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BRANCHED-CHAIN AMINO ACID METABOLISM
IN CLOSTRIDIUM SPOROGENES

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BRANCHED-CHAIN AMINO ACID METABOLISM IN *CLOSTRIDIUM SPOROGENES*

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ABSTRACT

BRANCHED-CHAIN AMINO ACID METABOLISM IN *CLOSTRIDIUM SPOROGENES*.

By

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Preliminary studies indicated that *C. sporogenes* (ATCC 7955, NCA PA 3679) made isoleucine in a novel pathway not involving threonine or threonine dehydratase. Data herein show that radioactivity from [3-^{14}C]pyruvate, [^{14}C]propionate and α-amino-[3-^{14}C] butyrate was incorporated into isoleucine. Increasing concentrations of α-methylbutyrate reduced the incorporation of [^{14}C]pyruvate. Crude extracts of the bacteria grown in a minimal medium were found to contain levels of α-acetohydroxyacid synthase activities comparable to those found in cells of *E. coli* K12 grown in minimal medium. Stepwise degradation of isoleucine labeled with specifically-labeled precursors supports the conclusion that *C. sporogenes* makes isoleucine via the reductive carboxylation of propionate to yield α-ketobutyrate which is metabolized to isoleucine in the classical fashion, or isoleucine can be made via the reductive carboxylation of α-methylbutyrate to α-keto-β-methylvalerate.

Clostridium sporogenes requires L-leucine and L-valine for growth in a minimal medium, although valine can be replaced by isobutyrate, and leucine by isovalerate. Cells

grown in minimal media incorporated significant ^{14}C-label from [^{14}C]valine into leucine and ^{14}C-label from [^{14}C]leucine into valine. These results indicate that these bacteria can interconvert leucine and valine.

Considerable label from [^{14}C]valine, [^{14}C]leucine and [^{14}C]isoleucine was incorporated into cellular lipid after growth of *C. sporogenes* in a minimal medium, with six times more radioactivity from labeled valine than leucine or isoleucine being incorporated. It appears that branched-chain amino acids were an important source of the branched-chain fatty acids in the cellular lipid. The branched-chain amino acids were shown to be subject to non-specific oxidation to volatile fatty acids in an assay with whole cells, and isoleucine oxidative activity appeared to be inducible to some extent.

DEDICATION

This dissertation is dedicated to my parents, William and Kathleen, my siblings Tom, John, Anne Marie, Mike, Jim, George and Bob, and especially my partner, Elizabeth. Their support, encouragement and diversionary tactics made the difficult times bearable, and the good times wonderful.

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SECTION IV

ORGANIZATION

This dissertation is organized into five sections. Section I consists of an introduction and review of the recent literature pertinent to the research discussed. Sections II and III consist of manuscripts for a paper and a note for publication. Section IV consists of results obtained which are related to the research but not included in manuscripts. A summary of all the research is presented in Section V.

SECTION I

INTRODUCTION

The proteolytic clostridia are a diverse group of saprophytic organisms and commensal inhabitants of the intestinal tract of animals. *Clostridium sporogenes* is often found in the soil, but is also encountered in contaminated food, and occasionally in necrotic tissue where *C. tetani* or *C. perfringens* have established an infections. *C. sporogenes* is physiologically indistinguishable from *C. botulinum* type A, a causative agent of botulism poisoning. These attributes have made *C. sporogenes* an important model for investigations of anaerobic physiology and energy metabolism.

It was in *C. sporogenes* that Stickland first demonstrated the coupled oxidation and reduction of paired amino acids which is the principle source of ATP in many clostridia and other anaerobes (24, 45). Much of our understanding of the unique strategies for amino acid catabolism employed by anaerobes comes from investigations with this species.

Studies of biosynthetic pathways in the anaerobes are infrequent, largely due to the difficulties which arise when using the complex media required by most anaerobes for growth. The development in our laboratory of a simple defined medium for *C. sporogenes* (R.S. Hadioetomo, 1980, Ph.D. dissertation, Michigan State University) has allowed us to investigate some biosynthetic activities of this organism.

Hadioetomo found that while _C_. _sporogenes_ requires leucine and valine for growth, it does not require isoleucine. This was a surprising observation since in most microbes these amino acids are produced via a common pathway (45). The threonine dehydratase (the first enzyme of isoleucine biosynthesis in _Escherichia coli_) activity in _C_. _sporogenes_ was found to be catabolic in nature, and was not regulated by isoleucine _in vitro_. Cells grown in a minimal medium incorporated label from $^{14}CO_2$, [^{14}C]serine and [3-^{14}C]pyruvate into isoleucine, but not from [^{14}C]threonine. In addition, radioactive carbon from α-[^{14}C]methylbutyrate was incorporated into isoleucine, presumably by reductive carboxylation to α-ketoβ-methylvalerate and subsequent transamination. These observations led Hadioetomo to propose that isoleucine was produced via a novel pathway in _C_. _sporogenes_.

The object of the experiments described in this dissertation was to elucidate branched-chain amino acid metabolism in _C_. _sporogenes_. The principal goal was to determine the nature of the unusual pathway for isoleucine synthesis. The results indicate that one pathway to isoleucine originates with propionate, which is reductively carboxylated to α-ketobutyrate. The condensation of α-ketobutyrate and pyruvate follows (catalyzed by acetohydroxyacid synthase), and isoleucine is then produced in the classical fashion. α-Methylbutyrate appears to be an alternate precursor of isoleucine. In addition, _C_. _sporogenes_ can interconvert leucine and valine when grown in appropriate media.

The picture of branched-chain amino acid metabolism which emerges is considerably more complex than that seen in *E. coli*. In addition to the drain on leucine, valine and isoleucine resulting from protein synthesis, *C. sporogenes* must also contend with continuous non-specific oxidation of these amino acids for ATP generation. α-Ketoisovalerate, the keto-acid analog of valine, is also the source of pantoate in the cell. All three branched-chain amino acids are liberally incorporated into cellular lipid as well. To some extent *C. sporogenes* takes advantage of its reduced environment to meet these special demands. The various reductive carboxylation reactions utilized are novel and important options for the supply and regulation of isoleucine, leucine and valine in these bacteria.

LITERATURE REVIEW

The Clostridia

The genus *Clostridium* encompasses a diverse group of anaerobic, spore-forming, rod-shaped bacteria (16, 21, 38). The clostridia are widely distributed in nature, and their natural habitats include soil, fresh and salt water, and the intestinal tracts of animals. Although usually considered gram positive, their reaction to this staining procedure is frequently variable. The bacteria are motile, relatively large rods which frequently are swollen when endospores form (hence, from the Greek *clostridium*; small spindle) although filamentous forms are sometimes observed. Spores are located centrally, terminally or subterminally while developing in the cell. A few species (including *C. perfringens*) have been observed to produce capsules. The bacteria possess flavoprotein enzymes which will readily reduce molecular oxygen (O_2) to hydrogen peroxide (H_2O_2) and to superoxide (O_2^-), but they lack catalase, peroxidase and superoxide dismutase activities which would protect them from these toxic products. Despite this inadequacy, different species show markedly different tolerances to oxygen; while most are strict anaerobes, *C. perfringens* can tolerate low O_2 levels, and *C. histolyticum* grows slowly on blood agar plates exposed to the air. Clostridial spores are notoriously resistant to heat, disinfectants, and lengthy exposure to air. Metabolically, the clostridia can be divided into two general physiological groups; the predominantly proteolytic or, the predominantly saccharolytic clostridia. Proteolytic clostridial fermentations usually result in the formation of volatile acids, ammonia, CO_2 and H_2. The ptomaines, putrescine and cadaverine, are produced by decarboxylation of ornithine and lysine, respectively.

The clostridia are generally thought of as saprophytic soil organisms and commensal inhabitants of the intestinal tracts of animals. However, several species are important pathogens, and are responsible for three major diseases in humans; botulism (C. botulinum types A, B, and E), tetanus (C. tetani), and gas gangrene (predominantly C. prefringens). None of these diseases are caused by the invasion of bacteria into healthy tissues. Botulism is a classic intoxication resulting from the ingestion of food containing preformed toxin. Tetanus and gas gangrene are usually the result of local clostridial growth in traumatized tissue, from which the soluble toxins are disseminated and a general toxemia results. Clostridium pasteurianum is a soil organism capable of fixing molecular nitrogen (N_2). Many other members of this genus manifest unusual and sometimes useful metabolic activities, and have been used in industrial fermentations for many years. The investigation of clostridial intermediary metabolism has revealed several novel pathways and activities, unique to these anaerobic bacteria.

Clostridium sporogenes

The most striking and significant feature of *Clostridium sporogenes* is its marked resemblence to *C. botulinum* type A. The sole reliable distinguishing property between these species is the elaboration of botulinum toxin by *C. botulinum* type A. Both species produce very resistant, oval, subterminal spores. They are proteolytic, and produce large amounts of butyric acid, and smaller amounts of acetic, isobutyric, isovaleric and isocaproic acids and propyl, isobutyl and isoamyl alcohols during fermentations. *C. sporogenes* and *C. botulinum* type A were found to ferment only fructose and glucose among 28 carbohydrates tested (38). The G + C content of both species is 26-28 moles %, and several strains show 100% DNA homology in DNA:DNA hybridization experiments (28). A pyrolysis gas-liquid chromatography study of *C. botulinum* and related organisms readily distinguished botulinum types A and B from types C and D, type E, and nonproteolytic types B and F (23), but failed to differentiate type A cultures from *C. sporogenes*. Recent investigations of the endproducts of aromatic amino acid fermentations (20), and branched-chain amino acids (19) show that the two species ferment these compounds in the same fashion. In a study of the quantitative utilization of amino acids by representative strains of the clostridia, *C. sporogenes* and *C. botulinum* type A were shown to utilize the amino acids to a similar extent (29).

The neurotoxin elaborated by C. botulinum type A is the most potent biological toxin known; 1 μg is sufficient to kill the average human. Not surprisingly, most investigators pursue their studies in the metabolically indistinguishable C. sporogenes, and subsequently confirm results in C. botulinum type A.

The precise nature of the relationship of C. sporogenes to C. botulinum is not clear. Ekland and Poysky (17) have demonstrated that when C. botulinum types C and D were cured of their prophages with mitomycin C, they concommitantly ceased to produce their dominant toxins. These cured, non-toxigenic strains of types C and D could then be converted to either toxigenic type C or D depending on the specific phage used. A similar interconversion of type C to C. novyi type A has been reported (18). Inducible bacteriophages have only recently been isolated from C. botulinum type A (43), but the relationship between toxigenicity and lysogeny, or phage conversion to toxigenicity has yet to be demonstrated. It is quite possible that C. sporogenes is an atoxigenic strain of C. botulinum types A by virtue of the loss of a temperate bacteriophage.

The intimate relationship of C. sporogenes to C. botulinum type A has led to considerable interest in the physiological characteristics of this species. Much of our current understanding of intermediary metabolism in anaerobes comes from studies with Clostridium sporogenes. The degradation of amino acids by anaerobic bacteria has recently been reviewed (4). These investigations have revealed several unique and unusual metabolic activities and pathways, found only in the anaerobes.

The important observation that many clostridia, while unable to grow with a single amino acid as a fermentable energy source would readily grow if provided with appropriate pairs of amino acids, was first made with C. sporogenes by Stickland in 1934 (4). The coupled oxidation-reduction of amino acid pairs (the Stickland reaction) has since been observed in many clostridia. C. sporogenes has an absolute requirement for selenium when using glycine as the oxidant in a Stickland reaction system (14). The bacteria oxidize the branched-chain amino acids valine, leucine, and isoleucine to isobutyrate, isovalerate and methylbutyrate in this fashion, although at least one strain converts leucine to isocaproate and isobutyrate utilizing a novel enzyme, leucine 2,3-aminomutase, which converts α-leucine to β-leucine (34). An investigation of ornithine metabolism in C. sporogenes has led to the elucidation of several novel enzymes, notably ornithine cyclase (deaminating) (31), ornithine 5,4-aminomutase (40), 2,4-diaminopentanoic acid C_4 dehydrogenase (39), and a reversible proline dehydrogenase which is regulated by glutamate (15, 30). The fermentation of the aromatic amino acids by C. sporogenes also proceeds in a novel fashion, and several unique enzyme activities have been identified including a 2-enoate reductase enzyme, which catalyzes the interconversion of E*-cinnamate to phenylpropionate in an NADH-dependent reaction (4, 11).

