EVIDENCE FOR A NOVEL PATHWAY FOR ISOLEUCINE BIOSYNTHESIS IN *CLOSTRIDIUM SPOROGENES*

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1980

ABSTRACT

EVIDENCE FOR A NOVEL PATHWAY
FOR ISOLEUCINE BIOSYNTHESIS
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Extracts of C. sporogenes (ATCC 7955, NCA PA 3679) were shown to contain an active biodegradative threonine dehydratase. The enzyme was constitutive and was markedly stimulated by ADP at low threonine concentrations. No stimulation occured at high substrate concentrations. The apparent K_m's for threonine in the presence and absence of ADP were 2.5 and 33.33 mM, respectively. No isoleucine sensitive threonine dehydratase activity was detected over a wide pH range. Threonine aldolase and threonine dehydrogenase activities were also not detected.

C. sporogenes grew well in a minimal synthetic medium containing 8 essential amino acids (L-serine, L-arginine, L-phenylalanine, L-tyrosine, L-tryptophan, L-valine, L-leucine, and L-methionine), salts and vitamins (medium B-10). The facts that exogenously added isoleucine was not required for growth and that isoleucine sensitive threonine dehydratase could not be detected prompted an investigation of isoleucine biosynthesis in this

organism by measuring ^{14}C incorporation from labeled individual components of medium B-10. Of the amino acids in medium B-10, only ^{14}C-serine was found to contribute a significant amount of ^{14}C to cellular isoleucine during growth. ^{14}C-Isoleucine was also found in hydrolysates of proteins from cells grown in the presence of [3-^{14}C]pyruvate and $^{14}CO_2$. Cells grown in the presence of either ^{14}C-threonine, ^{14}C-aspartate, or ^{14}C-glutamate did not contain labeled isoleucine. The amount of ^{14}C from [3-^{14}C]pyruvate and $^{14}CO_2$ found in isoleucine was lower than would be expected to account for all 6 carbons. In contrast, results of studies of the extent of ^{14}C incorporation and of $^{14}CO_2$ released by decarboxylation of radioactive alanine, aspartate, threonine, lysine, glutamic acid, serine, and glycine were consistent with established biosynthetic pathways.

Growth in medium B-10 was dependent on the presence of a low contaminating level of isoleucine or on the presence of substantial levels of 2-methylbutyric acid. The latter is the major product of isoleucine degradation by C. sporogenes. Protein from cells incubated in the presence of fermentation products of ^{14}C-isoleucine incorporated the label specifically into the isoleucine of cell protein. However, the specific activity of the isoleucine formed indicated an average of less than 4 carbons from ^{14}C-2-methylbutyric acid incorporated per isoleucine formed. This plus the facts that the C-3 carbon of pyruvate and the carbon from $^{14}CO_2$ are incorporated into carbons other than the carboxyl carbon of isoleucine indicates that at least part of the isoleucine formed is synthesized

by reactions more complex than the reductive carboxylation of 2-methylbutyric acid.

C. sporogenes grew well in medium B-10 without either leucine or isoleucine when 8 mM 2-methylbutyrate was added. Either there was sufficient contaminating leucine in the medium or this organism has the capacity to synthesize leucine. However, no label from any of the labeled substrates tested including 2-methylbutyric acid was found in the leucine isolated from cell proteins. Failure to incorporate significant ^{14}C from labeled pyruvate or CO_2 may have resulted from the high levels of leucine in the growth medium used in these experiments.

DEDICATION

To my husband,

IGNATIUS J. HADIOETOMO,

for his unfaltering support,

encouragement and understanding.

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ACKNOWLEDGEMENTS

I wish to thank my major professor, Dr. R. N. Costilow, for his guidance and assistance throughout the course of this work and during the preparation of this dissertation.

I would also like to express my thanks to Dr. H. L. Sadoff and Dr. C. A. Reddy for the use of their laboratory facilities.

Financial support for the program under which this study was conducted was provided by the Midwest Universities Consortium for International Activities under a contract with the Agency for International Development.

INTRODUCTION

In Escherichia coli, Salmonella typhimurium, and some other microorganisms (66), the first enzyme in the isoleucine biosynthetic pathway is threonine dehydratase. The latter is a pivotal enzyme, since, being subject to feedback inhibition by isoleucine, it regulates the flow of carbon over this pathway in these organisms. In E. coli and S. typhimurium, the formation of the biosynthetic threonine dehydratase is controlled by multivalent repression by isoleucine, valine, and leucine (65).

The biosynthesis of isoleucine via unusual pathways in a few bacteria is also well documented. Some rumen bacteria synthesize isoleucine by carboxylation of 2-methylbutyric acid (55). Some of these organisms (e.g. Methanobacterium ruminantium strain M-1 and Bacteroides ruminicola subsp brevis, 8, 20) require 2-methylbutyrate for growth, while some others do not. Certain species of rumen bacteria were found to preferentially synthesize amino acids de novo even when they are grown in a medium containing a complete mixture of amino acids (9, 10). While representative strains fo B. ruminicola were found to utilize peptide nitrogen or ammonia nitrogen, free amino acids would not serve as the nitrogen source for growth (52). A threonine dehydratase-less mutant of E. coli Crookes strain uses glutamate as a precursor of α-ketobutyrate (and isoleucine) (50). In Leptospira serotypes semaranga and tarassovi, isoleucine

biosynthesis via threonine dehydratase reportedly operates to a very limited extent; instead, most of the cellular isoleucine is synthesized from α-ketobutyrate which in turn is synthesized via an unusual pathway (15).

Preliminary studies in this laboratory showed that Clostridium sporogenes could utilize certain amino acids as the sole source of energy, carbon and nitrogen. The data indicated that neither isoleucine nor branched chain volatile fatty acid(s) were required for growth. The organism was found to possess a very active threonine dehydratase, suggesting that α-ketobutyrate is produced from threonine. However, the failure to detect (in vitro) an isoleucine-sensitive threonine dehydratase and the lack of repression of the latter in cells grown in the presence of isoleucine, leucine, and valine raised the possibility that threonine might not be the precursor of isoleucine. This was confirmed by the inability of the cells to incorporate label from L-[U-^{14}C] threonine into isoleucine, suggesting that in C. sporogenes isoleucine might be synthesized via a pathway different from those described above. Accordingly, experiments were designed to investigate the pathway for isoleucine biosynthesis in C. sporogenes by isotopic labeling with specific substrates, enzymological approaches, and other analytical methods. The results are described herein.

LITERATURE REVIEW

Nutritional requirements of C. sporogenes. The early literature describing nutritional requirements of C. botulinum and related clostridia was summarized by Mager et al (43). The majority of these studies resulted from the necessity to develop synthetic media suitable for investigating the physiology of toxin production, growth, and sporulation in C. botulinum. Most of the media so developed were chemically defined and consisted of mixtures of amino acids, salts, vitamins and, usually, glucose.

In general, excellent growth was usually obtained with media containing mixtures of 15 to 19 amino acids (13, 43, 48). Mager et al (43), Roessler and Brewer (56) and Campbell and Frank (13) identified the following amino acids as essential for growth of C. parabotulinum type A: tryptophan, threonine, valine, leucine, isoleucine, methionine, arginine, phenylalanine and tyrosine. The last three amino acids were required in unusually high concentrations (43), which is consistent with the data of Shull et al (61) for C. sporogenes. The requirement for excess arginine could be relieved in part by ornithine and lysine (43); however, higher concentrations of arginine in a complete mixture of amino acids resulted in sporulation (48) instead of lysis (43, 48) following maximal growth. The requirement for methionine could be partially relieved by cysteine but less than maximal growth was obtained. Leucine

and isoleucine could substitute for each other to some extent (43). Not all the strains require valine. Studies with media containing only the essential amino acids revealed that glycine would eliminate the requirement for threonine, and that serine could replace glycine. Among the various strains tested, some preferred glycine, and some others preferred serine (34). From a nutritional point of view, C. sporogenes is indistinguishable from C. parabotulinum types A and B (34, 43).

Campbell and Frank (13) found that 10 strains of a putrefactive anaerobe (PA 3679), although variable in their requirements for other amino acids, shared a common requirement for arginine, phenylalanine, tyrosine, valine, isoleucine, and serine. The latter amino acids, along with proline and histidine, were reported to be essential for C. sporogenes ATCC 7955 (13), the organism used throughout this investigation.

It should be noted that variation in growth conditions, reagent purity, etc. can result in erroneous conclusions about the nutrient requirements of a particular organism. It was demonstrated that decreasing the size of inoculum may necessitate the inclusion of an amino acid which is otherwise dispensible when a large inoculum is used (34). These and other inconsistencies (e.g. the level of growth that is scored as positive, etc.) have resulted in conflicting reports of the nutritional requirements of a given organism.

The vitamin requirements of C. botulinum vary among the individual strains. In C. sporogenes, biotin and p-aminobenzoate

are essential, whereas nicotinic acid and thiamine are merely stimulatory (34, 60). The strain used in these experiments (ATCC 7955) requires only thiamine and biotin (13).

Threonine dehydratase. Threonine dehydratase (L-threonine hydrolyase deaminating, E.C.4.2.1.16) is one of the enzymes involved in ghe degradation of threonine and catalyzes the conversion of threonine to α-ketobutyrate and ammonia (65). Two distinctly different forms of threonine dehydratases (termed biosynthetic and biodegradative, respectively) are known to be produced by E. coli (64, 74).

In E. coli, the biosynthetic form of threonine dehydratase is synthesized by cells growing aerobically in a glucose minimal medium. The enzyme is inhibited by isoleucine (64) and thus considered to participate in the initial step of isoleucine biosynthesis. Threonine dehydratase has been purified from various sources, including a yeast, S. typhimurium, E. coli, Rhodospirillum spheroides, and Rhodospirillum rubrum [for review, see Umbarger (65)]. While differences exist among the threonine dehydratases from various sources, the enzymes share several common features. In particular, biosynthetic threonine dehydratase is subject to inhibition by isoleucine which is reversed by valine. The degree of inhibition, however, varies with the source of the enzyme. For example, in R. rubrum, the enzyme in vitro is only slightly sensitive to isoleucine (28); however, there has been no report on the in vivo control over this enzyme in this organism.

