regulation of each pathway are common to all of the pathways. (a) It is interesting that most of the amino acid catabolic enzymes are inducible, but not necessarily by the initial substrate. However, the initial substrate induces some of the enzymes of each pathway. (b) In addition, each pathway has enzymes which are induced by sequential mechanisms.

The valine catabolic pathway also has two constitutive enzymes, branched chain amino acid transaminase and isobutyryl-CoA dehydrogenase.

Since branched chain amino acid transaminase is active with methionine and phenylalanine, as well as with the branched chain amino acids (27), it becomes more apparent why this enzyme is not induced by growth of P. putida on the branched chain amino acids. In addition, it is possible that the same transaminase is active in valine catabolism and anabolism. If this situation applies, it would seem advantageous for the

transaminase to be constitutive. The significance of the constitutivity of isobutyryl-CoA dehydrogenase is not understood.

The allosteric regulation of amino acid catabolic enzymes remains largely uninvestigated. Evidence was presented that urocanase was sensitive to feedback inhibition by succinate (11). This thesis provides evidence that the activity of branched chain oxo acid dehydrogenase was regulated allosterically by leucine and isoleucine. The regulatory effect of these amino acids on branched chain oxo acid dehydrogenase will be the subject of further research. Since isobutyryl-CoA dehydrogenase occurs at the convergence of two pathways, and is not inducible, it might be advantageous to the organism for isobutyryl-CoA dehydrogenase to be regulated allosterically.

The mutants of P. putida, C1S used in these studies were previously isolated and tentatively characterized by growth of the organism on several valine catabolic intermediates. Prior to this study there were no assays available to measure the oxidation of 2-oxoisovalerate, and the dehydrogenation of isobutyryl-CoA in bacteria. Therefore, a prerequsite to further investigation of the regulation of branched chain amino acid catabolism was the development of these assays. Once developed, the assays were employed to characterize the mutants obtained by Martin (20). P. putida, MUS 5 was characterized by growth as a branched chain amino acid transaminase mutant. The organism was able to grow on 2-oxoisovalerate, but grew sparsely on valine. The assay of Taylor and Jenkins (49) indicated that this mutant had only about one half of the transaminase activity of wild type.

The other mutant of P. putida, C1S used in this study was MUS 17.

Growth experiments (20) and studies employing the branched chain oxo acid dehydrogenase assay developed during the course of this research indicated that MUS 17 had a genetic block in the branched chain oxo acid dehydrogenase.

The present study suggested that P. putida may have two branched chain amino acid transaminases, catabolic and anabolic. MUS 5 was unable to grow well on valine, but could grow on all other valine catabolic intermediates. However, the organism was not a valine auxotroph. These observations suggested the possibility that MUS 5 lacks the catabolic branched chain amino acid transaminase, but has the anabolic counterpart of the enzyme. Branched chain oxo acid dehydrogenase was not synthesized when MUS 5 was grown in the presence of valine, but was formed when the mutant was grown in the presence of branched chain oxo acids. Since growth of MUS 5 in the presence of valine does not result in the induction of branched chain oxo acid dehydrogenase, it appears that the catabolic block is tight enough to interfere with the induction of branched chain oxo acid dehydrogenase by 2-oxoisovalerate. This observation tends to further support the premise that there is a separate catabolic transaminase.

These data do not preclude the possibility that the partially active transaminase was able to provide valine for anabolism, but was too inactive for the organism to grow on valine as the only substrate.

The inducible valine catabolic enzymes studied included D-amino acid dehydrogenase, branched chain oxo acid dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, and methylmalonate semialdehyde dehydrogenase. D-Amino acid dehydrogenase, the enzyme which catalyzes the alternate

first step of valine catabolism (reaction 1', Fig. 1), was induced in P. putida by several amino acids. P. putida, MUS 5 grown on L-glutamate and induced either by L-valine, L-isoleucine, L-leucine, or L-alanine synthesized D-amino acid dehydrogenase. The organism did not form the enzyme when grown on glutamate alone. These data indicated that the branched chain amino acids (and alanine), and not a catabolic intermediate of the amino acids induced the D-amino acid dehydrogenase.

The same experiment provided evidence that branched chain oxo acid dehydrogenase was induced sequentially by its own substrates. When MUS 5 was grown in the presence of valine no branched chain oxo acid dehydrogenase was detected; however, when branched chain oxo acids were substituted for valine in the medium the enzyme was formed at low levels.