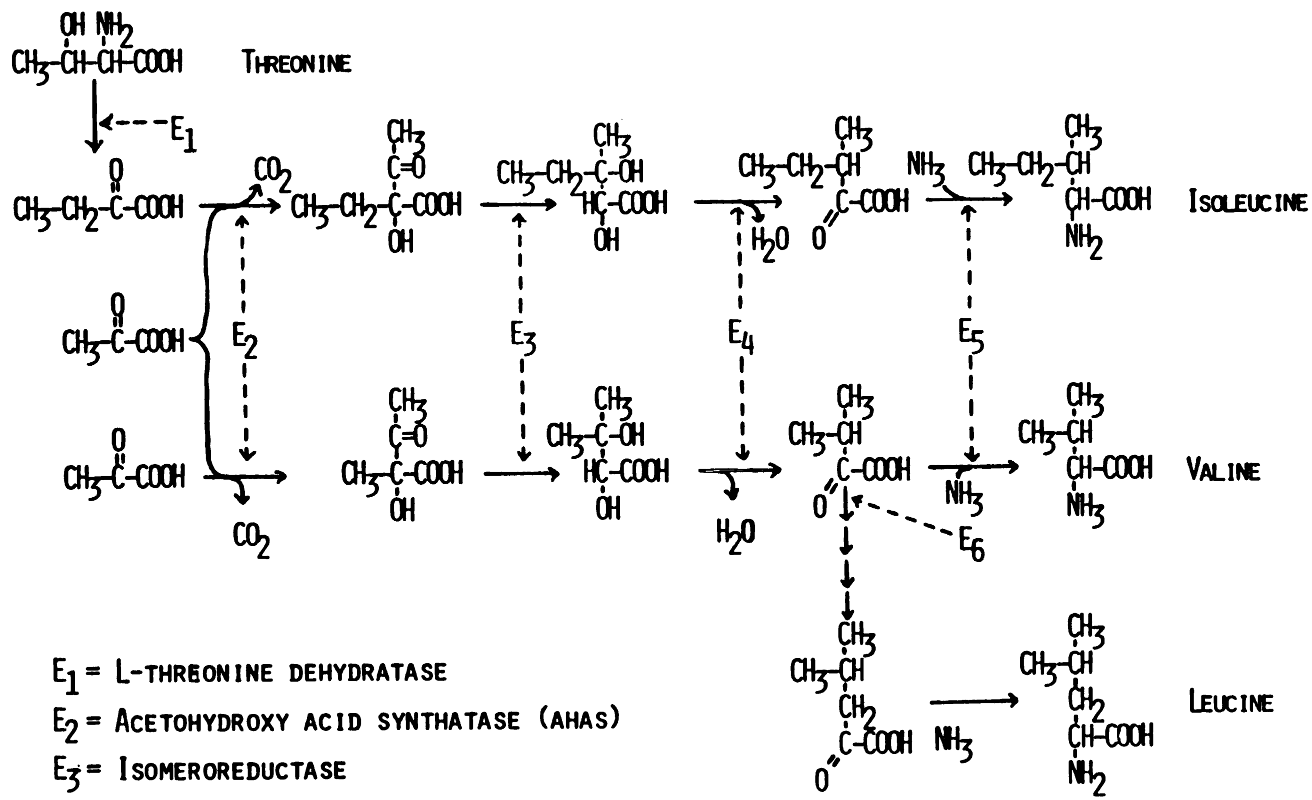
*The latest nomenclature for cis-trans isomers. E=entogen = trans; Z = zusammen = cis (4).

Investigations of amino acid metabolism in the clostridia have been hampered by the fact that these bacteria require a complex and ill-defined medium for good growth. Mead (29) was able to develop a reasonably well-defined medium for good growth. Mead (29) was able to develop a reasonably well-defined medium to study amino acid utilization, but a casein hydrolysate was always included. Such a medium is not suitable for investigations of amino acid synthesis. Perkins and Tsuji (32) developed a synthetic medium in which C. sporogenes could grow, although it could not sporulate well. The historical development of minimal media for the clostridia has recently been reviewed (R.S. Hadioetomo, Ph.D. dissertation, Michigan State University, 1980); in this investigation, a minimal medium for C. sporogenes was developed which includes only eight amino acids, salts, and vitamins.

Branched-chain Amino Acid Synthesis

The reactions involved in the biosynthesis of valine, leucine, and isoleucine were first elucidated over a quarter of a century ago by Strassman, Weinhouse, and co-workers (41, 42). In subsequent years, these reactions have been observed in every biological system in which branched-chain amino acids are synthesized from smaller (3-4 carbon) precursors (45). The most significant feature of these biosynthetic reactions is that they have been shown to be catalyzed by a set of "shared" enzymes which operate both in the synthesis of leucine and valine from pyruvate, and the synthesis of isoleucine from pyruvate and α-ketobutyrate (Fig. 1). The continued extensive investigation of this system in *Escherichia coli*, *Salmonella typhimurium*, *Neurospora crassa*, *Saccharomyces cerevisiae* and other species has been aimed at elucidating the unusual and complex regulatory phenomena associated with this multi-product system. Table 1 illustrates these regulatory relationships in wild-type *E. coli*. These dynamic interactions of several overlapping regulatory systems are viewed as a general model for the control of pathways with shared precursors, intermediates and enzymes.

Control of carbon flow over the isoleucine biosynthetic pathway is usually achieved at the first step in the pathway (Fig. 1). Threonine dehydratase catalyzes this conversion of L-threonine to α-ketobutyrate, ammonia and water. The enzyme and its regulation have been recently reviewed (R.S. Hadioetomo, 1980 Ph.D. dissertation, Michigan State University). Two distinctly different forms of the enzyme have been reported. A "biosynthetic" activity has been observed and purified from several sources (44, 45). Isoleucine is a negative effector and valine a positive effector that antagonizes isoleucine inhibition of this activity. Synthesis of threonine dehydratase is subject to multivalent repression in the presence of excess isoleucine, valine, and leucine (25, 45) in *E. coli* and *S. typhimurium*. A "catabolic" threonine dehydratase, which is not allosterically modified by branched-chain amino acids and is



E_1 = L-THREONINE DEHYDRATASE

E_2 = ACETOHYDROXY ACID SYNTHATASE (AHAS)

E_3 = ISOMEROREDUCTASE

E_4 = DIHYDROXYACID DEHYDRASE

E_5 = TRANSAMINASE

E_6 = ISOPROPYLMALATE SYNTHASE

Fig. 1. Biosynthesis of Branched-chain amino acids.

Table 1. Branch-point enzymes and their regulation in *Escherichia coli*.

Enzymes	Branch Product		Primary Effectors	Remote Effectors
L-threonine dehydratase	ILE		ILE (-)	LEU (-), VAL (+)
Acetohydroxy acid synthase	ILE, LEU, VAL	I	VAL (-)	LEU (-), ILE (-)
		II	?	
		III	VAL (-)	
Isopropylmalate	LEU		LEU (-)	ILE (-)

stimulated by ADP, has been observed in anaerobically-grown E. coli and other species including Clostridium tetanomorphum (46) and C. sporogenes (R. S. Hadioetomo, 1980 Ph.D. dissertation, Michigan State University).

The first "shared" enzyme activity in isoleucine synthesis is a α-acetohydroxy acid synthase (AHAS), which catalyzes the condensation of two pyruvate molecules to form α-acetolactate or pyruvate and α-ketobutyrate to form α-aceto α-hydroxybutyrate. E. coli and S. typhimurium possess three isoenzymes to catalyze this reaction, but their properties, and roles in the regulation of carbon flow through the pathway, are not well understood. Considerable controversy has arisen surrounding the nature of several valine-resistant mutants of E. coli K-12. This strain has the genetic potential to express all three AHAS isoenzymes (45) [ilvB (AHAS I), ilvG (AHAS II), and ilvHI (AHAS III)], but is unable to grow in minimal medium supplemented with valine. Since AHAS I and III are inhibited by valine, while AHAS II is not (Table 1), and mutants of E. coli K-12 without AHAS I and III require isoleucine, it has been proposed that the ilvG gene is cryptic in E. coli K-12 (22). Two markedly different explanations for the reversion of this strain to valine resistance have been proposed. Lawther et al. (27) have demonstrated that K-12 contains a frameshift mutation within ilvG and that valine resistant mutants have displaced this frameshift resulting, they say, in the expression of ilvG and valine resistance. However, Berg et al., contend that a truncated product of the ilvG gene is in fact made and that this protein may function in valine resistant mutants, perhaps as part of a slightly altered AHAS isoenzyme complex (6).

Carbon flow to leucine is regulated at the first enzyme in this branch of the pathway, α-isopropylmatate synthase. The enzyme catalyzes the initial step in the chain-elongation mechanism, the reaction of α-keto-isovalerate and acetyl-CoA to form α-isopropylmalate. Subsequent steps lead to the 1-carbon-longer homolog of α-keto-isovalerate, α-keto-isocaproate. This is transaminated to yield leucine. Usually this enzyme is quite specific for α-keto-isovalerate, but mutants have been obtained in Serratia marcescens in which other keto-acids are elongated (26). This leads to the production in these mutants of the abnormal amino acids norvaline, norleucine, homoisoleucine, homoleucine, and homovaline. A similar activity appears to be utilized in the spirochete Leptospira to produce α-ketobutyrate (via a citramalate intermediate) from the one-carbon-shorter homolog, pyruvate (13). It is not clear if this activity for isoleucine synthesis is "borrowed" from the leucine pathway or should be considered a "shared" enzyme, but the activity appears to be regulated by isoleucine suggesting the latter is the case. In this regard, this system is one of several ways to bypass the usual pathway of a α-ketobutyrate synthesis from threonine.

A variety of alternate pathways to form α-ketobutyrate have been observed (Figure 2). In addition to the pathway

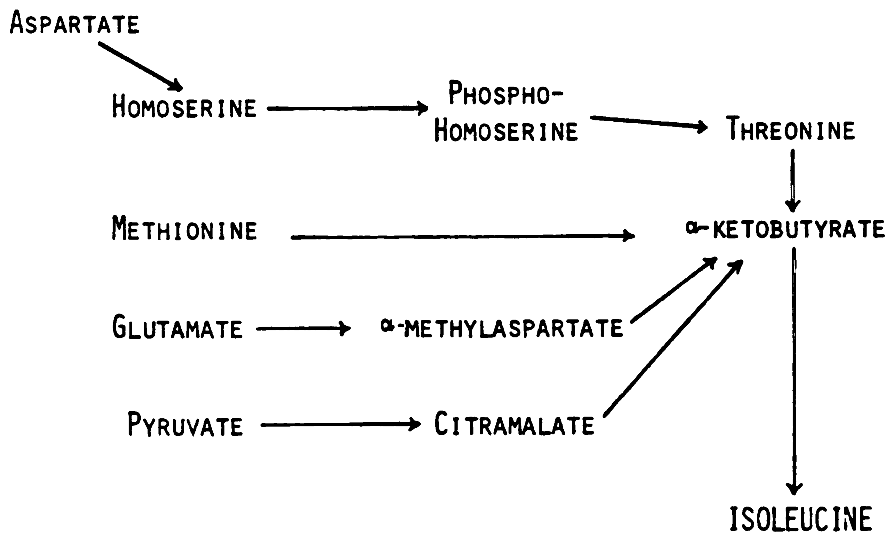


Figure 2. Known pathways to isoleucine via α-ketobutyrate

from threonine and the aforementioned elongation of pyruvate, α-ketobutyrate can reportedly be synthesized from phosphoserine, methionine, and glutamate. In *Bacillus subtilis*, phosphohomoserine is dephosphorylated and deaminated directly to α-ketobutyrate, without the usual threonine intermediate (37). Methionine is converted to α-ketobutyrate by some organisms (45). β-Methyl-aspartate (which can be synthesized from glutamic acid) is converted to isoleucine in *E. coli* W (33) and in *Acetobacter suboxydans* (5), probably via β-methyloxaloacetate and α-ketobutyrate.

The reactions following the condensation of pyruvate and α-ketobutyrate outlined in Figure 1 have been observed in all organisms studied which produce isoleucine from α-ketobutyrate. An alternate pathway to the penultimate product of isoleucine synthesis, α-keto β-methylvalerate, is via the reductive carbozylation of α-methylbutyric acid. Isoleucine biosynthesis from α-methylbutyrate has been demonstrated in anaerobic bacteria from the rumen (35), and in *C.* sporogenes (R.S. Hadioetomo, 1980 Ph.D. dissertation, Michigan State University). Many rumen bacteria require branched-chain volatile acids for growth (1) while for others they stimulate growth but are not requisite. There is no evidence to suggest that α-methylbutyrate is synthesized *de novo* in these bacteria.

Reductive Carboxylations

The reductive carboxylation of organic acids is an important mechanism for CO_2 assimilation in some anaerobic bacteria (8). While most microbes synthesize α-ketoglutarate utilizing the "forward" tricarboxylic acid cycle via isocitric dehydrogenase, some anaerobes produce this precursor for glutamate through the reductive carboxylation of succinate. The physiological importance of the α-ketoglutarate synthase activities was first demonstrated in the green photosynthetic bacteria *Chlorobium thiosulfatophilum* and *Rhodospirillum rubrum* (7), and has been shown to be the predominant pathway in several *Bacteriodes* species (2). In the photosynthetic bacteria, α-ketoglutarate synthase and α-ketopropionate (pyruvate) synthase have been shown to play central roles in the reductive carboxylation cycle utilized by these bacteria as the principal mechanism to produce the precursors for amino acids and other cellular constituents (9). One complete turn of this cycle results in the incorporation of four melecules of CO_2 and the net synthesis of one molecule of oxaloacetate.