In E. coli and S. typhimurium, synthesis of biosynthetic threonine dehydratase is subject to multivalent repression; i.e., repression which is mediated by the simultaneous presence of excess isoleucine, valine and leucine (65). However, this mechanism of regulation is not ubiquitous, since in Corynebacterium sp (6) and Pseudomonas multivorans (39) multivalent repression of the enzyme by isoleucine, leucine and valine does not occur. Moreover, in the latter two organisms, the biosynthetic enzyme is the only form of threonine dehydratase found, it is synthesized constitutively, and is sensitive to feedback inhibition by isoleucine. In contrast to the P. multivorans enzyme, which has no direct role in threonine catabolism (39), threonine dehydratase from Corynebacterium sp was shown to have a catabolic function which is dependent on the concomitant catabolism of branched-chain amino acids (6).

In E. coli and S. typhimurium, the biodegradative form of threonine dehydratase is formed under anaerobic conditions, is induced by threonine and serine, requires adenylic acid for optimal activity, and is sensitive to catabolite repression (41, 49, 78). More recently, Yui et al (77) reported that threonine, serine, aspartic acid, and methionine collectively function as inducers of the biodegradative threonine dehydratase in the Crookes strain of E. coli and proposed the term "multivalent induction." Valine, leucine and arginine are amplifiers of enzyme production. In contrast, Egan and Phillips (22) reported that omission of serine from a complete synthetic medium containing 19 amino acids resulted in only a minor reduction in the synthesis of the enzyme by the same

organism. Similarly, enzyme induction was not affected by omission of aspartate and methionine from the medium. Unfortunately, the requirement for aspartate could not be objectively assessed owing to the experimental conditions employed. It had been reported that omission of arginine curtailed induction of the enzyme (77). However, this was interpreted by Egan and Phillips (22) as resulting from a lack of an energy source capable of maintaining adequate arginine levels for protein synthesis. However, in spite of these apparent contradictions, the available data (22, 77) point to the conclusion that in the Crookes strain of E. coli, threonine, leucine, and valine are essential components for induction of the biodegradative form of threonine dehydratase.

Anaerobiosis is required for optimal synthesis of biodegradative threonine dehydratase in E. coli (22, 64, 74). Oxygen was found to cause a rapid inactivation of purified threonine dehydratase in the absence of reducing agent (71). However, oxygen did not affect the enzyme stability in vivo under conditions where further protein synthesis and growth was inhibited (22). This suggests that oxygen causes a metabolic condition that prevents further enzyme formation.

Shizuta and Hayaishi (59) observed that cyclic adenosine 3',5'-monophosphate (cAMP) reverses the catabolite repression caused by glucose and promotes the appearance of biodegradative threonine dehydratase in resting cells of E. coli, and concluded that this represents a transcriptional effect similar to that reported for the cAMP stimulation of β-galactosidase. The absolute requirement for

cAMP was later confirmed by Phillips, Egan and Lewis (51) in their studies using an adenylate cyclase mutant. cAMP was also required for the synthesis of the catabolic threonine dehydratase by S. typhimurium. This enzyme is immunologically different from the biosynthetic dehydratase in this organism (41).

The proposed role of biodegradative threonine dehydratase is to provide ATP formation from threonine during anaerobic growth in an amino acid rich medium when ATP cannot be produced by means of oxidative phosphorylation (22). Such a role is further implied by the dehydratase activation by AMP (74). The mechanism of activation has been shown to involve both changes in quaternary structure of the enzyme and specific facilitation of an early step in the reaction mechanism (53, 71). Presumably homologous with the effect of AMP on the E. coli enzyme is the effect of ADP on the enzyme from C. tetanomorphum (27). ADP promotes and helps to maintain the enzyme in aggregated state; however, the aggregation and dissociation appear to be a slower process with the ADP-activated enzyme than they are with the AMP-activated enzyme. C. tetanomorphum dehydratase was shown to breakdown threonine into propionate and CO_2 as terminal products via the intermediates α-ketobutyrate and propionyl phosphate, and the process was linked to ATP generation (62, 63, 65).

Phillips, Egan and Lewis (51) recently showed that when an exponentially growing culture of E. coli Crookes strain was made anaerobic, a sharp increase in internal cAMP was noted, and the synthesis of biodegradative threonine dehydratase was detected immediately after the attainment of the peak cAMP levels and

continued for several generations. Pyruvate addition at the time of anaerobic shock severely affected both cAMP accumulation and threonine dehydratase synthesis; however, externally added cAMP could partially counter the pyruvate effect on enzyme synthesis. It was thus concluded that conditions which resulted in a temporary energy deficit brought about the major accumulation of cAMP, and this elevated level served as a signal for initiation of threonine dehydratase synthesis to supply energy by the non-oxidative degradation of threonine.

Isoleucine biosynthesis. Several pathways for isoleucine formation are known to occur in microorganisms (Fig. 1). These have been reviewed by Umbarger (66). The biosynthesis of isoleucine in most organisms initially involves the conversion of threonine to α-ketobutyrate by threonine dehydratase. The condensation of α-ketobutyrate with a two-carbon fragment followed by a succession of four reactions results in the formation of isoleucine. The enzymes of the latter four reactions are shared in valine biosynthesis.

Exceptions to the threonine dehydratase pathway have been reported for a number of organisms (Fig. 1). These include rumen bacteria (3, 55), Bacillus subtilis (57), E. coli grown with β-methylaspartate (1), E. coli Crookes strain (50), Acetobacter suboxydans (7), Leptospira (15), and Serratia marcescens (36). Except for the rumen bacteria, α-ketobutyrate from sources other than threonine is the precursor for isoleucine synthesis in all cases.

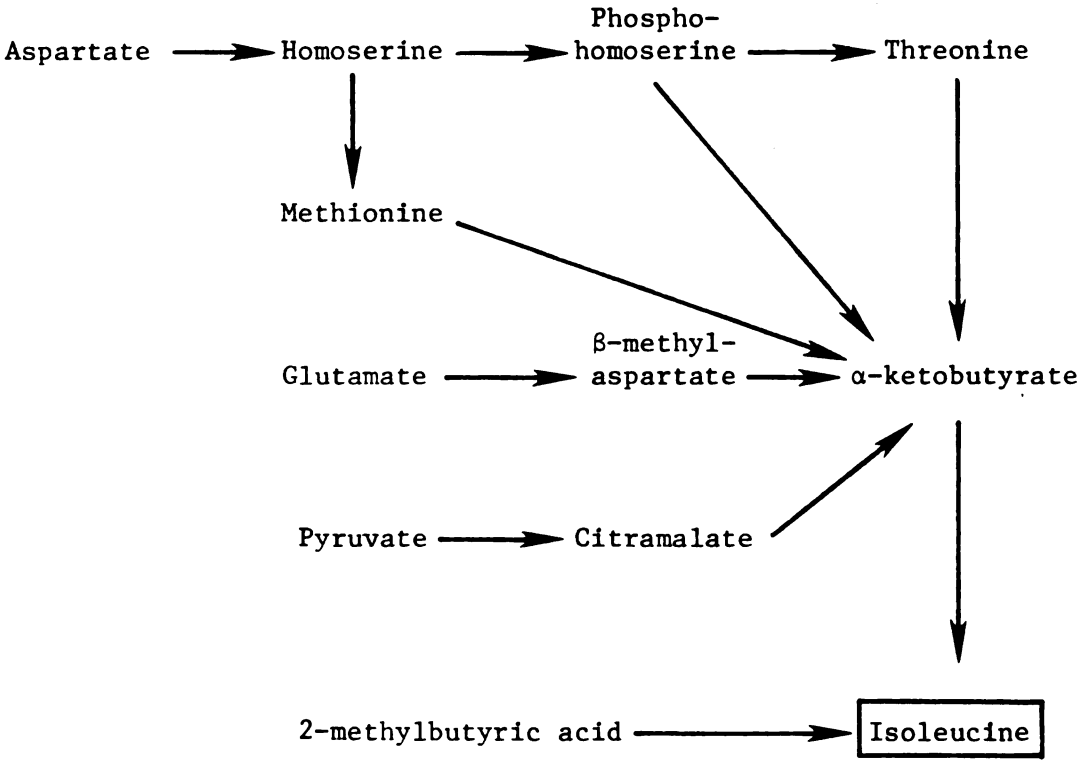


FIG. 1. Various pathways of isoleucine biosynthesis by micro-organisms.

While some species of rumen bacteria use exogenous amino acids (11), amino acids in peptides are more efficiently utilized for growth than free amino acids (52). Certain species utilize ammonia in preference to preformed exogenous organic nitrogen (3). Branched-chain volatile fatty acids are nutritional requirements for several groups of important ruminal bacteria. The acids are used for synthesis of branched-chain amino acids, branched-chain fatty acids and aldehydes, and probably for other cellular constituents possessing branched-carbon chains. Some representatives of the more important rumen bacteria were reported to synthesize isoleucine from 2-methylbutyric acid (55), presumably via reductive carboxylation followed by amination reactions. Similar reactions are operative in the synthesis of leucine, valine, phenylalanine, and tryptophan from isovalerate, isobutyrate, phenylacetate, and indole acetic acid, respectively (3). However, not all of these bacteria require 2-methylbutyric acid for growth. For instance, Bacteroides ruminicola strain 23 was stimulated by a mixture of volatile fatty acids including 2-methylbutyric acid but these acids were not essential (10). The organism must be able to synthesize its branched-chain carbons, including isoleucine, since it was found to grow well when acetate was the only volatile fatty acid added to the medium (55). Isoleucine biosynthesis from 2-methylbutyric acid is subject to end-product control by isoleucine from casein peptides (55). The synthesis of amino acids from volatile fatty acids by rumen bacteria is a reflection of their habitat in which the concentration of amino acids is usually relatively low (free amino

acids are rapidly catabolized), whereas the concentrations of straight- and branched-chain fatty acids are high, these having been generated from the catabolism of peptides and amino acids (42).