Growth in the presence of valine was shown to effect the induction of 3-hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase over the transaminase block in MUS 5, and over the branched chain oxo acid dehydrogenase block in MUS 17. In both experiments MUS 5 and 17 were grown on glutamate and valine. These experiments indicated that valine induces 3-hydroxyisobutyrate dehydrogenase, and methylmalonate semialdehyde dehydrogenase. It is interesting that these enzymes were also induced by growth on isobutyrate.

The induction of D-amino acid dehydrogenase, branched chain oxo acid dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, and methylmalonate semialdehyde dehydrogenase was studied by adding valine as the inducer to cells growing on glucose at the middle of the logarithmic phase of growth. Upon the addition of valine, the activities of these enzymes increased linearly with increased protein which indicated enzyme induction.

Branched chain amino acid transaminase and isobutyryl-CoA dehydrogenase were formed when P. putida was grown on glucose and succinate to the same levels as when the organism was grown on valine-isoleucine. This indicated that these enzymes were synthesized constitutively.

It is interesting that isoleucine, and to some extent leucine induced 3-hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase, since reactions comparable to these do occur in the leucine and isoleucine catabolic routes. However, it may be significant that cells grown on propionate have 3-hydroxyisobutyrate dehydrogenase activity comparable to cells grown on leucine. It may be that propionyl-CoA, an intermediate of leucine and valine catabolism, exerts an inductive effect on these enzymes. In addition, when P. putida was grown on a combination of valine and isoleucine, these enzymes were induced to higher levels than when cells were grown on valine-isoleucine. The significance of leucine and isoleucine as inducers, either individually or in combination with valine, of 3-hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase is not understood.

Since a single mutation (MUS 17) blocks the oxidation of all three of the branched chain oxo acids, it is likely that one enzyme or enzyme complex catalyzes this reaction. This thesis presents evidence that branched chain oxo acid dehydrogenase is induced in P. putida grown on the branched chain amino acids. Highest enzyme levels were seen when cells were grown in the presence of a mixture of valine and isoleucine, both in high concentration. Branched chain oxo acid dehydrogenase activity was greater in cells grown on high valine and isoleucine in combination than the cumulative activities of cells grown on valine and isoleu-

cine individually (Table 18). These findings may represent a multivalent induction mechanism for branched chain oxo acid dehydrogenase. In addition, isoleucine and leucine were shown to stimulate branched chain oxo acid dehydrogenase activity.

P. putida grown on valine and isoleucine synthesized several-fold more branched chain oxo acid dehydrogenase than when grown on valine-isoleucine. In addition, isoleucine or leucine in the reaction mixture stimulated the activity of branched chain oxo acid dehydrogenase. Isoleucine's role in the regulation of branched chain oxo acid dehydrogenase may be related to its own conservation when cells are grown in the presence of valine. Leucine's role in allosteric regulation is not understood.

Although assays for crotonase and 3-hydroxyisobutyryl-CoA deacylase were modified for use with Pseudomonas, these assays were not employed in the regulatory study, because of the time envolved in assaying the other six enzymes.

CHAPTER V

SUMMARY

A study of the regulation of branched chain amino acid catabolism was made in P. putida. To accomplish this study an assay was developed to measure the oxidation of the branched chain oxo acids, and assays for the dehydrogenation of isobutyryl-CoA, the hydration of methacrylyl-CoA, and the deacylation of 3-hydroxyisobutyryl-CoA, previously measured only in animal tissue, were modified for use with P. putida.

D-Amino acid dehydrogenase, 3-hydroxyisobutyrate dehydrogenase, and methylmalonate semialdehyde dehydrogenase were shown to be induced by valine. In addition, the D-amino acid dehydrogenase was induced by the other branched chain amino acids and by alanine. 3-Hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase were also induced by isobutyrate, and to some extent by growth on isoleucine, leucine and propionate. Branched chain oxo acid dehydrogenase was sequentially induced by its substrates, the branched chain oxo acids. Branched chain oxo acid dehydrogenase was apparently induced by a multivalent induction mechanism, since this enzyme was induced to higher levels in cells grown on a combination of valine and isoleucine than the cumulative level of cells grown individually on valine and isoleucine. Two of the valine catabolic enzymes, branched chain amino acid transaminase and isobutyryl-

CoA dehydrogenase were synthesized constitutively.

The oxidation of the branched chain oxo acids in P. putida is apparently achieved by a single enzyme or enzyme complex, since a single mutation blocks the oxidation of all of the branched chain oxo acids.

Branched chain oxo acid dehydrogenase activity was stimulated by leucine and isoleucine. Isoleucine's role in the induction and stimulation of branched chain oxo acid dehydrogenase may reflect a control mechanism geared to maintain the isoleucine concentration at a critical level to overcome valine toxicity.

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