α-Keto acid synthases catalyze reactions which rarely occur in aerobic cells. Pyruvate synthase and α-ketoglutarate synthase each reverse reactions associated with the tricarboxylic acid cycle which are irreversible in aerobic cells; the decarboxylation of pyruvate to yield acetyl-CoA and CO_2, and the decarboxylation of α-ketoglutarate to yield succinyl-CoA and CO_2. These reversals are made possible by the strongly electronegative potential of ferredoxin found in many anaerobic bacteria which overcomes the energy barrier for carboxylation (8). The potential of reduced ferredoxin is approximately equal to that of hydrogen gas (H_2). α-Ketoglutarate synthase activity has been demonstrated in several genera of rumen bacteria including Veillonella, elenomonas, and Bacteriodes (2) although the exact nature of the low potential electron carrier is not known.

In recent years it has become clear that the reductive carboxylation reaction is not limited to acetate and succinate substitutes. The reactions below illustrate the presumed steps in the reductive carboxylation, although the exact mechanism is not well understood.

$$\text{i)}\quad R - COOH + ATP \rightarrow R - \overset{\overset{\displaystyle O}{\|}}{C} - Pi + ADP$$

$$\text{ii)}\quad R - CO - Pi + HSCoA \rightarrow R - CO - SCoA + Pi$$

$$\text{iii)}\quad R - CO - SCoA + CO_2 + \text{Ferredoxin}_{red} \xrightarrow{TPP} R - CO - COOH + HSCoA + \text{Ferredoxin}_{ox}.$$

The reductive carboxylation of α-methylbutyrate to yield α-keto-β-methylvalerate for isoleucine synthesis has been demonstrated in rumen bacteria (35) and C. sporogenes (R.S. Hadioetomo, 1980 Ph.D. dissertation, Michigan State University). Isobutyrate and isovalerate have been shown to be precursors of valine and leucine in some rumen bacteria preparations (1, 10, 36). The synthesis of α-ketobutyrate via the reductive carboxylation of propionate has been observed in <u>Chromatium</u>, <u>Clostridium pasteurianum</u>, and <u>Desulfovibrio</u> (7), and in mixed rumen bacterial preparations (10, 2).

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SECTION II

Isoleucine synthesis from α-methylbutyrate or propionate by *Clostridium sporogenes*

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Running head: Isoleucine synthesis in *C. sporogenes*

ABSTRACT

Preliminary studies showed that *C. sporogenes* (ATCC 7955, NCA PA 3679) made isoleucine in a novel pathway not involving threonine or threonine dehydratase. Radioactivity from [3-^{14}C]pyruvate, [^{14}C]propionate and α-amino-[3-^{14}C] butyrate was incorporated into isoleucine, and increasing concentrations of α-methylbutyrate were found to reduce the incorporation of [^{14}C]pyruvate. Crude extracts of the bacteria grown in a minimal medium were found to contain levels of α-acetohydroxyacid synthase activities comparable to those found in cells of *E. coli* K12 grown in minimal medium. Stepwise degradation of isoleucine labeled with specifically-labeled precursors supports the conclusion that *C. sporogenes* can make isoleucine via the reductive carboxylation of propionate to yield α-ketobutyrate which is metabolized to isoleucine in the classical fashion, or isoleucine can be made via the reductive carboxylation of α-methylbutyrate to α-keto-β-methylvalerate.

INTRODUCTION

The pathway for branched-chain amino acid synthesis is well established in many aerobic organisms (32) and the regulation is known in some detail (15). Little consideration has been given to the synthesis of these molecules by strictly anaerobic bacteria, due mainly to the lack of a simple, defined medium for growth of these bacteria. The recent development in our lab of such a medium for *Clostridium sporogenes* (R. S. Hadioetomo, 1980, Ph.D. dissertation, Michigan State University) revealed that this species requires valine or leucine for growth, but not isoleucine. Further investigation showed that these bacteria synthesize isoleucine in a novel fashion from pyruvate and CO_2, and that α-[^{14}C]methylbutyrate is incorporated exclusively into isoleucine. Label from [^{14}C]threonine was not incorporated into isoleucine, and the threonine dehydratase in these organisms was found to be catabolic in nature.

We have therefore sought to determine the nature of isoleucine biosynthesis in *C. sporogenes*. Here we report that these bacteria incorporate [^{14}C]propionate into isoleucine, probably via the reductive carboxylation of propionate to α-ketobutyrate. Crude extracts possess significant levels of α-acetohydroxyacid activity, and presumably metabolize α-ketobutyrate to isoleucine via the classical pathway. Isotope competition experiments and degradation of isoleucine isolated from cells labeled with specifically-labeled precursors indicate the *C. sporogenes* produces isoleucine either from the reductive carboxylation of propionate to α-ketobutyrate, or from the reductive carboxylation of α-methylbutyrate to α-keto-β-methylvalerate, which is transaminated to form isoleucine.

MATERIALS AND METHODS

Culture and growth medium. *Clostridium sporogenes* (ATCC 7955, National Canners Association PA 3679) was used in all experiments. Procedures for maintaining the culture and for preparation of inocula were as previously described (12). The minimal medium used in these experiments is a modification of the synthetic medium of Perkins and Tsuji (23) containing salts, vitamins and eight amino acids (L-serine, 25 mM; L-arginine, 20 mM; L-phenylalanine, 10 mM; L-tyrosine, 2 mM; L-tryptophan, 2 mM; L-methionine, 10 mM; L-valine, 10 mM and L-leucine, 10 mM). The bacteria were grown in an anaerobic chamber (Coy Mfg., Ann Arbor, MI) at 37°C in an atmosphere of 5% carbon dioxide and 10% hydrogen in nitrogen.

Labeling and fractionation of cells. [^{14}C]-Labeled compounds were added to exponentially growing cells (10 ml cultures, O.D. ∿0.3) which were grown for 4 h (1 generation) and then harvested by centrifugation. Typically, the cell pellet was extracted twice with 5 ml of 10% trichloroacetic acid (TCA) for 15 min, followed by 30 min of extraction in 10% TCA at 90°. Following this extraction, the pellet was washed once with hot 10% TCA, suspended in 1.5 ml 6 N HCl and hydrolysed at 110° for 24 h in an argon-flushed ampuole. The hydrolysate was evaporated to dryness, dissolved in distilled water and adsorbed onto a cation exchange resin (AG 50w-x4, hydrogen form; Biorad) packed in a pasteur pipette. The resin was washed with 15 ml of distilled water, and the amino acids eluted with 1 N ammonium hydroxide. The eluate was evaporated to dryness, resuspended in 100 μl of water and stored at -20°.

Acetohydroxyacid synthase assay. Enzyme activity was assayed in crude extracts of *C. sporogenes* and *E. coli* K12 (source R. R. Brubaker). *E. coli* K12 was grown at 37° on a rotary shaker in the minimal medium (without citrate) of Davis and Mingoli (13). *C. sporogenes* was grown in the minimal medium. Crude extracts of these organisms were made from cells harvested from 500 ml of exponentially growing cultures. Pelleted, washed cells were suspended in 3 ml of the buffer used for washing (50 mM potassium phosphate, 10% glycerol and 0.5 mM dithiothreitol, pH 7.5), and sonicated for 3 x 30 sec, with 1 min cooling intervals. Cellular debris was removed by centrifugation, and the extract used immediately.

Acetolactate synthase activity in crude extracts was determined by the method of Størmer and Umbarger (29). Typical reaction mixtures contained 1 M potassium phosphate, pH 8.0, 0.1 ml; 1 mM thiamine pyrophosphate, 0.1 ml; 0.2 mg/ml flavin adenine dinucleotide (FAD), 0.1 ml; 0.1 M $MgCl_2$, 0.1 ml; 0.2 M sodium pyruvate, 0.2 ml; crude extract and water to a final volume of 1 ml. The reaction was stopped after various intervals with 0.1 ml 50% H_2SO_4. The concentration of acetoin was determined with the addition of 1.0 ml 0.5% creatine hydrate and 1.0 ml 5% α-naphthol in 10%

NaOH. After 1 h at room temperature, absorbance at 540 nm was measured, and the concentration of acetoin determined from a standard curve.

A similar reaction mixture was used for the determination of acetohydroxybutyrate synthase activity, except 0.1 ml of 0.2 M sodium pyruvate and 0.1 ml of 0.2 M α-ketobutyrate acid were used and the reaction stopped with 0.1 ml of 10% (w/v) $ZnSO_4$ and 0.1 ml of 1 N NaOH. Enzyme activity was determined using the microbiological assay developed by Leavitt and Umbarger (17), which is based on the fact that the inhibition of the growth of _E. coli_ K12 by L-valine is reversed by any six-carbon precursor of is oleucine. Growth of _E. coli_ K12 in the minimal medium supplemented with 50 ug/ml L-valine and various dilutions of the reaction mix was compared with tubes with known concentrations of isoleucine. The growth response of _E. coli_ K12 in this medium with added isoleucine is very similar to that with added acetohydroxybutyrate (18).

Specific activity is expressed as μmoles of acetolactate or acetohydroxybutyrate formed per g of protein per hour. Protein concentrations were determined by the method of Lowry _et al_. (19). All enzyme reactions were performed at 37° in the anaerobic chamber.

Separation of amino acids. Amino acids were separated by either thin layer or descending paper chromatography, and high-voltage paper electrophoresis. Whatman LK-5-D linear K preadsorbant plates (Whatman, Inc., Clifton, NJ) developed with a methylethylketone-pyridine-water-glacial acetic acid (73:5:20:2) solvent system (20) were used to separate isoleucine from all other amino acids. L-aspartate and L-glutamate were separated from other amino acids and each other by paper electrophoresis on Whatman No. 1 paper in 0.25 M sodium acetate buffer at pH 4.3. Ten μl samples of protein hydrolysates were spotted on the paper and electrophoresed at 17-35 volts/cm for 90 min. Isoleucine was readily obtained for degradation experiments utilizing descending paper chromatography with a butanol-acetic acid-water (120:30:50) solvent system. Hydrolysates were spotted in 5 μl amounts across sheets of Whatman No. 1 paper (46 x 57 cm). Following development, the isoleucine/leucine region of the chromatogram was identified and removed, and the amino acids eluted. The coelution of isoleucine and leucine in this solvent system was not a problem, since leucine was never significantly labeled. Amino acids were located by comparison with identical adjacent lanes sprayed with ninhydrin (0.1% in acetone).

Isoleucine degradation. The location of ^{14}C incorporated into isoleucine from labeled precursors was determined with a stepwise degradation of isoleucine from protein hydrolysates. The procedure is essentially that of Strassman *et al.* (30) with some modifications. Isoleucine was decarboxylated with ninhydrin and the α-methylbutyraldehyde formed distilled into 20 ml of 5 M CrO_3 in 25% (v/v) H_2SO_4 and directly oxidized to α-methylbutyrate (14). The α-methylbutyrate was recovered by steam distillation and degraded to sec-butylamine via the Schmidt reaction (24). This was oxidized to methylethylketone and subsequently degraded to propionate and CO_2. The CO_2 was trapped in 1 N NaOH and precipitated as $BaCO_3$. The $BaCO_3$ was washed with distilled water, suspended in 5 ml of water, and 5 ml conc. H_2SO_4 was added in a closed, evacuated 500 ml jar with five vials containing 1 ml of hydroxide of hyamine to trap the CO_2. After 12 h, 10 ml of toluene-based scintillation fluid was added to each of the vials, and the radioactivity determined in a scintillation counter.

Materials. ^{14}C-Labeled compounds were obtained from New England Nuclear, Boston, MA. Hydroxide of hyamine was purchased from Packard Instrument Co., Inc., Downers Grove, IL. Aqueous scintillation cocktail was obtained from New England Nuclear and the toluene-based fluid prepared by mixing 6.0 g of 2,5-diphenyloxasole and 0.01 g 1,4-bis-2-(5-phenyloxazoyl)-benzene in 1 liter of toluene; both chemicals were obtained from Research Products International Corp., Elk Grove Village, IL.

RESULTS

__Effect of α-methylbutyrate on [U-^{14}C]pyruvate and $^{14}CO_2$ incorporation into isoleucine.__ α-Methylbutyrate can be converted to isoleucine by _C. sporogenes_ (R. S. Hadieotomo, Ph.D. dissertation, Michigan State University). Cells were grown in the minimal medium with 0, 5, 10 or 15 mM α-methylbutyrate, and the incorporation of label from exogenous [U-^{14}C]pyruvate into isoleucine and aspartate in the protein determined. [U-^{14}C]Pyruvate (10 μCi) and α-methylbutyrate were added to 10 ml cultures with optical densities of about 0.3, which then were allowed to grow for 4 h. The protein was extracted from the cells, hydrolysed, and aspartate and isoleucine separated and the radioactivity determined. An identical experiment was performed with ^{14}C-$NaHCO_3$ (10 μCi/10 ml culture). Figure 1 shows that increasing concentrations of α-methylbutyrate lead to a significant decrease in the incorporation of label from [U-^{14}C]pyruvate into isoleucine relative to aspartate. No such change is seen with the $^{14}CO_2$ label.