An enzyme found in B. subtilis, termed phosphorine deaminase, catalyzes the dephosphorylation and deamination of phosphohomoserine to α-ketobutyrate without the intermediate formation of threonine (57). The deaminase activity was found to be associated with threonine synthetase since both activities were shown to be affected by a single mutational event and coordinately derepressed. Growth experiments with a strain deficient in biosynthetic threonine dehydratase conducted in the presence of ^{14}C-L-homoserine plus excess unlabeled threonine or in ^{14}C-L-threonine plus increasing concentrations of unlabeled homoserine showed the synthesis of isoleucine from homoserine without the intermediate formation of threonine.

Abramsky and Shemin (1) demonstrated the conversion of β-methylaspartate exclusively to isoleucine in E. coli W. since all the radioactivity in the isoleucine synthesized from [CH_3-^{14}C] β-methylaspartate was in C-5, they suggested that β-methylaspartate is converted to α-ketobutyrate. Such labeling pattern would be obtained upon the conversion of [4-^{14}C] α-ketobutyrate to isoleucine by the established pathway (66). Their suggestion was supported by the facts that: (a) a cell extract catalyzed the transamination of β-methylaspartate to its corresponding β-keto acid, which on decarboxylation yielded α-ketobutyrate; and (b) β-methylaspartate supported the growth of an isoleucine auxotroph of E. coli W blocked

between threonine and α-ketobutyrate. However, it was not clear if the above mechanism of isoleucine synthesis occurred only on the addition of β-methylaspartate to the growth medium.

Phillips et al (50) demonstrated α-ketobutyrate formation from glutamate via β-methylaspartate. A threonine dehydratase minus mutant of E. coli, Crookes strain, was shown to grow without added isoleucine in certain media containing glutamate or in media which presumably allowed for the production of appreciable intracellular glutamate. When both a wild type culture and a threonine auxotroph blocked between homoserine and threonine were grown on [1-^{14}C] glutamate, the label was almost completely retained in C-1 of isoleucine; and all the radioactivity in isoleucine was recovered in CO_2 upon decarboxylation with ninhydrin. These findings are convincing evidence for a β-methylaspartate route. It was further supported by the presence of glutamate mutase and β-methylaspartate aminotransferase activities in the same organism. However, the physiological significance of this pathway is unclear.

Charon et al (15) proposed two possible pathways of isoleucine biosynthesis in the spirochete Leptospira involving citramalate as an intermediate (Fig. 2). Only a trace amount of radioactivity from [4-^{14}C]aspartate and [U-^{14}C]threonine was incorporated into isoleucine suggesting that the threonine dehydratase pathway operates only to a minor extent. [3-^{14}C]Pyruvate and [1-^{14}C]pyruvate incorporation experiments showed that apparently two C-3's but not C-1's of pyruvate contributed to the carbon skeleton of isoleucine. One molecule of acetate was also found to

contribute to the carbon skeleton of isoleucine in Leptospira. Exogenous isoleucine inhibited the incorporation of radioactivity from [3-^{14}C]pyruvate into isoleucine by 98% which implies the regulation of the isoleucine pathway by isoleucine in Leptospira. However, attempts to determine the precise intermediate in the pathway by isotope competition studies with [3-^{14}C]pyruvate and some of the proposed radioactive intermediates were not successful. Furthermore, since the above studies deal with the synthesis of α-ketobutyrate by a pathway other than threonine dehydratase pathway, it is unfortunate that threonine dehydratase activity of the microorganism studied was not reported.

The sequence of reactions of β-methylaspartate synthesis from pyruvate plus acetyl coenzyme A via citramalate and mesaconate (Fig. 2A) was demonstrated enzymatically in Acetobacter suboxydans (7). When the latter organism was grown in glycerol basal-salts medium containing [1-^{14}C]acetate, label was incorporated exclusively into the C-1 position of isoleucine. Thus the participation of β-methylaspartate in the synthesis of isoleucine was suggested. The second pathway (Fig. 2B) was reported to operate in leucine-accumulating isoleucine revertants of S. marcescens (36). The first enzyme in the pathway is isopropylmalate synthase which is the first enzyme in the leucine biosynthetic pathway. The latter enzyme from a variety of sources has been shown to have only a limited specificity; it can transfer the acetyl group not only to α-ketoisovalerate but also to pyruvate, α-ketobutyrate, α-ketovalerate, and even to α-ketoisocaproate itself (66). It was

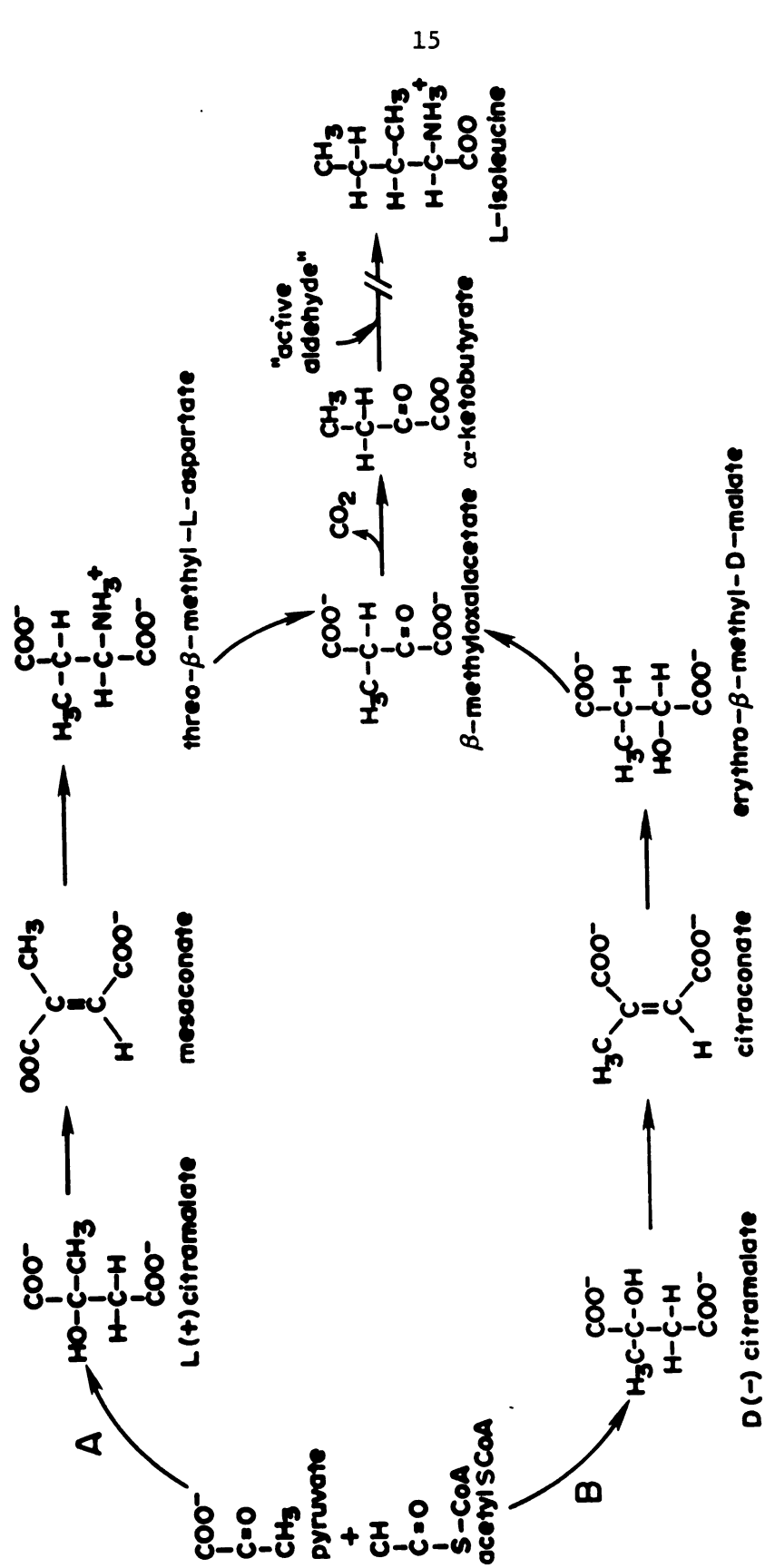


FIG. 2. Proposed pathways of isoleucine biosynthesis in Leptospira (15).

suggested that this lack of specificity does not seem to matter in wild type organisms because either the enzyme does not encounter significant amount of secondary substrate or the K_m for α-ketoisovalerate makes it the favored substrate. Most of the isoleucine revertants accumulated large amounts of leucine in the medium due to both constitutive synthesis of isoleucine resulting from α-aminobutyric acid resistance and desensitization of α-isopropylmalate synthase resulting from isoleucine auxotrophy (35). It was found that partial reversion of isoleucine auxotrophy in leucine-accumulating revertants did not depend on the restoration of L-threonine dehydratase activity (36). The growth of the revertants was stimulated by β-methylaspartate, D(-)-citramalate or citraconate but not by glutamate, L(+)-citramalate or mesaconate. Also, no glutamate mutase was found in the cell extracts. It was therefore suggested that isoleucine is formed in the revertants from pyruvate by the leucine biosynthetic enzyme via citramalate and citraconate as intermediates of α-ketobutyrate formation. The cell extracts of the revertants were shown to catalyze the formation of citramalate from pyruvate plus acetyl coenzyme A and the isomerization of D(-)-citramalate to erythro-β-methyl-malate via citraconate. The formation of α-ketobutyrate from citraconate and not from mesaconate seemed to confirm the proposed pathway.

MATERIALS AND METHODS

Cultures and cultural methods. C. sporogenes (ATTC 7955, National Canners Association PA 3679) was used in all experiments.

The growth media used were:

(1) Medium A: "standard trypticase medium" consisted of 4.0% trypticase, 2 ppm thiamine hydrochloride, and 0.05% sodium thioglycolate.

(2) Medium B: a synthetic medium containing salts, vitamins, and amino acids adapted from the medium of Perkins and Tsuji (48). Modifications of medium B (designated B-1 through B-10; Table 1) were prepared by varying the amino acid composition.

All media were prepared with deionized distilled water and adjusted to pH 7.4 before autoclaving.

The culture was maintained by refrigeration of a culture that had sporulated in medium A. 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 for 6-8 h. All growth experiments were performed in an anaerobic chamber (Coy Mfg., Ann Arbor, MI) at 37°C. For growth in various synthetic media, the cells were preadapted in the corresponding medium.

Table 1. Compositions of various synthetic media used for the cultivation of C. sporogenes[a]

Component	Concentration (mM) in media									
	B-1	B-2	B-3	B-4	B-5	B-6	B-7	B-8	B-9	B-10
L-tyrosine	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	2.00
L-phenylalanine	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	10.00
L-tryptophan	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	2.00
L-leucine	11.50	11.50	11.50	15.00	11.50	11.50	11.50	11.50	11.50	11.50
L-valine	17.00	17.00	15.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00
L-methionine	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	10.00
L-arginine	13.40	13.40	13.40	13.40	20.00	5.00	5.00	13.40	13.40	20.00
L-serine	9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50	--	25.00
L-histidine	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30	--
glycine	1.60	1.60	1.60	1.60	1.60	1.60	1.60	--	1.60	--
L-alanine	4.50	4.50	4.50	4.50	4.50	4.50	4.50	--	--	--
L-cysteine	14.00	14.00	7.00	7.00	7.00	7.00	7.00	--	--	--
L-glutamate	0.70	0.70	7.00	7.00	7.00	7.00	7.00	--	--	--
L-ornithine	10.00	10.00	10.00	10.00	--	--	15.00	--	--	--
L-proline	4.30	4.30	4.30	4.30	4.30	15.00	--	--	--	--
L-threonine	8.40	15.00	8.40	8.40	--	--	--	--	--	--
L-isoleucine	3.80	--	--	--	--	--	--	--	--	--
D-glucose	--	--	--	--	--	--	--	--	20.00	--

[a]All media contained the following components in a final volume of 10 ml: 0.02 mg $FeSO_4 \cdot 7\ H_2O$, 0.02 mg $CaCl_2 \cdot 2\ H_2O$, 0.10 mg $MnSO_4 \cdot H_2O$, 0.40 mg $MgSO_4 \cdot 3\ H_2O$, 0.02 mg NaCl, 10 mg K_2HPO_4, 10 mg KH_2PO_4, 0.005 µg biotin, 4.0 µg thiamine, 0.1 µg p-aminobenzoic acid, and 5.0 mg sodium thioglycolate.

Preparation of cell extracts. Exponential phase cells were harvested by centrifugation at 18,000 X g, washed once with cold 0.1 M potassium phosphate buffer (pH 7.5), and resuspended in the same buffer (0.5 g of packed cells per ml). Cell extracts were prepared by ultrasonic oscillation of 1.5 to 2.0 ml of cell suspension for four 15-sec intervals in a 100-W ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London) while the temperature was maintained below 20°C in an ice bath. The cell debris was removed by centrifugation at 20,000 X g for 20 min at 4°C. In all cases the cell extracts were passed through a 1.2 X 7.0 cm column of Sephadex G-15 to remove low molecular weights compounds. The extracts were tested immediately for enzyme activity.

Enzyme assay. Threonine dehydratase was assayed by a modification of the lactic dehydrogenase coupled assay of Dunne et al (21). The incubation mixture (1.0 ml final volume) in a cuvette with 1-cm light path contained: 5 μmoles of dithiothreitol, 5 μmoles of ADP (pH 8.0), 0.15 μmoles of NADH, 15 μg of rabbit muscle lactic dehydrogenase (Type II), 75 μmoles of potassium phosphate buffer (pH 8.0), 20 μmoles of L-threonine (pH 8.0) and cell extract (∿0.2 mg protein). The assay mixture was preincubated at 37°C for 5 min. The reaction was initiated by the addition of substrate and the rate of oxidation of NADH was followed at 340 nm with a Gilford 2000 recording spectrophotometer with the cuvette chamber maintained at 37°C by means of a Haake constant temperature regulator. One enzyme unit deaminates 1 μmoles of threonine per min.

Threonine aldolase activity was assayed spectrophotometrically by a modification of the method described by Dainty (19). The reaction mixture (3.0 ml total volume) contained 100 µmoles potassium phosphate buffer (pH 7.0, 7.5 or 8.0) or Tris.HCl buffer (pH 8.5 or 9.0); 20 µmoles of L-threonine; 0.3 µmoles of NADH; 10 units of alcohol dehydrogenase; and cell extracts (0.2-1.0 mg protein). Following a 5 min preincubation of the assay mixture at 37°C, the reaction was initiated by addition of substrate and the rate of oxidation of NADH at 37°C was followed at 340 nm as described above.

Threonine dehydrogenase activity was measured colorimetrically by a modification of the method described by Komatsubara et al (37). The reaction mixture (1.0 ml total volume) contained 100 µmoles of potassium phosphate (pH 7.0, 7.5 or 8.0) or Tris.HCl buffer (OH 8.5 or 9.0); 0.5 µmoles NAD^+ or $NADP^+$; 20 µmoles of L-threonine; 100, 200 or 300 µmoles KCl and cell extract (0.2-1.0 mg protein). The reaction was initiated by addition of threonine and all samples were incubated at 37°C. After 30 min, the reaction was stopped by the addition of 1.0 ml of 0.3 M trichloroacetic acid (TCA) and deproteinized by centrifugation. Amino acetone in the supernatant was assayed by the method of Urata and Granick (67).

The spectrophotometric assay for threonine dehydrogenase was performed with the same reaction mixture as described above. After a 5 min preincubation at 37°C, the reaction was initiated by the addition of substrate, and the NADH/NADPH formed was measured

by the increase in absorbance at 340 nm with a Gilford 2000 recording spectrophotometer described above.

Fractionation of isotopically labeled cells. Cultures used for measurement of $^{14}CO_2$ uptake into cell protein were grown in Hungate tubes to prevent equilibration of label with atmospheric CO_2. All additions to or withdrawals from the tubes were made through a rubber septum using a sterile disposable syringe. All other labeling experiments were performed in 16 X 125 mm screw capped tubes. Six identical cell cultures (without label) were grown along with each culture that contained labeled substrates. Of the former, three were used to monitor growth, and three for measurement of cell protein.

Incorporation of label into protein was determined by quantitation of radioactivity in individual amino acid fractions isolated following protein hydrolysis. The latter was performed by a modification of the method of Roberts et al (54). Cultures were harvested by centrifugation at 18,000 X g for 15 min. The cell pellets were then extracted twice with the same volume (2.5 ml) of 5% TCA. The first extraction was performed at 0°C for 15 min and the second at 90°C for 30 min. The pellets were washed and resuspended in 1.5 ml of 6 N HCl and transferred to ampoules. The ampoules were flushed with argon, sealed and incubated at 110°C for 24 h. The hydrolysates were dried under a stream of nitrogen at 80°C, dissolved in distilled water and adsorbed onto a cation exchange resin (AG 50W-X4, hydrogen form; Bio-Rad) packed in a

disposable pipette (1.6 ml resin bed). The resin was washed with 10 ml water, and the amino acids eluted with 1 M ammonium hydroxide. The eluates were dried as described above, dissolved in 200-500 µl water and stored at -10°C for further analysis.

Fractionation of amino acids for protein hydrolysates. Three different methods were used to separate the amino acids in the radioactive hydrolysates, i.e. (a) thin layer chromatography (TLC); (b) paper electrophoresis; and (c) amino acid analysis using an automatic amino acid analyzer.

Amino acids were resolved by ascending chromatography on Whatman LK-5-D linear K preadsorbent thin layer plates (Whatman, Inc., Clifton, N.J.). The solvent system used was methyl ethyl ketone:pyridine:water:acetic acid (70:15:15:2, v/v). Although it did not separate leucine from isoleucine, this solvent system did resolve these two amino acids from all others in the protein hydrolysates. Unlabeled amino acid standards were spotted along with samples on each plate. To correct for losses in recovery of label, a non-radioactive protein hydrolysate to which known amounts of ^{14}C-isoleucine were added was applied to each plate along with the sample being analyzed. The plates were developed 3-4 times at room temperature for 1.5 h each with adequate drying in between developments. A guide strip (containing amino acid standards) on the chromatograms was sprayed with a solution of 0.1% ninhydrin in acetone and heated for 15-30 min in a drying oven. Areas of the unstained portion of the chromatograms corresponding to the leucine

and isoleucine spots on the guide strip were scraped with a spatula and the loosened adsorbent was collected in scintillation vials. The sample was extracted with 1 ml of distilled water, then diluted with 10 ml of aqueous scintillation fluid (Formula 963) and the radioactivity was counted.

Electrophoresis of amino acids in protein hydrolysates was performed on Whatman no. 1 paper. Buffer system A (0.25 M sodium acetate, pH 4.3) was used to separate acidic from neutral and basic amino acids; buffer system B (0.05 M potassium phosphate, pH 7.5) was used to resolve lysine from all other amino acids except arginine. However, since C. sporogenes cannot synthesize arginine, unless radioactive arginine is added to the culture the arginine in the protein hydrolysate is not labeled.