__Incorporation of α-amino-[3-^{14}C]butyrate into isoleucine.__ Although _C. sporogenes_ cells grown in the minimal medium do not incorporate L-[^{14}C]threonine into isoleucine, we wished to determine if alternate sources of α-ketobutyrate might be incorporated. A 10 ml culture was labeled for one generation with α-amino-[3-^{14}C]butyrate (25 μCi). The radioactive amino acids present in the protein hydrolysate migrated with the adjacent isoleucine control spot (Fig. 1). These data

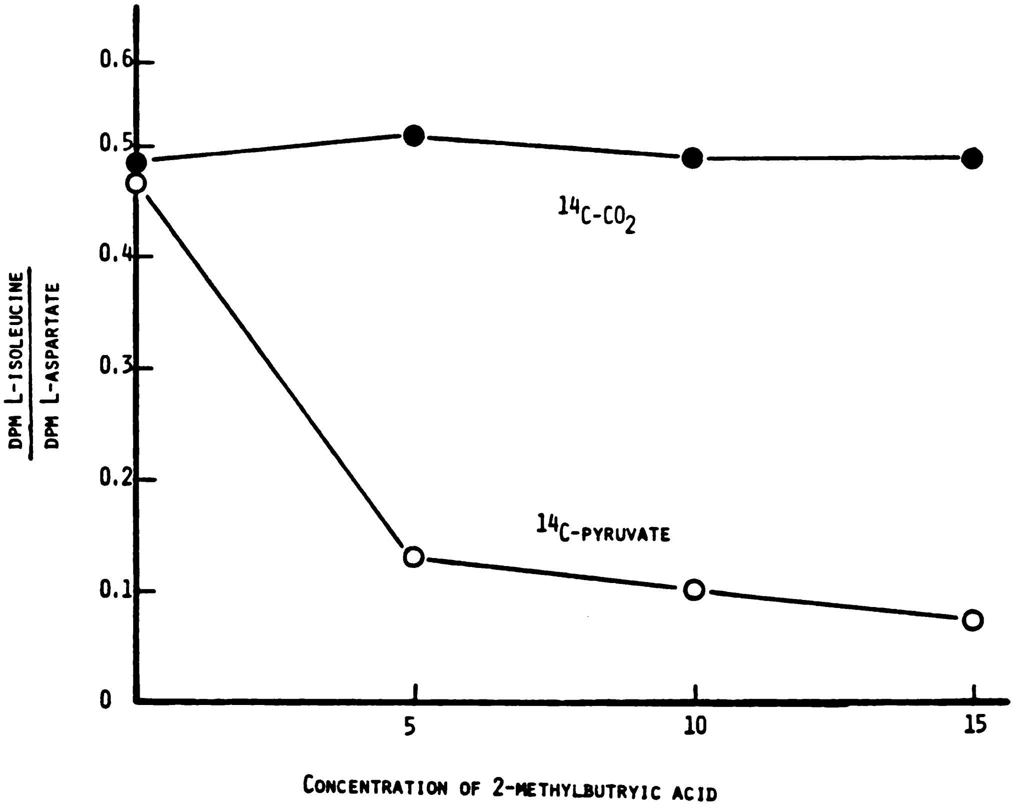

Fig. 1. Effect of α-methylbutyrate on the incorporation of [U-^{14}C]pyruvate and $^{14}CO_2$ into isoleucine. C. sporogenes was grown for one generation in a minimal medium supplemented with 0, 5.0, 10 or 15 mM α-methylbutyrate and the ^{14}C-labeled compounds indicated for one generation. Since the specific activity of aspartate did not change, the ratio of cpm in isoleucine to cpm in aspartate is shown on the abcissa.

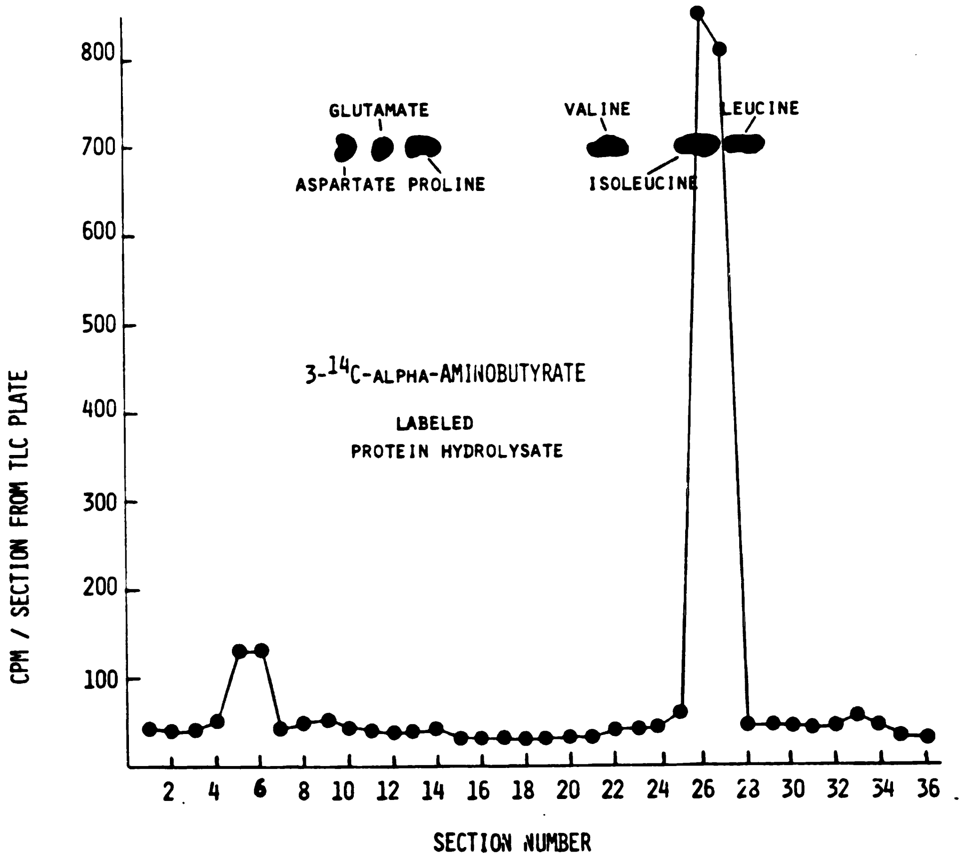

Fig. 2. Incorporation of α-amino-[3-^{14}C]butyrate into amino acids. 10 ul samples of the protein hydrolysate were applied to TLC plates. After development, 5 mm fractions were scraped from the lanes and counted. The location of amino acid standards chromatographed on an adjacent lane are shown at the top of the figure.

show that the C-3 carbon of α-aminobutyrate is incorporated into isoleucine, since essentially all the ^{14}C-label is in this spot.

α-Acetohydroxyacid synthase activity in crude extracts. Since α-ketobutyrate appeared to be a precursor of isoleucine, we assayed crude extracts for the first enzyme in the classical pathway for branched-chain amino acid synthesis, α-acetohydroxyacid synthase (AHAS). The AHAS isoenzymes in *E. coli* catalyze two reacions; the condensation of two pyruvate molecules to form α-acetolactate and CO_2 (for valine and leucine synthesis) and the condensation of pyruvate and α-ketobutyrate to form α-aceto-α-hydroxybutyrate and CO_2. In crude extracts of *C. sporogenes* and *E. coli* K12 cells grown in minimal media, the specific activities of α-acetolactate synthase were 2.2 μmoles/min/g protein and 3.0 μmoles/min/g protein respectively. The α-aceto-α-hydroxybutyrate synthase specific activities were 2.4 μmoles/min/g and 6.1 μmoles/min/g protein. These data show that when *C. sporogenes* is grown in the minimal medium, it possesses considerable AHAS activity but somewhat less than that found in minimal medium grown *E. coli* K12.

Incorporation of ^{14}C-propionate into isoleucine. Our finding that α-ketobutyrate was a precursor of isoleucine in *C. sporogenes*, although L-threonine and L-glutamate are not (R. S. Hadioetomo, 1980, Ph.D. dissertation, Michigan State University), led us to look for other sources of α-ketobutyrate in the cell. The fact that *C. sporogenes* can

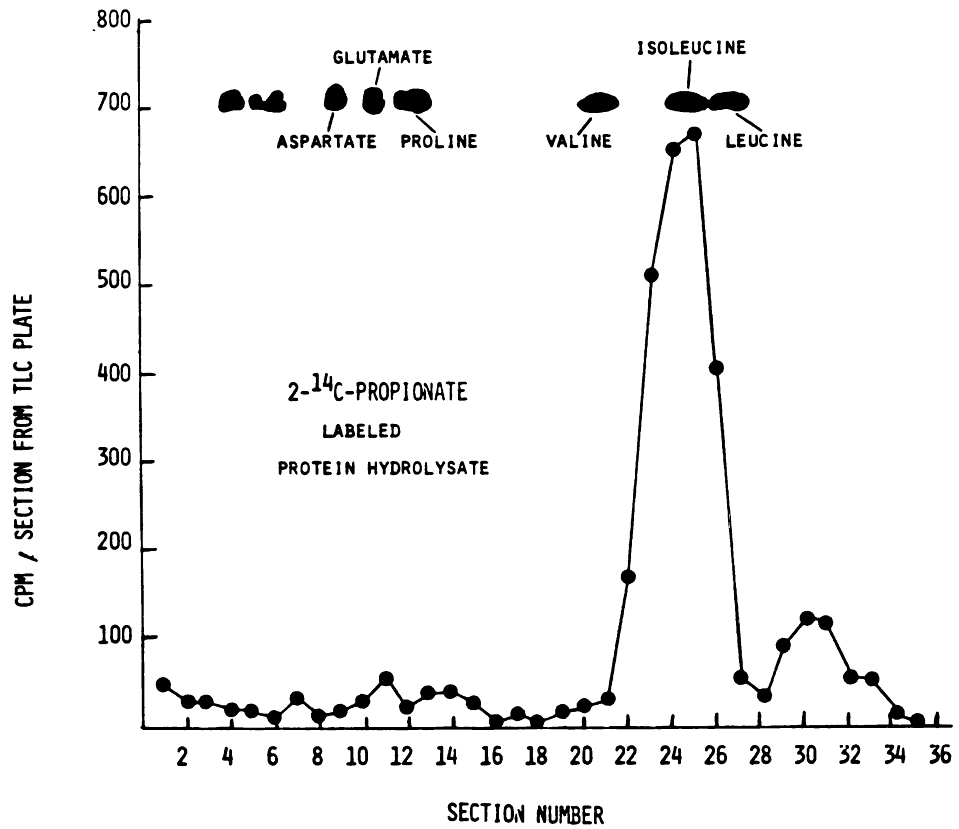

Fig. 3. Incorporation of [2-^{14}C]propionate into amino acids. 10 ml samples of the protein hydrolysate were applied to TLC plates. After development, 5 mm fractions were scraped from the lanes and counted. The location of amino acid standards chromatographed on an adjacent lane are shown at the top of the figure.

reductively carboxylate α-methylbutyrate to form α-keto-β-methylvalerate suggested that these cells might also reductively carboxylate propionate to form α-ketobutyrate. A 10 ml culture was labeled for one generation with 50 μCi of [2-^{14}C]propionate, and the amino acids in the protein hydrolysate separated on TLC plates (Fig. 3). Most of the label in the protein hydrolysate migrated with the adjacent isoleucine control spot. These data show that propionate is also a precursor of isoleucine.

When the supernatent fluid from [1-^{14}C], [2-^{14}C] and [3-^{14}C]propionate labeled cultures were analyzed by paper chromatography, a significant amount of ^{14}C-label was found in α-aminobutyrate (Table 1). The amino acids from the supernatent media were separated in n-butanol:acetic acid:water (120:30:50), methanol:water:pyridine (160:40:8) and n-butanol:pyridine:water (60:60:60). In each case, the ^{14}C-label amino acid had the same Rf as α-aminobutyrate. No other amino acid had a similar Rf in all three cases.

<u>Effect of propionate on [U-^{14}C] serine incorporation into isoleucine.</u> Previous data showed that *C. sporogenes* incorporates a significant amount of ^{14}C from [^{14}C]-serine into isoleucine (R. S. Hadioetomo, 1980, Ph.D. dissertation, Michigan State University). To examine the effect of exogenous propionate on [U-^{14}C]serine incorporation into isoleucine, 10 ml cultures and *C. sporogenes* in the basal medium were supplemented with 10 μCi of [U-^{14}C]serine and 1.0, 2.0 or 3.0 mg of sodium propionate. The cultures were

TABLE 1. Incorporation of ^{14}C-propionate into α-amino butyrate

Amino acid	Rf in solvent[a]		
	BuA	BuP	MeP
Radioactive spot	0.45	0.38	0.60
L-α-aminobutyrate	0.44	0.38	0.61
L-proline	0.37	0.39	-
L-methionine	0.50	0.50	0.60
L-aspartate	0.24	0.14	-
L-tyrosine	-	0.61	-
L-tryptophan	0.50	0.62	-
L-leucine	0.69	0.60	0.68
L-valine	0.51	0.47	0.64
L-serine	-	0.31	-
L-threonine	-	0.31	0.57
L-alanine	0.34	0.35	-
L-phenylalanine	0.59	0.62	0.61

[a]Amino acids were separated by descending paper chromatography. Solvents: BuA; n-butanol:acetic acid:water (120:30:50), BuP; n-butanol:pyridine:water (60:60:60) and MeP; methanol:water:pyridine (160:40:8).