Protein hydrolysates were spotted on paper, along with amino acid standards, and electrophoresed at 35 volts/cm. The paper was then dried and a guide-strip containing the amino acid standards was stained with ninhydrin as described above. Areas of the unstained portion of the electrophoretogram corresponding to standards in the guide strip were cut out, folded, immersed in toluene-based scintillation fulid in glass vials and counted for radioactivity. Recovery of label in samples resolved by electrophoresis was only 63-65% as determined with labeled amino acid standards separated by the same procedure.

Amino acids from protein hydrolysates were quantitated on an analytical scale using a Beckman Model 121 programmable amino acid analyzer (Beckman Instrument, Inc., Palo Alto, CA). Program I,

with 0.2 M sodium citrate buffer, pH 2.99, containing 3% by volume of n-propanol, was used to separate aspartate, threonine, serine, glutamate, glycine, and alanine. Program II with 0.2 M sodium citrate buffer, pH 3.39, containing 3% by volume of methanol was used to separate valine, methionine, isoleucine, and leucine. All separations were performed at 52°C on a 0.9 X 65 cm column of spherical sulphonated styrene copolymer resin (Type AA-15, 8% crosslinkage; Beckman Instruments, Inc.). The flow rates for Program I and II were 28 ml/h and 70 ml/h respectively. Before each run, the column was flushed with 0.2 N NaOH for 8 min (at 70 ml/h), then equilibrated for 35 min (at 70 ml/h) with the appropriate program buffer. After injection of the sample (600 µl), the flow rate was adjusted to that required for each program.

For analysis of radioactive samples, the elution stream from the analyzer was interrupted before reaction with ninhydrin and diverted to a Gilson Microfractionator Type FC-80K fraction collector (Gilson Medical Electronics, Inc., Middleton, WI). The point at which the eluant stream was diverted was determined by reference to a calibration mixture elution profile which was used as a guide. Fractions were collected in the time mode at one-minute intervals. Every second or third fraction was quantitatively transferred to a glass vial, mixed with aqueous scintillation fluid (Formula 963) and the radioactivity was counted. In all cases, plots of radioactivity vs elution time gave discrete peaks which coincided to those obtained with calibration standards (Fig. 3). Fractions of individual peaks were pooled and the total label was

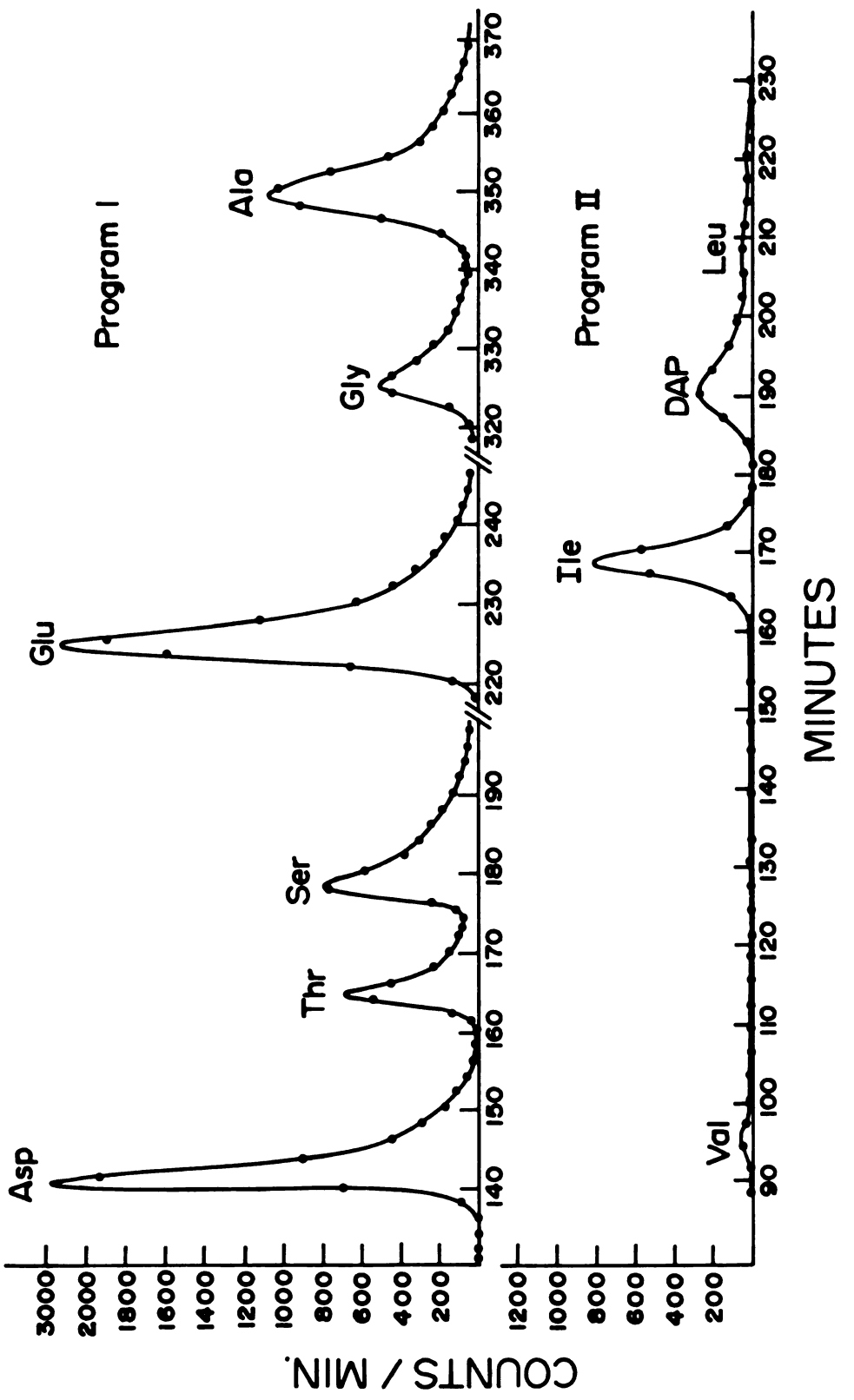


FIG. 3. Distribution of ^{14}C in amino acids of hydrolyzed protein from C. sporogenes grown in medium B-10 containing L-[U-^{14}C]serine.

quantitated by counting duplicate aliquots. The average value was used to calculate the total radioactivity in each amino acid pool. All values were corrected for quenching caused by the elution buffer; the counting efficiency was 80.0-83.5% as measured against an internal standard. The recovery of label by this method based on analysis of a mixture of radioactive amino acid standards was 98-100%.

Determination of intracellular amino acid pools. Cells grown in 50 ml medium B-10 were harvested at a culture density of 0.3 O.D. units by vacuum filtration through Metricel membrane filters (25 mm diameter and 0.2 μm pore size; Gelman Instrument Co., Ann Arbor, MI), and each filter washed with 5% TCA in the cold as described above. The solution was extracted five times with equal volumes of ether to remove TCA, and adsorbed on a cation exchange resin packed in a disposable pipette. After washing, the amino acids were eluted with 1 M ammonium hydroxide as described above. The eluate was dried as described previously and the crystalline amino acids were dissolved in a small volume of water. Amino acid concentrations were determined with an amino acid analyzer.

Decarboxylation of radioactive amino acids. Monocarboxylic amino acids and glutamic acid were decarboxylated with chloramine-T according to the procedure of Kemble and McPherson (33). The radioactive sample was placed in a Warburg flask with 0.5 ml of 12% chloramine-T solution in the side arm and 0.4 ml of hydroxide of hyamine in the center well to trap the $^{14}CO_2$. The reaction was initiated by tipping the chloramine-T solution into the sample. The

reaction was allowed to proceed for 2 h at 30°C. At the end of incubation, the contents of the center well were transferred to a glass vial; the well was rinsed with 1 ml of methanol and the washings added to the same vial. Ten ml of toluene-based scintillation fluid was added to the vial and the radioactivity was counted. A vial containing 0.4 ml of hydroxide of hyamine and 1 ml of methanol in 10 ml of the same scintillation fluid served as a reagent blank.

Aspartic acid was decarboxylated with ninhydrin by a modification of procedure of Greenberg and Rothstein (26). The $^{14}CO_2$ produced in the reaction vessel was driven by a stream of nitrogen into two receiving vessels containing barium hydroxide solution. The precipitated barium carbonate was collected and transferred to a Warburg flask containing 0.5 ml of 2 N HCl in the side arm and 0.4 ml of hydroxide of hyamine in the center well. The flask was sealed, and the acid tipped into the sample. The flask was held at room temperature for 1-2 h to allow for complete absorption of the liberated $^{14}CO_2$ by the alkali. The rest of the procedure was as described above.

In both procedures, the buffer was that in which the individual radioactive amino acids were eluted from the amino acid analyzer since the molarities and the pH of the buffers were suitable for this purpose.

Incorporation of the degradation product(s) of L-[U-^{14}C] isoleucine into isoleucine. Ten μCi of L-[U-^{14}C]isoleucine was added to a 10 ml culture of C. sporogenes as soon as growth was observed, and the culture incubated until it reached a turbidity of

0.3 O.D. units. The cells were harvested by centrifugation and discarded. The supernatant was acidified with HCl to pH $\sim$ 3.8-4.0, and shaken vigorously to remove all the CO_2. The solution was passed through 6 columns, one after the other, of cation exchange resin (AG 50W-X4, hydrogen form, 9.6 ml total volume) packed in disposable pipettes to remove all amino acids and other amines. A medium was prepared with the resulting solution by adding a mixture of amino acids, salts, vitamins, and sodium thioglycollate in the amounts and proportions corresponding to medium B-10 (Table 1). After the pH was adjusted to 7.4, the medium was flushed with argon for 10 min, stoppered, placed in the anaerobic chamber and sterilized by filtration. This medium was used for culturing cells which were harvested at a culture turbidity of 0.6 O.D. units. Cell protein was isolated and hydrolyzed as described above and the hydrolysate analyzed by means of an amino acid analyzer.