TABLE 2. Effect of propionate on incorporation of [U-^{14}C]-serine into isoleucine.

Propionate (mg/ml)	cpm				fraction of total in:		
	Total	ASP	GLU	ILE	ASP	GLU	ILE
0	6430	500	966	109	0.088	0.15	0.017
0.1	5490	555	778	28	0.10	0.14	0.005
0.2	3860	276	558	25	0.07	0.15	0.006
0.3	2590	208	432	16	0.08	0.17	0.006

[a]10 μl of the protein hydrolysate was used. Aspartate and glutamate were separated by paper electrophoresis, isoleucine by thin layer chromatography.

allowed to grow for three generations and were then harvested and the protein extracted. When the ratios of cpm in aspartate/total cpm in amino acids and cpm in glutamate/ total cpm in amino acids were examined (Table 2), increasing concentrations of propionate in the medium were found to have no effect on the percent fraction incorporated into these amino acids. In contrast, the cpm in isoleucine/total cpm in amino acids ratio was found to decrease significantly (by about 65%) with added propionate (0.1 mg/ml). Incorporation of [U-^{14}C]serine into isoleucine was reduced to a very low level, but not zero. This isotope competition of [^{14}C]serine incorporation by propionate indicates that these two substrates provide some common element in isoleucine biosynthesis. The fact that some minimal level of [^{14}C]serine incorporation was attained suggests that serine may provide some element not produced from propionate.

Incorporation of L-[U-^{14}C] threonine into isoleucine. Although previous results indicated that L-[U-^{14}C]threonine is not incorporated into isoleucine by C. sporogenes grown in the minimal medium (R. S. Hadioetomo, 1980, Ph.D. dissertation, Michigan State University), the fact that α-ketobutyrate is incorporated into isoleucine prompted us to reexamine this phenomenon. In the previous study, cells growing in the minimal medium were labeled with very high specific activity [^{14}C]threonine and a very low total threonine concentration (25 μCi of label with a specific activity of 210 μc/μmole). In this experiment, the minimal medium was supplemented with 10 mM L-threonine. After several generations of growth in this medium, 25 μCi of L-[U-^{14}C]-threonine were added, and the cells grown for one generation. As in the previous study, most of the label in the protein hydrolysate was in threonine. However, while no significant label was recovered in isoleucine in the previous study (less than 150 dpm from a total of 124,000 dpm), there was significant incorporation of ^{14}C from L-[U-^{14}C]threonine into isoleucine (over 270 cpm of 13,200 cpm incorporated) in this experiment.

Degradation of isoleucine labeled with [3-^{14}C]pyruvate, [1-^{14}C]propionate and [2-^{14}C]propionate. Specifically labeled isoleucine from *C. sporogenes* was degraded in a stepwise fashion to locate the position of the label in the isoleucine molecule (Tables 3 and Fig. 4). The recoveries of various carbons by this procedure were low, therefore the results must be compared to appropriate controls. When the isoleucine was labeled with [3-^{14}C]pyruvate, no significant incorporation occurred in C-1 or C-2. All of the label was incorporated into the last four carbons. Cleavage of *sec*-butylamine results in the formation of propionate (C-3, -4, -5) and iodoform (C-6) and the ^{14}C-label was found in both fractions. In the classical pathway, the C-6 of isoleucine arises from pyruvate, and carbons 4 and 5 arise from α-ketobutyrate. The degradation results with [3-^{14}C]pyruvate labeled isoleucine show that if isoleucine is

TABLE 3. Degradation[a] of isoleucine from C. *sporogenes* grown in the presence of [3-^{14}C]pyruvate, [1-^{14}C]-propionate and [2-^{14}C]propionate

Degradation product	U-^{14}C-isoleucine control degradation	^{14}C-growth substrate [3-^{14}C]-pyruvate	[1-^{14}C]-propionate	[2-^{14}C] propionate
Isoleucine	55,000	5,600	1,300	2,200
C-1	4,900 (53)[b]	56 (1)	None	140 (6)
α-methyl butyrate	15,000 (32)	4,400 (79)	560 (43)	1,600 (73)
C-2	170 (2)	None	290 (22)	60 (3)
Sec-butyl amine	3,600 (10	900 (16)	None	230 (10)
Propionate	750 (3)	390 (7)	-	92 (4)
Iodoform	140 (2)	140 (2.5)	-	-

[a]See Figure 4.

[b]Percent theoretical recovery.

[c]Percent recovery.

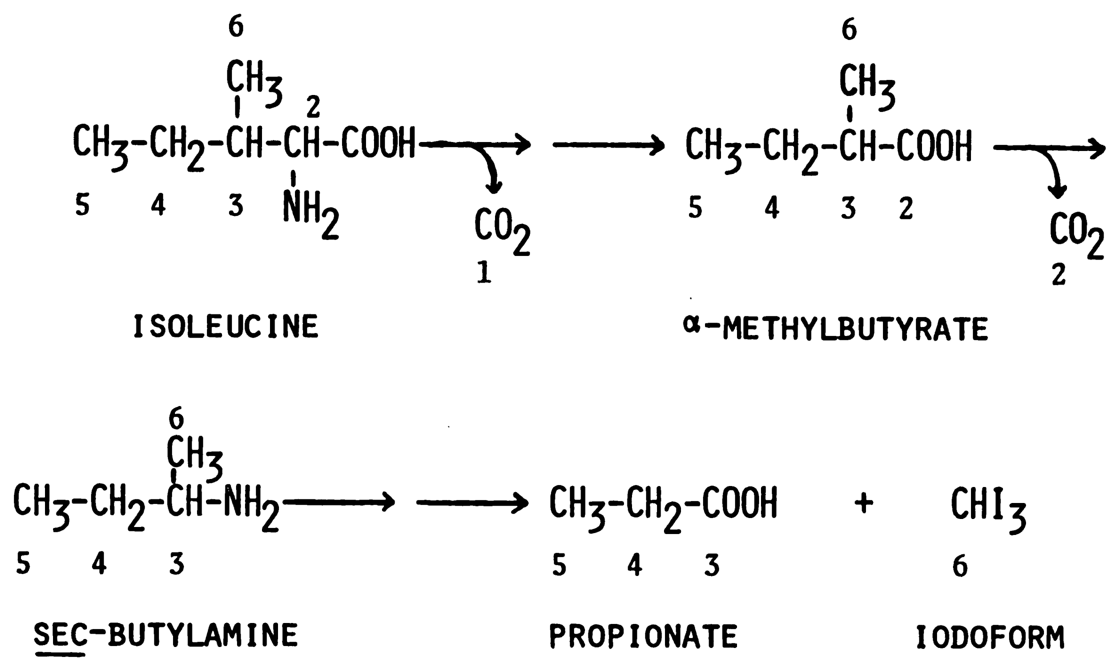

Fig. 4. A scheme for the degradation of isoleucine. See Materials and Methods for experimental details.

made via the classical condensation of pyruvate and α-ketobutyrate, pyruvate must also be incorporated into α-ketobutyrate prior to this step.

All the label from [1-^{14}C]propionate in isoleucine appears to be in C-2. No label is released during decarboxylation of isoleucine, but over 65% of the radioactivity in α-methylbutyrate was recovered as CO_2. These results are as expected if isoleucine is made from α-ketobutyrate, which is in turn formed by the reductive carboxylation of propionate. Although some label was released with the C-1 and C-2 carbons of [2-^{14}C]propionate labeled isoleucine, most of the label remains in the <u>sec</u>-butylamine and a significant amount was recovered in the propionate fraction. These results support the conclusion that α-ketobutyrate is made from propionate, and is subsequently incorporated into isoleucine in the classical manner.

DISCUSSION

These results offer a good explanation of the unusual ^{14}C-labeling of isoleucine by C. sporogenes reported by Hadioetomo (1980, Ph.D. dissertation, Michigan State University). She observed that ^{14}C-label from [^{14}C]threonine was not incorporated into isoleucine, while radioactivity from [^{14}C]pyruvate, [^{14}C]serine, $^{14}CO_2$ or [^{14}C]-α-methylbutyrate was incorporated. She concluded that isoleucine was made in a novel fashion. The evidence presented here indicates that the synthesis of isoleucine in the minimal medium proceeds via the classical pathway (30) but that the α-ketobutyrate used is derived from a source(s) other than threonine.

The results clearly show that α-ketobutyrate is a precursor of isoleucine. When C. sporogenes was grown with α-amino-[3-^{14}C]butyrate, the ^{14}C-label was recovered predominantly (almost exclusively) in isoleucine. It is probably converted to isoleucine via the classical pathway, since the first enzyme in the pathway, acetohydroxyacid synthase (AHAS) is present in crude extracts in levels comparable to those found in E. coli. The data from the degradation of specifically-labeled isoleucine supports this conclusion, and indicates that propionate is an important source of α-ketobutyrate in C. sporogenes. If propionate were carboxylated to α-ketobutyrate, most of the radioactivity from [2-^{14}C]propionate labeled isoleucine would be expected in the C-4 position. When isoleucine was labeled with this substrate, the bulk of the label was recovered in the carbon 3-5 fragment of isoleucine. Similarly, ^{14}C-label from [1-^{14}C]propionate was found in C-2 of isoleucine, which is the ultimate location of the C-2 of α-ketobutyrate in the classical pathway. Finally, ^{14}C-label from [3-^{14}C]pyruvate was found in two locations in isoleucine, which suggests that pyruvate is not only condensed with α-ketobutyrate as in the classical pathway, but also that pyruvate is a precursor of α-ketobutyrate. There are probably other sources of α-ketobutyrate in the medium besides propionate, since both L-methionine and L-threonine can be converted to α-ketobutyrate by C. sporogenes growing in complex media (4). When C. sporogenes was grown with 10 mM L-threonine in the minimal medium, a small amount of radioactivity from [^{14}C]threonine was recovered in isoleucine.

The unusual aspect of isoleucine synthesis in C. sporogenes is that the predominant source of α-ketobutyrate appears to be propionate, not threonine. An important source of propionate is L-serine, which is present in high concentration (25 mM) in the medium. The fact that increasing concentrations of propionate in the medium decreases the incorporation of [^{14}C]serine into isoleucine supports this proposal, as does the observation that some ^{14}C-label from [3-^{14}C]pyruvate appears to be incorporated into isoleucine through α-ketobutyrate. Serine is readily converted to alanine by C. sporogenes growing in the minimal medium (R. S. Hadioetomo, 1980, Ph.D. dissertation, Michigan State University), via pyruvate. In this fashion, serine and alanine could be fermented to propionate via pyruvate. The fermentation probably proceeds by the acrylate pathway observed in C. propionicum or by the succinate-propionate pathway employed by other propionate producing bacteria (13). Hadioetomo showed that pyruvate in the cell was subject to extensive CO_2 exchange at the C-1 position. Label from $^{14}CO_2$ in the medium was incorporated into the C-1 of serine and alanine, and therefore would also be found in propionate. Since the C-1 of propionate eventually becomes the C-2 of isoleucine (Fig. 5) this may explain Hadioetomo's observation that $^{14}CO_2$ was incorporated into at least one carbon other than the C-1 of isoleucine. The reductive carboxylation of propionate to α-ketobutyrate which is proposed here has been observed in a number of other anaerobic bacteria, including C. pasteuranum (3,6).

α-Methylbutyrate is an alternate precursor of isoleucine for C. sporogenes growing in the minimal medium. Hadioetomo showed that [^{14}C]-α-methylbutyrate was incorporated exclusively into isoleucine, and no radioactivity was released upon decarboxylation. We have shown here that increasing concentrations of α-methylbutyrate in the medium decreased the incorporation of [^{14}C]pyruvate but did not change the relative incorporation of radioactivity from $^{14}CO_2$ into isoleucine. If propionate is reductively carboxylated to α-ketobutyrate and processed to isoleucine in the classical fashion, or α-methylbutyrate carboxylated directly to α-keto-α-methylvalerate (the keto-acid analog of isoleucine),

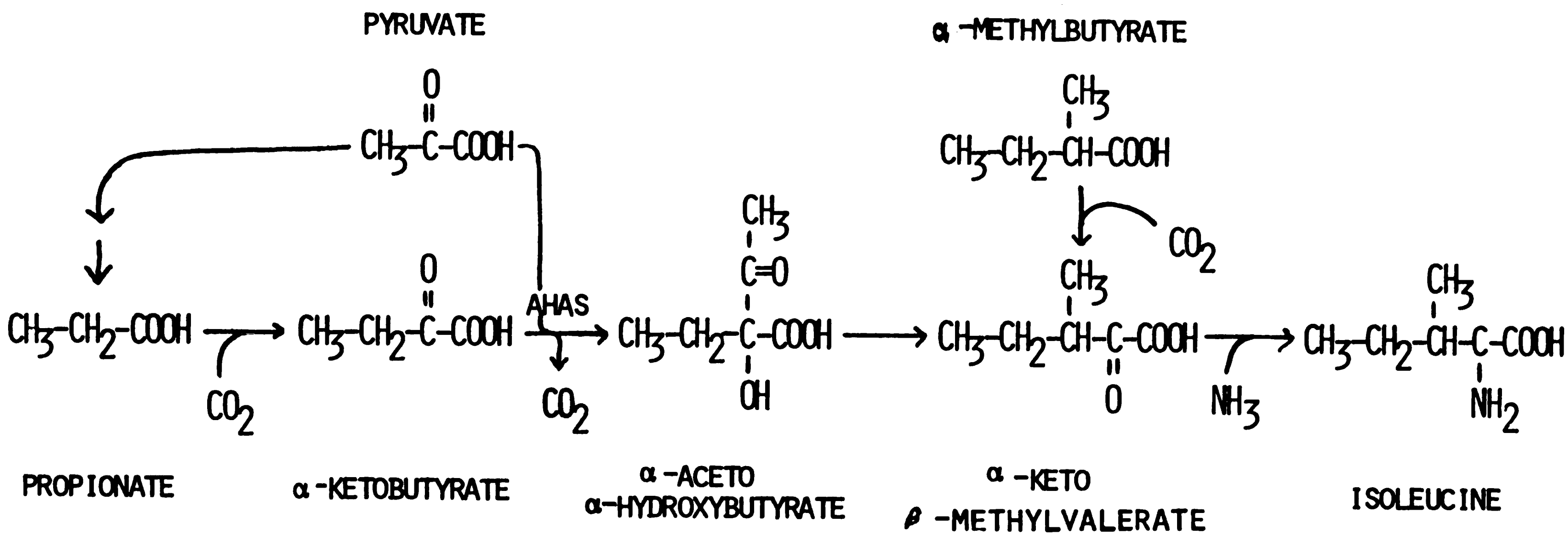

Fig. 5. Proposed pathway for isoleucine synthesis in <u>Clostridium</u> <u>sporogenes</u>.

the net incorporation of $^{14}CO_2$ would not change regardless of the proportion of isoleucine made from α-methylbutyrate. The data presented here support the proposal that propionate and α-methylbutyrate are alternate sources of isoleucine. The carboxylation reactions which occur are presumably similar to the reductive carboxylation reactions which produce α-ketobutyrate from propionate (3,6), α-ketoglutarate from succinate (2,7) and α-ketoisocaproate from isovalerate and α-ketoisovalerate from isobutyrate (1,8) in other anaerobic bacteria. The incorporation of [^{14}C]-α-methylbutyrate into isoleucine in this fashion has been demonstrated in the rumen species *Bacteroides ruminicola* (26).

When *C. sporogenes* was grown in the minimal medium considerable α-ketobutyrate was produced, leading to the accumulation of α-amino-[^{14}C]butyrate in the medium when the cells were grown with [1-^{14}C]propionate, [2-^{14}C]propionate or [3-^{14}C]propionate. *C. sporogenes* also accumulates α-aminobutyrate when grown in a complex medium (21). In *E. coli*, α-ketobutyrate is usually produced from threonine, and the levels of α-ketobutyrate in the cell are strictly controlled by feedback regulation of threonine dehydratase by isoleucine and to a lesser extent valine and leucine (16). Threonine is not the primary source of α-ketobutyrate in *C. sporogenes*, nor is threonine dehydratase activity regulated by isoleucine (R. S. Hadioetomo, 1980, Ph.D. dissertation, Michigan State University). The accumulation of α-aminobutyrate in the medium is of special significance since it has been shown to be an antimetabolite of valine in *Bacillus anthracis* (12) and *E. coli* K-12 (25). Gladstone (12) observed that the growth of *B. anthracis* in a minimal medium was inhibited by α-aminobutyrate in concentrations as low as 0.04 mM, and that the inhibition could be overcome by the addition of L-valine to the medium. The inhibitory effects of α-aminobutyrate may be responsible for the inability of *C. sporogenes* to grow in the minimal medium without valine and leucine. α-Aminobutyrate inhibition may be the result of reduced acetolactate production by the cell. In *E. coli* the condensation of two pyruvate molecules to form acetolactate (for valine and leucine) and the condensation of α-ketobutyrate to form α-aceto-α-hydroxybutyrate (for isoleucine) are catalyzed by the same AHAS isoenzymes. Consequently, increased concentrations of α-ketobutyrate will competitively inhibit the formation of acetolactate. In experiments with purified AHAS I from *E. coli*, Grimminger and Umbarger (14) showed that as the pyruvate/α-ketobutyrate ratio in their assay was changed from 10/1 to 1/1, the ratio of acetolactate/α-aceto-α-hydroxybutyrate formed changed from 5.3/1 to 1/1.5. When α-aminobutyrate resistant mutants were selected from *E. coli* K-12 they were found to have derepressed levels of AHAS. Increasing enzyme concentration is one way to overcome competitive inhibition. Amino acid tRNA-syntheses play a role in the regulation of branched-chain amino acid synthesis

(16), and although α-aminobutyrate is activated by valyl-tRNA synthase (5), it is not transferred to $tRNA^{val}$ and has no effect on the formation of the isoleucine-valine biosynthetic enzymes in *E. coli* (11,31). Regardless of the mechanism of inhibition, it seems likely that the same system which allows *C. sporogenes* to make isoleucine from propionate via α-ketobutyrate may also be indirectly responsible for the cell's inability to make valine and leucine, since the accumulation of α-aminobutyrate may preclude growth without exogenous valine and leucine.

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Interconversion of Valine and Leucine by

Clostridium sporogenes

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Running head: Interconversion of Valine and Leucine

SECTION III

ABSTRACT

Clostridium sporogenes has been found to require L-leucine and L-valine for growth in a minimal medium, although valine can be replaced by isobutyrate, and leucine by isovalerate. Cells grown in minimal media were found to incorporate significant ^{14}C-label from [^{14}C]valine into leucine and ^{14}C-label from [^{14}C]leucine into valine. These results indicate that these bacteria can interconvert leucine and valine.

It is well-established that many microbes can convert L-valine to L-leucine (14). This is accomplished by the elongation of α-ketoisovalerate (the keto-acid analog of valine) to α-ketoisocaproate (the keto-acid analog of leucine). This chain-elongation is similar in function to the elongation system in the tricarboxylic acid cycle which converts oxaloacetate to α-ketoglutarate. Up to this point, there has been no evidence for the conversion of L-leucine to L-valine.

C. sporogenes (ATCC 7955, National Canners Association PA 3679) was used in all experiments. It was maintained by refrigeration of a culture that had sporulated in a medium containing 4% trypticase, 2 mg of thiamine hydrochloride per liter, and 0.05% sodium thioglycolate. Vegetative cultures for inoculation of experimental media were initiated by inoculating tubes of this same medium (10 ml/tube) with 0.1 ml of the sporulated culture. These were heat-shocked (60°C for 10 min) and incubated at 37°C in an anaerobic chamber (Coy Manufacturing Company, Ann Arbor, MI). After growth for 6 to 8 h, tubes with 10 ml of the basal medium containing 10 mM valine and 10 mM leucine were inoculated with 0.1 ml of this culture. When this second culture had growth to late exponential phase, 0.05 ml was used to inoculate experimental growth media. In the labeling experiments, cells were preadapted to the appropriate medium before addition of label. The basal medium used in these experiments was a modification of the synthetic medium of

Perkins and Tsuji (11). It contained salts, vitamins, 0.05% sodium thioglycolate and six amino acids - L-tyrosine (2 mM), L-tryptophan (2 mM), L-phenylalanine (10 mM), L-methionine (10 mM), L-arginine·HCl (20 mM) and L-serine (25 mM). For growth experiments optical densities at 600 nm were measured with a mini-spec 20 spectrophotometer (Bausch and Lomb Optical Co., Rochester, NY).

C. sporogenes grew well in the basal medium supplemented with 10 mM valine and 10 mM leucine; 10 mM valine and 10 mM α-methylbutyrate; 10 mM leucine and 10 mM α-methylbutyrate or 10 mM leucine and 10 mM isobutyrate (Table 1). These results encouraged us to attempt to grow the bacteria without either valine or leucine. Some growth occurred when 10 mM isobutyrate and 10 mM α-methylbutyrate, were added to the medium (Table 2). Slightly more growth was achieved by adding 10 mM isovalerate along with the other two volatile acids. These results suggested that the keto-acid analogues of leucine (α-ketoisocaproate) and valine (α-ketoisovalerate) can be produced by C. sporogenes from isovalerate and isobutyrate, respectively.

Individual lots of cells were grown for one generation (∿4 h) in the basal medium supplemented as follows: (a) 50 μCi L-[U-^{14}C]leucine; 10 mM leucine, 10 mM isoleucine, and 10 mM α-methylbutyrate; (b) 50 μCi L-[U^{14}C]valine, 10 mM valine and 10 mM α-methylbutyrate; and (c) 10 μCi [U-^{14}C]pyruvate or 10 μCi $^{14}CO_2$ in the medium with 10 mM valine and 10 mM leucine. Cells were harvested, extracted, and

Table 1. Maximum growth in basal medium[a] supplemented with amino acids and volatile fatty acids.

Concentrations (mM)					
L-Valine	L-Leucine	Isovalerate	Isobutyrate	α-Methyl-butyrate	Final O.D.
0	0	0	0	0	0.039
10	0	10	0	0	0.081
10	0	0	10	0	0.13
10	0	0	0	10	0.95
0	10	10	0	0	0.086
0	10	0	10	0	0.34
0	10	0	0	10	0.75
10	10	0	0	0	0.92

[a]The basal medium is described in the text. Triplicate 10 ml cultures were grown at 37° in the anaerobic chamber, and the optical density (O.D.) monitored at 2 h intervals.

Table 2. Maximum growth in basal medium supplemented with volatile fatty acids.

Concentrations (mM)			
Isobutyrate	Isovalerate	α-Methyl-butyrate	Final O.D.
0	0	0	0.039
10	0	0	0.066
10	10	0	0.066
10	10	10	0.40
0	10	0	0.056
0	10	10	0.056
0	0	10	0.080
10	0	10	0.30

protein hydrolysates prepared as described elsewhere (Monticello and Costilow, manuscript submitted). Aspartate, glutamate, valine, and leucine were separated from the protein hydrolysates and the specific radioactivity of each amino acid determined. Whatman LK-5-D linear K preadsorbant thin layer plates (Whatman, Inc., Clifton, NJ) developed with a methylethylketone-pyridine-water-glacial acetic acid (73:5:20:2) solvent system (9) were used to separate isoleucine, leucine, and valine. L-Aspartate and L-glutamate were separated from other amino acids and each other by paper electrophoresis on Whatman No. 1 paper in 0.25 M sodium acetate buffer at pH 4.3. Samples (10 μl) were spotted on the paper and electrophoresed at 17-35 volts/cm for 90 min. Amino acids were located by comparison with identical adjacent lanes sprayed with ninhydrin (0.1% in acetone). The relative abundance of each of these amino acids in the protein from C. sporogenes grown in the basal medium is known (R. S. Hadioetomo, 1980 Ph.D. dissertation, Michigan State University), so the specific activity (cpm/nmole) of each amino acid could be estimated. Table 3 shows the results of these incorporation experiments.

A significant amount of label was incorporated into valine when the cells were labeled with [^{14}C]-leucine, and into leucine when the cells were labeled with [^{14}C]-valine. When amino acids are oxidized by C. sporogenes the carboxyl-group is released into the medium, and other amino acids may become labeled in this way. To determine the influence of

Table 3. Incorporation of ^{14}C into aspartate, glutamate, leucine, and valine.

Growth Substrate	cpm/10 μl Protein Hydrolysate Aspartate	Glutamate	Leucine	Valine	Sp. Act. Ratios Leu/Asp	Val/Asp
[^{14}C]-Leucine	30 (0.67)[a]	50 (0.98)	3200 (94)	160 (4.4)	140	6.6
[^{14}C]-Valine	140 (3.0)	110 (2.1)	370 (11)	3400 (91)	3.7	30
[3-^{14}C]-Pyruvate	2700 (60)	4400 (86)	20 (0.6)	0 (0)	0.01	0
$^{14}CO_2$	3400 (75)	2800 (54)	580 (17)	440 (12)	0.23	0.16

[a]Estimated specific activities (cpm/nmole).

such reactions on the incorporation of label from [^{14}C]-leucine into valine, cells were labeled with $^{14}CO_2$ and the amino acids examined. The ratio of radioactivity in valine/aspartate should remain roughly equal if the ^{14}C-label in the valine is from exogenous $^{14}CO_2$, or $^{14}CO_2$ released from [^{14}C]-leucine. In fact, the ratio of the specific activities of valine/aspartate was 41 times larger with [^{14}C]-leucine as compared to $^{14}CO_2$ as substrate (Table 3). This rules out $^{14}CO_2$ as the principle source of radioactivity from [^{14}C]-leucine which was found in valine. The same reasoning applies for the incorporation of [^{14}C]-valine into leucine; in this case, the ratio of specific activities in leucine/aspartate was 16 times that obtained when the cells were labeled with $^{14}CO_2$. As found in many other experiments (Hadioetomo, 1980 Ph.D. dissertation, Michigan State University) no significant amount of ^{14}C from [3-^{14}C] pyruvate was found in either leucine or valine.