Measurement of volatile fatty acids. Volatile fatty acids were extracted from acidified supernatant solutions (from either a growing culture or from cells incubated in a solution containing 100 mM L-isoleucine and L-proline in 0.2 M potassium phosphate buffer; pH 7.5), and were qualitatively identified and quantitated by gas chromatography according to the procedure as described in the Anaerobe Laboratory Manual (29). A varian model 1420 gas chromatograph, equipped with a thermal conductivity detector, was used. The column was stainless steel (0.125 inch by 6 feet [ca. 0.32 by 182.9 cm]) and contained 15% SP 1220-1% H_3PO_4 on Chromosorb W AW (100/120 mesh; Supelco, Inc., Bellefonte, PA). Helium was the

carrier gas (25 ml/min), and temperatures were: column, 135°C; injector and detector, 165°C each. Volatile fatty acids were applied to the column as ether solutions.

Preparation of [U-^{14}C]2-methylbutyric acid. [U-^{14}C] 2-Methylbutyric acid was prepared by fermenting L-[U-^{14}C]isoleucine in the presence of L-proline using C. sporogenes cells. The cells were grown in 100 ml medium-A, harvested in the exponential phase of growth and washed twice with 0.1 M potassium phosphate buffer, pH 7.4. The cells were then suspended (1.35 g of packed cells/ml) in a 2.0 ml solution containing 10 mM L-isoleucine, 20 μCi L-[U-^{14}C]isoleucine (sp.act. 360 μCi/ mole), and 20 mM L-proline. The whole mixture was allowed to incubate in the anaerobic chamber at 37°C for 4 h. Following centrifugation, the clear supernatant fluid was passed through a column of cation exchange resin (AG 50W-X4, hydrogen form) packed in a disposable pipette (1.6 ml resin bed) to remove the remaining amino acids. The fluid that came through (containing the volatile fatty acids) was collected and the total radioactivity was determined. Although the latter fraction may have contained traces of other volatile fatty acids, practically all of the radioactivity was expected to reside in 2-methylbutyric acid since this is the major volatile fatty acid obtained from the fermentation system employed (see Table 21). The yield of the latter was 28% based on radioactivity counting corresponding to a 2.8 mM concentration in 2.25 ml volume with a specific activity of 0.967 μCi/μmole.

Analytical methods. Growth of C. sporogenes was estimated by measuring optical density (O.D.) at 600 nm using a Mini Spec 20 spectrophotometer (Bausch and Lomb Optical Co., Rochester, N. Y.). The protein content of cell extracts and cell pellets obtained after cold TCA extraction were determined according to the methods of Kalb and Bernlohr (32) and Lowry et al (40), respectively. All radioactive counting was performed on a Packard Tri-Carb liquid scintillation spectrometer, model 3320 (Packard Instrument Co., Inc., Downers Grove, IL).

Chemicals. Amino acids of the highest purity available were purchased from several different companies; those that were used in medium B-10 were all purchased from Sigma Chemical Co., St. Louis, MO. Dithiothreitol was obtained from Aldrich Chemical Co., Milwaukee, WI; ADP was from General Biochemicals; NAD^+, $NADP^+$, NADH, rabbit muscle lactic dehydrogenase enzyme (Type II), alcohol dehydrogenase enzyme, and chloramine-T were from Sigma Chemical Co., St. Louis, MO. Volatile fatty acids were purchased from Eastman Kodak Co., Rochester, N.Y. and Fisher Scientific Co., Fairlawn, N.J.

The following radioactive compounds were purchased from New England Nuclear, Boston, MA: [U-^{14}C]acetate, L-[U-^{14}C]methionine, L-[U-^{14}C]arginine, L-[U-^{14}C]orthinine, L-[U-^{14}C]histidine, L-[U-^{14}C]valine, L-[U-^{14}C]aspartate, L-[U-^{14}C]threonine, L-[U-^{14}C]glutamate, L-[U-^{14}C]isoleucine, [3-^{14}C]pyruvate, ^{14}C-$NaHCO_3$, and ^{14}C-toluene. L-[U-^{14}C]phenylalanine, and L-[U-^{14}C]tyrosine were purchased from International Chemical and Nuclear Corp., Irvine, CA. [U-^{14}C]glucose, L-[U-^{14}C]proline, and L-[U-^{14}C]leucine were purchased from Amersham Corp., Arlington Heights, IL.

CA. [U-^{14}C]glucose, L-[U-^{14}C]proline, and L-[U-^{14}C]leucine were purchased from Amersham Corp., Arlington Heights, IL.

Hydroxide of hyamine was purchased from Packard Instrument Co., Inc., Downers Grove, IL. The aqueous scintillation fluid (Formula 963) was obtained from New England Nuclear. Toluene-based scintillation fluid was prepared by mixing 6.00 g of 2,5-diphenyloxasole and 0.01 g 1,4-bis-2-(5-phenyloxazoyl)-benzene in one liter of toluene; both chemicals were obtained from Research Product International Corp., Elk Grove Village, IL.

RESULTS

Threonine Metabolism

Threonine dehydratase activity. If both biodegradative and biosynthetic threonine dehydratases are present in C. sporogenes, it is reasonable to expect differences to occur in the specific activity of the enzyme from cells grown in media of varying composition. Table 2 shows that none of the growth media tested produced significant variation in the threonine dehydratase activity as compared to that in cells grown in complete synthetic medium (control). Omission of isoleucine failed to increase the activity as one might expect if a derepression of biosynthetic enzyme had occurred. Furthermore, the presence of excess isoleucine along with high concentrations of valine and leucine (medium B-1 contains 17 mM L-valine and 11.5 mM L-leucine) did not reduce the enzyme activity as would be expected for a system in which multivalent repression of enzyme synthesis was operational. Moreover, the enzyme is apparently not subject to catabolite repression, since no variation in activity was caused by glucose. No difference in specific activity was observed in cells grown in the presence of excess threonine or in the absence of threonine and/or serine, in contrast to the result expected if the catabolic enzyme were inducible by either or both amino acids (medium B-1 contains 8.4 mM

Table 2. Effect of growth medium on threonine dehydratase activity[a]

Growth Medium		Specific Activity (units/mg protein)
Standard trypticase (medium A)		0.725
Synthetic:	complete (medium B-1)	0.951
	no isoleucine	0.721
	+ 30 mM L-isoleucine	0.879
	+ 30 mM glucose	0.856
	no threonine	1.146
	no threonine and no serine	0.962
	+ 22 mM L-threonine	1.009
	+ 42 mM L-threonine	0.988

[a]Assay procedure is as described under Materials and Methods.

Table 3. The effect of AMP and ADP on threonine dehydratase activity at high and low substrate concentrations.[a]

Nucleotide	Specific Activity (units/mg protein)	
	20 mM L-thr	4 mM L-thr
None	1.050	0.431
+ 5 mM AMP	1.050	0.431
+ 5 mM ADP	1.050	0.812

[a]Assay mixture was as described in Materials and Methods. The cells were grown in synthetic medium (B-1) and the crude extract was desalted as described.

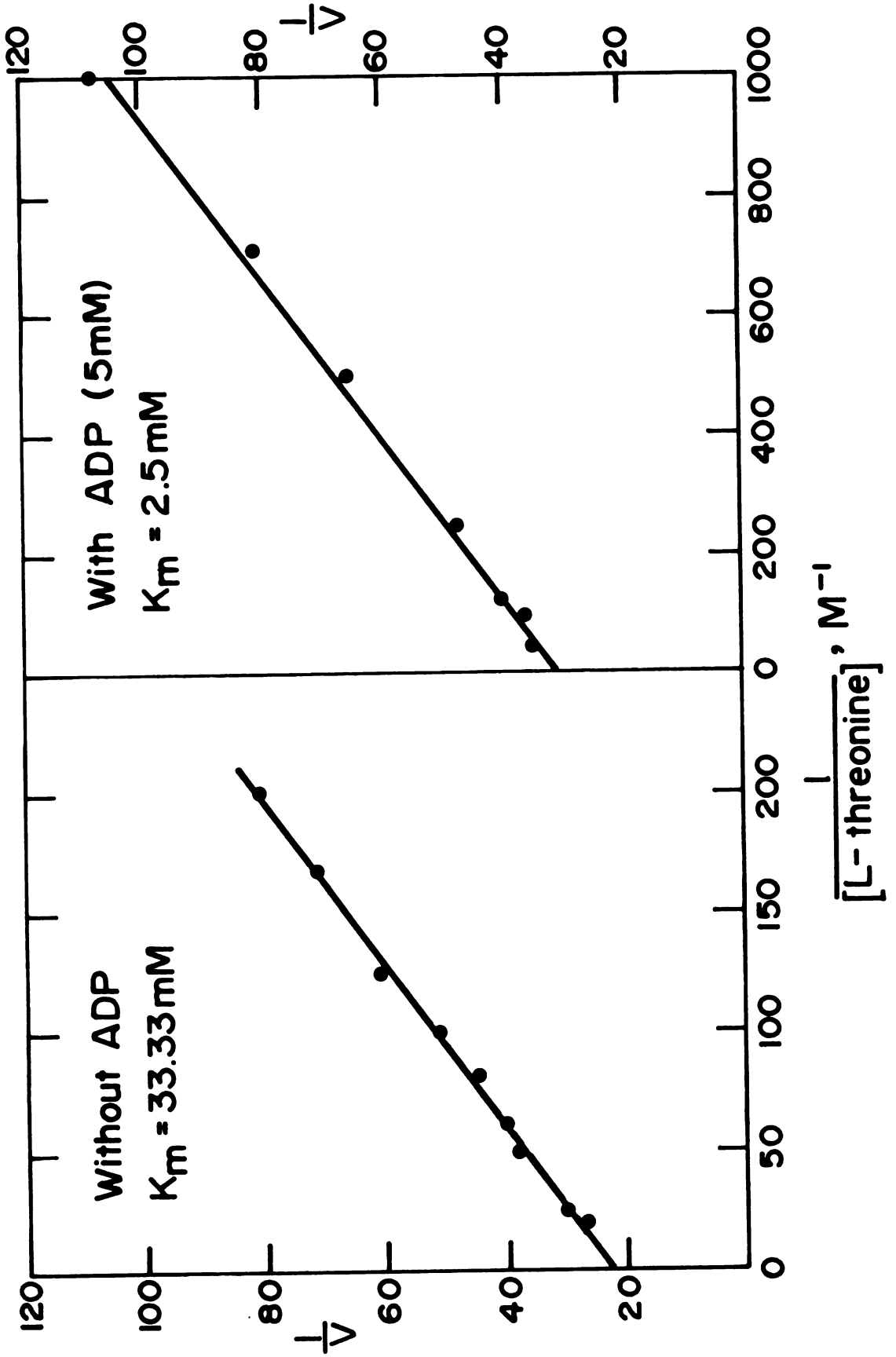
L-threonine and 9.5 mM L-serine). The role of leucine and/or valine in the induction of the enzyme could not be assessed since both amino acids were essential for growth in the medium used.