Branched-chain amino acid synthesis and its regulation in Escherichia coli, Salmonella typhimurium, Neurospora crassa, Saccharomyces cerevisiae and several other organisms have been the objects of intense investigation (15). Little work has been done in this area with the anaerobes, however. The results presented here indicate that although C. sporogenes usually requires L-valine and L-leucine for growth in a minimal medium, the bacteria can interconvert leucine and valine.

The production of isobutyrate, acetate, and isocaproate from cultures grown with leucine has been observed in several strains of C. sporogenes [NCIB 10696 (10), EC-9 (12) and ATCC7956 (our unpublished results), and other clostridia (4,10,12)]. The oxidation of leucine to isobutyrate by C. sporogenes has been shown to proceed via an unusual series of reactions; α-leucine is converted to β-leucine, which is oxidized to isobutyrate and acetate (12). It is likely that ^{14}C-leucine is converted to ^{14}C-isobutyrate in this fashion, and the ^{14}C-isobutyrate is reductively carboxylated to α-[^{14}C]-ketoisovalerate, the keto-acid precursor of valine. The proposed pathway is shown in Fig. 1.

α-Keto-acid synthase catalyzed reductive carboxylations are relatively common among the anaerobes. The reductive carboxylation of α-methylbutyrate to α-keto β-methylvalerate has been demonstrated in C. sporogenes (R. S. Hadioetomo, 1980 Ph.D. dissertation, Michigan State University) and Bacteriodes ruminicola (13). The reductive carboxylation of isobutyrate for valine synthesis, and isovalerate for leucine synthesis has been observed in extracts prepared from mixed rumen bacteria (1) and photosynthetic bacteria (8). The formation of α-ketobutyrate from propionate has been demonstrated in cell-free preparations from Chromatium, Clostridium pasteurianum, Desulfovibrio desulfuricans (5), mixed rumen extracts (7) and Veillonella alcalescens (3), which along with other rumen bacteria (2) and photosynthetic bacteria (6) produces α-ketoglutarate from succinate.

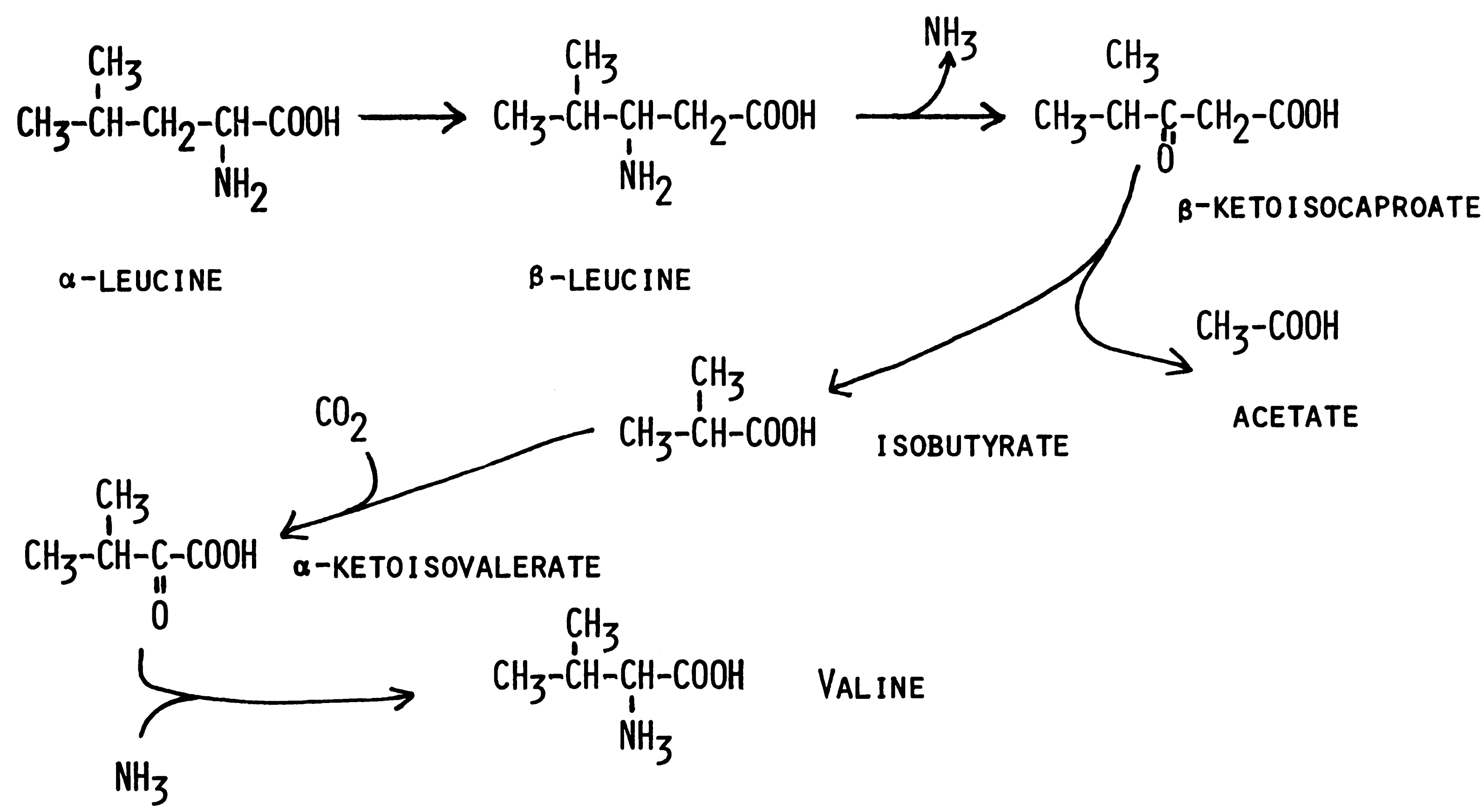

Fig. 1. Proposed pathway for the conversion of leucine to valine.

Reductive carboxylation reactions have only been observed in anaerobic heterotrophs from the rumen and other anaerobic habitats. The requirement for a very electronegative electron donor such as reduced ferredoxin to overcome the energy barrier for carboxylation essentially precludes this activity from aerobically grown organisms.

In light of the apparent ubiquity of the keto-acid synthase activity among the anaerobes, many of these bacteria may be capable of interconverting leucine and valine. The other key enzyme activity, leucine 2,3-amino mutase, which converts α-leucine to β-leucine, has been found in several clostridia, in rats, sheep, rhesus and African green monkey livers, and in human leukocytes (12). The overall roles of such reactions in the branched-chain amino acid metabolism by <u>Clostridium</u> <u>sporogenes</u> is not clear. Further experimentation is required to determine this, and the role of α-methylbutyrate in this process.

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SECTION IV

Incorporation of Branched-chain Amino Acids into Cellular Lipid

Many gram positive bacteria produce branched-chain fatty acids which are incorporated into cellular lipid. The initial step in this synthesis is the production of branched-chain acyl-CoA molecules derived from branched-chain amino acids. A characterization of the cellular fatty acids from *C. sporogenes* showned that these bacteria produce significant amounts of 15:0 (from leucine), 16:0 (from valine) branched-chain fatty acids, and perhaps some anti-iso 16:0 fatty acids (from isoleucine)* These fatty acids were present in the cellular lipid from cells of various ages grown in various media. Since the production of these fatty acid entails a significant drain on the pools of isoleucine, leucine and valine in the cell, the extent to which these amino acids were incorporated into cellular lipid during growth in a minimal medium was examined.

To determine the relative incorporation of valine, leucine and isoleucine into cellular lipid, the incorporation of ^{14}C-label from these amino acids into lipid was determined. The incorporation of [3-^{14}C] pyruvate, which

*Moss, C.W., and J.L. Vester. 1967. Characterization of clostridia by gas chromatography. I. Differentiation of species by cellular fatty acids. Appl. Microbiol. 15:390-397.

should be incorporated to a large extent, and [U-^{14}C] lysine, which is not readily incorporated into lipid was also examined. Cells were grown in the basal medium (section II, this dissertation) with 10 mM leucine and 10 mM valine, and labeled with [U-^{14}C]isoleucine (10 μCi/10 ml), [U-^{14}C]lysine (10 μCi/10 ml) or [3-^{14}C] pyruvate (10 μCi/10 ml). For labeling with [U-^{14}C]leucine, cells were grown in the basal medium with 10 mM valine and 10 mM α-methylbutyrate, and with 10 μCi/10 ml [U-^{14}C]leucine. For [U-^{14}C] valine labeling, cells were grown with 10 mM leucine and 10 mM isobutyrate. The cells were grown with the label for four hours (about 1 generation) and then harvested and fractionated as described elsewhere (section I, this dissertation). After extraction of the cellular material at 90°C for 30 min, the material was centrifuged. The supernatent contained cellular lipid and nucleic acids. Lipid was extracted for this solution with and equal volume of chloroform:methanol (1:1). The misture was shaken for 30 min, left overnight at 4°C, and the lipid fraction drawn off, dried, and counted in a toluene based scintillation cocktail (section I).

Table 1 shows that significant amounts of ^{14}C-label from [U-^{14}C]isoleucine, [U-^{14}C]leucine and [U-^{14}C]valine were incorporated into cellular lipid. The ratios of the cpm in lipid/protein indicate that approximately six times more valine than leucine or isoleucine was incorporated into cellular lipid.

Table 1. The incorporation of ^{14}C-label into cellular lipid and protein.

SUBSTRATE[a]	LIPID	PROTEIN	$\frac{\text{LIPID}}{\text{PROTEIN}}$
[U-^{14}C]isoleucine	42,000	15,000,000	o.0028
[U-^{14}C]leucine	64,000	30,000,000	0.0021
[U-^{14}C]valine	13,000	840,000	0.015
[3-^{14}C]pyruvate	180,000	4,600,000	0.039
[U-^{14}C]lysine	590	180,000	0.0032

[a] 10 μCi of ^{14}C-labeled substrate was added to a 10 ml culture (optical density = 0.3). The cells were grown for 4h (about 1 generation), harvested, and the total radioactivity in cellular lipid and protein determined.

Oxidation of Branched-chain Amino Acids by Whole Cells.

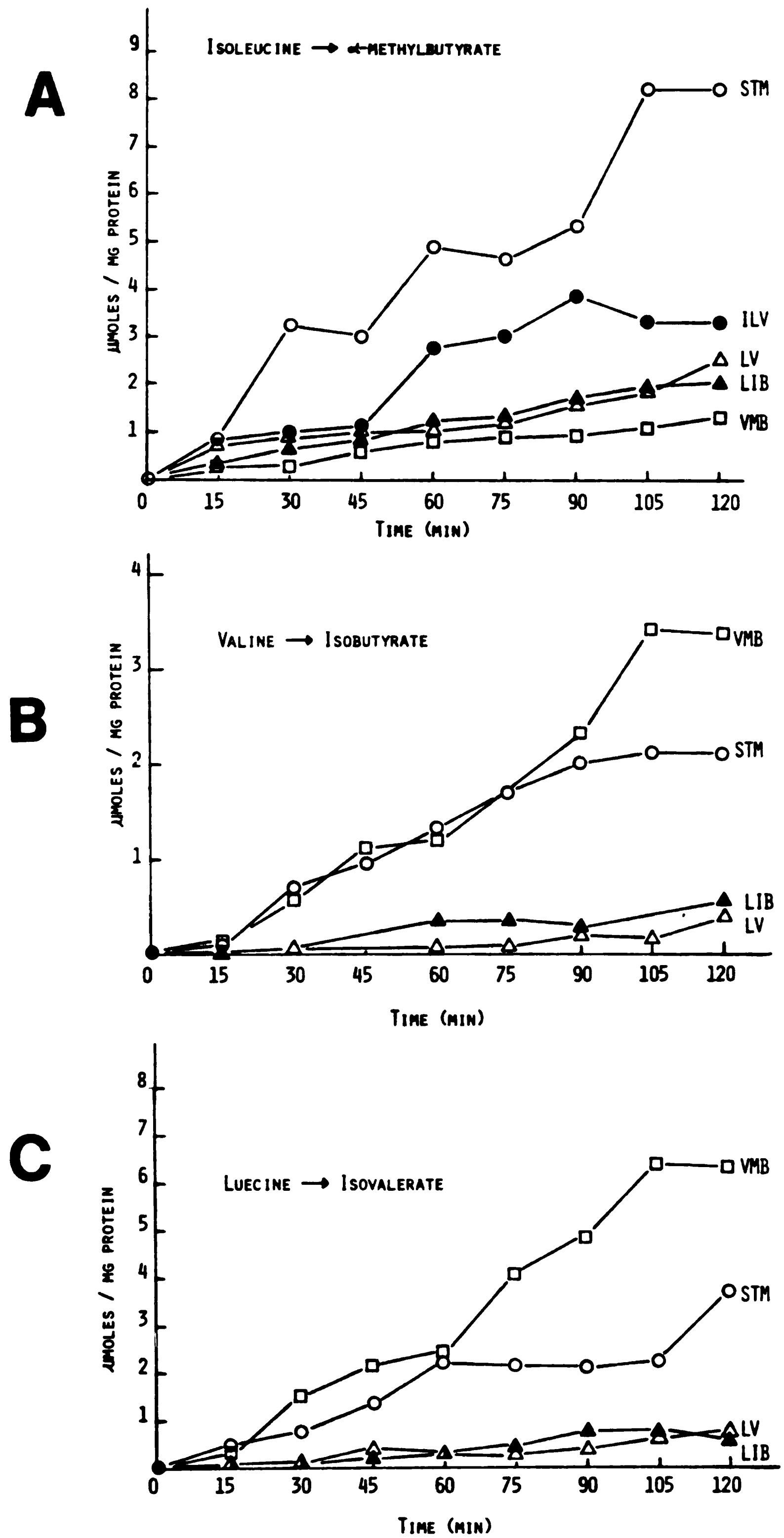
One mechanism utilized by *C. sporogenes* for ATP generation is the coupled oxidation and reduction of amino acid pairs (the Stickland reaction). An attempt was made to evaluate the contribution of these oxidations to the overall disposition of the branched-chain amino acids in cells grown in various minimal media. In some media, this drain on the amino acid pools may play an important part in diminishing the isoleucine, valine and leucine levels in the cell.

Cells were grown in the basal medium described in Section II, with 10 mM leucine and 10 mM valine (designated medium LV in figures 1-3); 10 mM leucine and 10 mM α-methylbutyrate (medium VMB); 10 mM isoleucine, 10 mM leucine and 10 mM valine (ILV) or the standard Trypticase medium (STM). 50 ml cultures of late exponential phase cells were harvested, washed with buffer (50 mM potassium phosphate, pH 7.5), and suspended in 20 ml of the same buffer with 50 mM L-proline and 25 mM L-isoleucine, L-leucine or L-valine. The suspensions were incubated at 37° in an anaerobic chamber. At 15 min intervals, 0.20 ml samples were removed and 0.05 ml of 41 mM n-valerate (final concentration 1 mM) added to them as an internal standard. Samples without n-valerate showed that no valerate was produced in these experiments. After acidification with 9 N H_2SO_4, the volatile fatty acids were extracted with 1 ml of ether, and the preparation frozen overnight.

Volatile fatty acid concentrations were determined by comparison with standards after separation by gas-liquid chromatography. Separations were effected on a 15% SP-1220/ 1% H_3PO_4 on 100/120 Chromosorb W AW, 6ft. x 4mm ID glass column, column temp. 145°C, flow rate 70ml/min. Volatile fatty acids were detected by flame ionization with an F&M Research Chromatogram.

The results of these in vitro oxidations of isoleucine, leucine and valine are shown in Fig. 1. Regardless of the medium the cells were grown in , they retained the capacity to oxidize all three amino acids to some extent. This suggests the presence of a relatively nonspecific amino acid oxidation system in these bacteria. Fig. 1-A shows that cells grown in STM could oxidize isoleucine significantly better than those grown in the other media. Since the complex STM medium contains considerable isoleucine, this may indicate the presence of an inducible isoleucine degradation system in the bacteria. A similar result was not observed for leucine and valine oxidation. However, cells grown in the VMB medium oxidized both valine (Fig. 1-B) and leucine (Fig. 1-C) better than cells grown in any other medium, including STM. The role of α-methylbutyrate in stimulating the valine and leucine oxidation system is not clear.

Figure 1. The oxidation of L-isoleucine (A), L-valine (b) and L-leucine (C) to α-methylbutyrate, isobutyrate or isovalerate. Cells were harvested from basal media supplemented as described in the text, and resuspended in buffer with 50 mm proline and 25 mm L-isoleucine, L-valine or L-leucine.


A
ISOLEUCINE → α-METHYLBUTYRATE
µMOLES / MG PROTEIN
TIME (MIN)
STM
ILV
LV
LIB
VMB
B
VALINE → ISOBUTYRATE
µMOLES / MG PROTEIN
TIME (MIN)
VMB
STM
LIB
LV
C
LUECINE → ISOVALERATE
µMOLES / MG PROTEIN
TIME (MIN)
VMB
STM
LV
LIB

Effect of α-Methylbutyrate on Total Growth

It has been shown that *C. sporogenes* grows well in the basal medium with 10 mM valine and either 10 mM leucine or 10 mM α-methylbutyrate (R.S. Hadioentomo, 1980, Ph.D. dissertation, Michigan State University). To further examine the role of α-methylbutyrate, cells were grown in the basal medium (section III) with 10 mM valine and increasing concentrations of either leucine, isoleucine or α-methylbutyrate (Figure 2.). The cells grew significantly better with added α-methylbutyrate than they did with added isoleucine. When the α-methylbutyrate was analyzed by separation by gas chromatography, it was found to be contaminated with approximately 10% isovalerate (Figure 3). Two different lots of this product (Eastman Co.) were similarly contaminated.

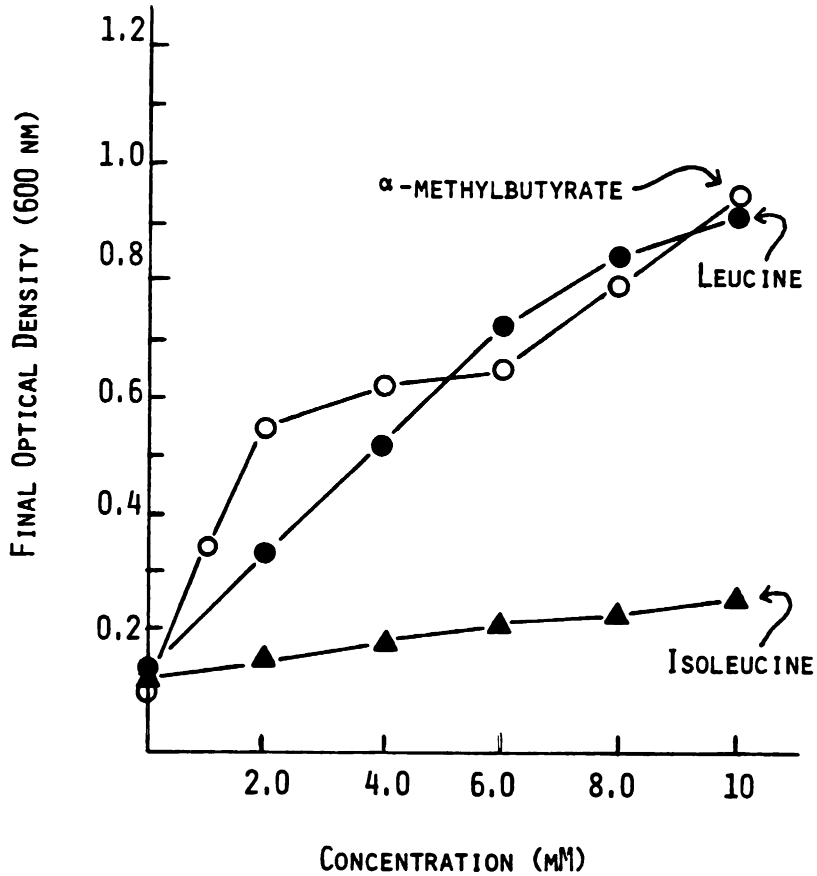


Figure 2. The effect of α-methylbutyrate on the final optical density (total growth) of cultures in 3 different media. All of the media include the basal medium and 10 mM L-valine, with α-methylbutyrate, L-leucine or L-isoleucine.

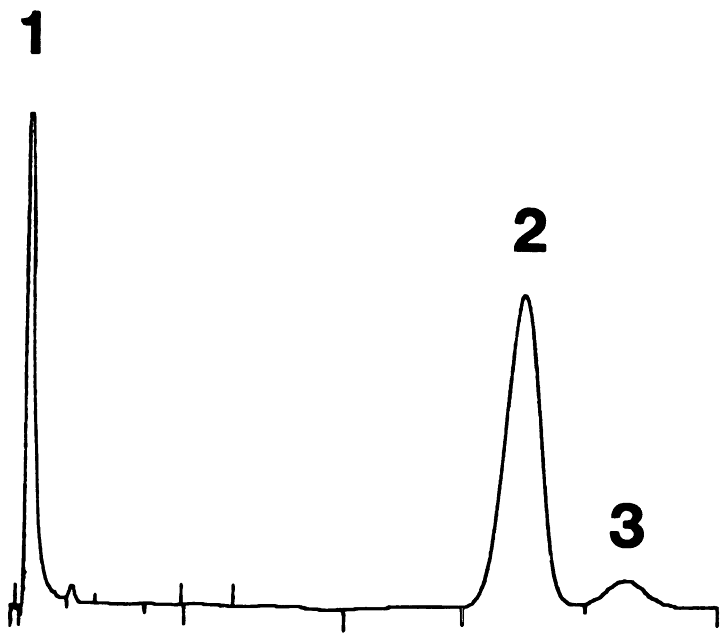


Figure 3. Separation of α-methylbutyrate and isovalerate by gas chromatography. Shown above is the recorder printout following the injection of a "10 mm α-methylbutyrate" sample. Peak 1 is the solvent front, peak 2 α-methylbutyrate, and peak 3 is isovalerate. Column and Conditions; Varian model 2400 equipped with flame ionization detector, 6ft x 2 mm glass column packed with carbopak c coated with 0.3% carbowax and 0.1% H_3PO_4. Injector temp - 175°, Detector temp. - 175°, oven temp. - 135°, H_2 carrier at 30 cc/min, sample size 1 μl.

SECTION V

SUMMARY

The results presented illustrate the complexity of branched-chain amino acid metabolism in *Clostridium sporogenes*. This investigation was initiated by the observation that *C. sporogenes* required valine and leucine for growth in a minimal medium, but not isoleucine.

There is considerable evidence (section II) that isoleucine is made by the classical pathway (4) using propionate as a precursor of α-ketobutyrate, or that isoleucine can be produced directly from α-methylbutyrate. In an isotope competition experiment, exogenous α-methylbutyrate was shown to decrease the incorporation of [^{14}C]pyruvate into isoleucine, while having no effect on $^{14}CO_2$ incorporation. Since L-threonine is not a major source of isoleucine, cells were labeled with α-amino-[3-^{14}C]butyrate to determine if α-ketobutyrate was produced from some other source. The incorporation of considerable ^{14}C-label, and the demonstration of AHAS activity (the first enzyme in the classical apthway) suggested that α-ketobutyrate was a precursor of isoleucine. Further labeling experiments showed that [2-^{14}C]propionate was also a precursor of isoleucine, and an isotope competition experiment demonstrated that exogenous propionate reduced the incorporation of

[^{14}C]serine into isoleucine. These experiments prompted the labeling of isoleucine with [1-^{14}C]propionate, [2-^{14}C]-propionate and [3-^{14}C]pyruvate and subsequent sequential degradation. It was found that the ^{14}C-label from [1-^{14}C]-propionate was in the C-2 of isoleucine, [2-^{14}C]propionate was in the carbon 3-5 fragment, and [3-^{14}C]pyruvate in the carbon 3-5 fragment and C-6. These results support the conclusion that propionate is reductively carboxylated to α-ketobutyrate in the cell, which is then converted to isoleucine via the classical pathway, and that α-methylbutyrate is an alternate source of isoleucine.

α-Amino-[^{14}C]butyrate was found to accumulate in the medium following labeling with [^{14}C]propionate. Since α-aminobutyrate is a potent inhibitor of valine synthesis in _B. anthracis_ (1) and _E. coli_ (3), this may explain _C. sporogenes_' inability to grow in the minimal medium without exogenous valine and leucine.

Further investigations of the reductive carboxylation capabilities of _C. sporogenes_ showed that these bacteria could make valine from isobutyrate and probably leucine from isovalerate. The results presented in section III indicate that _C. sporogenes_, like some other bacteria (4), is capable of the conversion of valine to leucine via the chain elongation pathway. This organism probably converts leucine to valine by first forming β-leucine using a leucine 2,3-aminomutase enzyme, which is then oxidized to acetate and isobutyrate (2). The isobutyrate can then be reductively carboxylated to the keto-acid precursor of valine.

The branched-chain amino acid pools in C. sporogenes were shown to be subject to several unusual activities not observed in the enteric bacteria (4). Isoleucine, valine and leucine are subject to non-specific oxidation, presumably by the cellular machinery for ATP synthesis via the coupled oxidation/reduction of amino acid paris (the Stickland reaction). ^{14}C-Labeled branched-chain amino acids were also incorporated into cellular lipid, especially valine. Branched-chain fatty acids have been shown to represent a considerable portion of the fatty acids in C. sporogenes lipid.

It is clear that the substitution of α-methylbutyrate for leucine in the minimal medium is possible because of the considerable amounts of isovalerate in the α-methylbutyrate preparation. Media which were believed to contain 10 mM α-methylbutyrate actually contained almost 1 mM isovalerate. Reductive carboxylation of this fatty acid yields the keto-acid precursor of leucine.

The results presented here illustrate the unique strategies and mechanisms employed by anaerobic bacteria to survive in their highly reduced environment. The reductive carboxylations observed here for the production of isoleucine from propionate or α-methylbutyrate, valine from isobutyrate and leucine from isovalerate can only proceed under such reduced conditions. These reactions and their regulation represent a unique system for branched-chain amino acid synthesis and utilization in Clostridium Sporogenes.

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