Table 3 shows that threonine dehydratase is activated by adenosine diphosphate (ADP) but not by adenosine monophosphate (AMP); however, the activation by ADP was only observed at low substrate concentration. From the Lineweaver-Burk plot shown in Fig. 4, the K_m values for the enzyme in the presence and absence of ADP were calculated to be 2.50 mM and 33.33 mM, respectively; under these conditions the V_{max} values were 0.033 and 0.045, respectively. Thus, at least one effect of ADP on the enzyme is a 13-fold reduction in its K_m for threonine.

Since threonine dehydratase has been studied extensively in other organisms including Clostridium tetanomorphum (65), the investigation of this enzyme in C. sporogenes was not pursued any further. However, the failure to detect an isoleucine-sensitive enzyme (addition of L-isoleucine up to 5 mM to the assay mixture at pH 7.0, 8.0, and 9.5 did not affect the specific activity) prompted the investigation of the isoleucine biosynthetic pathway in this organism.

Threonine aldolase and dehydrogenase. No threonine aldolase or threonine dehydrogenase activities were observed in C. sporogenes cell extract (cells were grown in medium A) under the assay conditions employed. Addition of 5 mM dithiothreitol to the assay mixture did not result in the detection of threonine aldolase

FIG. 4. Velocities of threonine dehydratase as a function of L-threonine concentration in the presence and absence of ADP.


With ADP (5mM)
K_m = 2.5mM
Without ADP
K_m = 33.33mM
$\frac{1}{V}$
$\frac{1}{[L\text{-threonine}]}$, M^{-1}
120
100
80
60
40
20
0
50
100
150
200
400
600
800
1000

activity. Cell extracts obtained from C. sporogenes grown in the synthetic media (medium B-1 with or without the addition of 28.5 mM L-leucine) did not show any threonine dehydrogenase activity. In E. coli K-12, threonine dehydrogenase is induced by L-leucine and not by its substrate, L-threonine (47).

Amino Acid Composition of Cells and Media

Composition of cell protein. The amino acid compositions of proteins from cells grown in either B-8 or B-10 media were essentially the same (Table 4). The concentration of aspartate constitutes that of both aspartate and asparagine; similarly, the concentration of glutamate constitutes that of glutamate and glutamine. It is probable that both glutamate and alanine from peptidoglycan in the cell wall are included in these values. Calculation of specific activities of radioactivity incorporated into the individual amino acids are based on the data obtained with cells grown in medium B-10.

Free amino acids in cell. During the exponential phase, most amino acids in the intracellular pool were present in relatively low concentrations except for proline, glutamate, and valine (Table 5). The unusually high concentration of the latter three amino acids might be due to the necessity of the cells to balance the osmotic pressure exerted by the growth conditions in medium B-10 (45).

Amino acids in growth medium. The concentrations of some amino acids in the growth medium at various stages of growth in

Table 4. Amino acid composition of protein from cells grown in various synthetic media[a]

Amino Acid	Concentration (nmoles/mg protein) Medium B-8	Medium B-10
Aspartate	771	752
Threonine	365	352
Serine	314	381
Glutamate	930	853
Proline	139	244
Glycine	533	522
Alanine	942	939
Valine	629	615
Methionine	346	unresolved
Isoleucine	593	518
Leucine	628	568
Tyrosine	182	291
Phenylalanine	255	337
Lysine	625	586
Histidine	140	157
Arginine	265	218

[a]Cells grown in medium B-8 and B-10 were harvested at the culture density of 0.7 and 0.6 O.D. units respectively. Proteins were isolated and hydrolyzed as described in Materials and Methods.

Table 5. Intracellular concentrations of amino acids in C. sporogenes during exponential growth[a]

Amino Acid	Concentration (nmoles/mg protein)
Proline	67
Glutamate[b]	52
Valine	38
Serine	13
Alanine	12
Tyrosine	9
Leucine	6
Aspartate	6
Glycine	5
Lysine	3
Threonine[b]	1
Methionine	1
Histidine[b]	1
Isoleucine[b]	1
Arginine	undetectable
Phenylalanine	unresolved[c]

[a]Cells were harvested when the turbidity of the culture was 0.3 O.D. units. Amino acids were resolved and quantitated by means of an amino acid analyzer.

[b]Estimated by comparing the area under their peaks (by weighing method) to those of known concentrations in the same chromatogram.

[c]Due to the presence of an unidentified ninhydrin-positive substance that overlapped with phenylalanine in the chromatogram.

medium B-10 from three separate experiments are presented in Table 6. Although there is discrepancy on the rate of serine degradation among the different determinations, most of serine was consumed before the culture reached a density of 0.6 O.D. units. Both serine and arginine seem to be limiting at higher O.D. values. A significant amount but not all of leucine was consumed during the growth period. Of particular interest is the accumulation of alanine in the growth medium accompanying the rapid rate of serine utilization during the exponential phase (notice that alanine was initially absent).

Isoleucine Biosynthesis

Compounds contributing no significant carbon to isoleucine.

The first attempt to determine the precursor of isoleucine was made by growing C. sporogenes in the presence of L-[U-^{14}C]threonine, and then measuring the amount of radioactivity in the isoleucine fraction isolated from whole-cell protein hydrolysates. No ^{14}C was found in isoleucine which indicated that this amino acid is not synthesized via the usual pathway. Therefore, other labeled components were added to the medium one at a time to find the precursor of isoleucine. Table 7 shows that there was substantial incorporation of radioactivity into cell protein from all ^{14}C-compounds tested, but negligible incorporation of label into isoleucine and/or leucine. The low level of radioactivity recovered in the isoleucine + leucine fraction from a few substrates may have come from $^{14}CO_2$ released on degradation of the labeled amino acids.

Table 6. Amino acid concentrations in the supernatant liquids of cultures grown in medium B-10 at various stages of growth

	Initial mM	Exp. 1 at O.D.$_{600}$ of:		Exp. 2 at O.D.$_{600}$ of:			Exp. 3 at O.D.$_{600}$ of:				
		0.300	0.600	0.187	0.347	0.620	0.200	0.284	0.432	0.585	0.77
		mM concentrations		mM concentrations			mM concentrations				
Serine	25.0	0.83	0.61	>15.00	>10.00	0.64	11.47	2.16	0.55	0.38	0.23
Valine	17.5	15.62	14.22	15.26	14.86	9.20	13.87	9.39	9.50	8.17	5.76
Methionine	10.0	8.36	7.45	8.64	8.30	5.70	8.93	6.05	5.96	4.33	3.76
Leucine	11.5	8.04	7.86	10.18	9.69	5.11	9.47	6.41	6.40	4.86	2.69
Tyrosine	2.0	1.90	1.24	1.66	1.39	0.85	1.99	1.31	1.07	0.49	0.37
Phenylalanine	10.0	NR[a]	NR[a]	8.71	6.42	5.47	9.16	6.25	6.09	5.16	NR[a]
Arginine	20.0	6.28	3.28	>14.00	9.99	0.40	13.46	6.89	3.73	0.64	0.00
Alanine	0.0	6.31	4.42	1.81	2.62	2.23	1.54	3.18	4.12	2.77	1.67

[a]NR = not resolved

Table 7. Recovery of radioactivity from protein hydrolysates of C. sporogenes grown in the presence of various labeled substrates[a]

Growth Medium	L-[U-^{14}C] Substrate	mM Substrate	Specific Activity (μCi/μmole)	Radioactivity dpm/mg Protein	Radioactivity dpm/10μl of hydrolysate Total	Radioactivity dpm/10μl of hydrolysate ile+leu[c]
B-2	Threonine	15.0	0.053	70,340	7,034	49
B-3	Valine	15.0	0.053	36,906	3,543	78
B-4	Leucine	15.0	0.053	48,219	4,629	107[d]
B-5	Arginine	20.0	0.050	58,375	3,736	213
B-7	Ornithine	15.0	0.033	7,656	245	33
B-6	Proline	15.0	0.033	11,719	375	15
B-9	Glucose	20.0	0.050	310,094	16,435	<100
B-8	Histidine	1.3	0.100	45,727	1,573	<100
B-10	Acetate[b]	--	--	115,993	5,413	60
B-10	Methionine	10.0	0.100	66,848	4,100	<100
B-10	Phenylalanine	10.0	0.100	94,377	5,002	<100
B-10	Tyrosine	2.0	0.100	97,174	5,960	<100

[a]In each experiment, isotope was present in the culture throughout the entire growth period.

[b]A total of 5 μCi was added.

[c]Except for culture labeled with L-[U-^{14}C]leucine (for which isoleucine was separated from leucine by buffered paper chromatography), it was not necessary to separate isoleucine from leucine. Isoleucine+leucine was separated from other amino acids by thin layer chromatography; Materials and Methods.

[d]dpm/10 μl in isoleucine only.

Some of these labeling experiments were conducted in media containing a number of amino acids not required for growth of C. sporogenes (media B-2 through B-7, see Table 1). This could reduce the potential for incorporation due to dilution of some common intermediate. In addition, there could be a reduction in the ^{14}C found in isoleucine from rapidly metabolized substrates such as threonine, arginine, valine and leucine due to protein turnover in stationary phase cells. This is particularly likely of a spore forming bacterium such as C. sporogenes. Therefore, further ^{14}C incorporation experiments were performed with some of the amino acids in the minimal medium (B-10) and the cells incubated with labeled substrate for only one generation.

Table 8 shows that under these conditions, uptake of label into protein was very high, but there was negligible incorporation into the isoleucine/leucine fraction except when leucine was the labeled substrate. Further analysis of the hydrolysate from cells labeled with L-[U-^{14}C]leucine showed that at least 98% of the label in the isoleucine + leucine fraction was in leucine.

Both threonine and glutamate provide α-ketobutyrate for isoleucine biosynthesis in some bacteria. Therefore, the protein hydrolysates from cells labeled with these amino acids were examined in more detail. Practically all of the label from L-[U-^{14}C]threonine was in the threonine fraction (Table 9). However, a small but significant amount was present in glycine. All of the label from L-[U-^{14}C]glutamate was found in the glutamic acid present in the protein hydrolysate (Table 10). These data support the conclusion

Table 8. Incorporation of radioactivity into isoleucine of cell protein by C. sporogenes labeled for one generation with various ^{14}C-amino acids[a]

L-[U-^{14}C] Substrate[b]	mM Substrate	Specific Activity (μCi/μmole)	Radioactivity Incorporated dpm/mg Protein	dpm/10μl hydrolysate Total ile+leu	[c]
Arginine	25.0	0.1	76,195	3,200	<100
Valine	17.0	0.1	59.079	2,580	<100
Leucine	11.5	0.1	95,524	4,000	3,800
Aspartate	--	--	887,550	29,370	<100
Threonine	--	--	3,406,100	114,010	<100
Glutamate	--	--	402,650	13,480	<100

[a]In each experiment the label was added to 10 ml culture grown in medium B-10 at a culture density of 0.3 O.D. units.

[b]A total of 25 μCi of each of the following were added: L-[U-^{14}C] aspartate (spec. act. 221 μCi/μmole), L-[U-^{14}C]threonine (spec. act. 210 μCi/μmole) and L-[U-^{14}C]glutamate (spec. act. 266 μCi/μmole).

[c]Isoleucine+leucine was resolved by thin layer chromatography (Materials and Methods).

Table 9. Distribution of radioactivity in amino acids from protein hydrolysate of C. sporogenes cells labeled for one generation with L-[U-^{14}C]threonine[a]

Amino Acid[b]	Total nmoles	Radioactivity (dpm)	Specific Activity (dpm/nmole)
Glycine	17	4,818	283
Aspartate	25	346	14
Threonine	12	121,505	10,125
Lysine	23	211	9
Glutamate	28	314	11
Isoleucine	17	40	8
Leucine	19	128	7
Total recovered		127,362 (>100%)[c]	

[a]25 μCi L-[U-^{14}C]threonine (specific activity 210 μCi/μmole) was added to 10 ml culture in medium B-10 at cell density of 0.3 O.D. units.

[b]Lysine was separated on paper electrophoresis; all other amino acids were separated and quantitated with an amino acid analyzer (Materials and Methods).

[c]Total radioactivity in the amount of hydrolysate analyzed was 123,537 dpm.

Table 10. Distribution of radioactivity in amino acids from protein hydrolysate of C. sporogenes cells labeled for one generation with L-[U-^{14}C]glutamate[a]

Amino Acid[b]	Total nmoles	Radioactivity (dpm)	Specific Activity (dpm/nmole)
Glutamate	34	16,335	480
Aspartate	30	8	<1
Lysine	23	51	2
Neutrals	--	60	---

[a]25 μCi L-[U-^{14}C]glutamate (spec. act. 266 μCi/μmole) was added to 10 ml culture in medium B-10 at cell density of 0.3 O.D. units.

[b]The amino acids were separated by paper electrophoresis (Materials and Methods).

that isoleucine is not synthesized by C. sporogenes via a classical pathway. The specific activity of the ^{14}C-threonine incorporated into protein was about 20 times that of ^{14}C-glutamate even though the specific activities of both amino acids added to the medium (see Tables 9 and 10) were essentially the same. This may have resulted from a large dilution of the added labeled glutamic acid by pool levels (see Table 5) or by continuous rapid synthesis of glutamic acid during the one generation of growth.

Incorporation of ^{14}C from L-[U-^{14}C]serine into isoleucine and other amino acids. All the previous experiments designed to incorporate label into isoleucine from various radioactive substrates were without success. Since serine was the principal (if not the sole) source of pyruvate in the minimal growth medium (B-10) employed, one would expect the incorporation of label from L-[U-^{14}C]serine into most amino acids which the organism can synthesize. This proved to be true (Table 11). The relative specific activities of the individual amino acids per mole or per carbon atom are within reason. Serine had the highest specific activity as might be expected since it would not be diluted by CO_2 exchange or uptake reactions or by pyruvate from other sources. Thus, the lower specific activity of alanine compared to serine in the cell protein could result from dilution by alanine in the pool formed prior to addition of labeled serine to the culture (see Table 5). The specific activity of glycine is less than two thirds that of serine, suggesting that glycine is not synthesized exclusively from serine. The specific activity

Table 11. Distribution of radioactivity in amino acids from protein hydrolysates of C. sporogenes cells labeled for one generation with L-[U-^{14}C]serine[a]

Amino Acid[b]	Total nmoles	Radioactivity (dpm)	Specific Activity dpm/nmole	Specific Activity dpm/C atom
Alanine	82	11,631	142	47
Serine	33	7,827	237	79
Glycine	46	4,753	103	51
Aspartate	66	12,127	184	46
Threonine	31	4,053	131	33
Lysine	51	11,775	231	39
Glutamate	75	18,359	245	49
Isoleucine	45	5,411	120	20
Valine	54	442	8	--
Total recovered 76,337 (87%)[c]				

[a]25 μCi L-[U-^{14}C]serine (spec. act. 162 μCi/μmole) was added to a 10 ml culture in medium B-10 at a culture density of 0.3 O.D. units.

[b]Lysine was separated on paper electrophoresis; all other amino acids were resolved by means of an amino acid analyzer (Materials and Methods).

[c]The total radioactivity in the sample of hydrolysate analyzed was 88,051 dpm.

Table 12. Decarboxylation of amino acids from protein hydrolysate of C. sporogenes cells grown in the presence of L-[U-^{14}C]serine

Amino Acid	Radioactivity (dpm) Initial	Radioactivity (dpm) $^{14}CO_2$	Percent Label Lost
Alanine	1,468	508	34.6
Serine	1,111	281	25.3
Glycine	728	415	57.0
Aspartate	2,160	1,073	49.7
Threonine	822	204	24.8
Lysine	2,599	333	12.8
Glutamate	2,310	418	18.1
Isoleucine	250	66	26.4

of aspartate is consistent with its synthesis from oxalacetate. Furthermore, the approximately equal distribution of specific activity among aspartate, threonine, and lysine suggests that the latter two amino acids are synthesized from aspartate via the usual pathway as described in E. coli (66). The specific activity of glutamate suggests its synthesis from α-ketoglutarate rather than from proline.

Isoleucine contained significant levels of label derived from the ^{14}C-serine. Its specific activity was only slightly less than that of alanine and was approximately equal to that of threonine. No significant amount of radioactivity was found in valine and the same was true for leucine although the latter amino acid was not completely resolved. Small amounts of label might be incorporated into valine and leucine by CO_2 exchange reactions. These data provide good evidence that there is some synthesis of isoleucine but no significant synthesis of the other two branched chain amino acids under the conditions employed.

Decarboxylations of the individual radioactive amino acids from cell protein was performed to help determine which carbon atom(s) had been labeled. With the exception of isoleucine, the results (Table 12) are not different from what would be expected if all of the amino acids examined were uniformly labeled. This would be predicted if all were formed from labeled pyruvate, acetate, and CO_2 from ^{14}C-serine. The amount of radioactivity lost during isoleucine decarboxylation (26.4%) suggests that there are 2 or 3 carbons which are not labeled.

Incorporation of radioactive carbon from $^{14}CO_2$ into isoleucine and other amino acids. To determine the incorporation pattern of CO_2 into cellular protein, two cultures of C. sporogenes were labeled for one and two generations respectively, in medium B-10 containing ^{14}C-$NaHCO_3$. In both cases, incorporation of total label per mg protein and the distribution of label among the amino acids was essentially the same (Table 13). In both experiments, the starting $^{14}CO_2$ specific activity was subject to dilution by the pre-existing non-labeled CO_2 resulting from catabolism prior to adding the label, and would be further diluted during growth. However, the observation that the amino acid labeling pattern was unchanged by doubling the incubation period to two generations indicates that the amount of dilution during growth had little effect.

The overall distribution of radioactivity among the individual amino acids in the protein hydrolysate obtained from the culture labeled for one generation was determined with the aid of an amino acid analyzer (Table 14). The nearly equal specific activities in alanine, serine, and glycine suggest that the label originated from an exchange reaction of pyruvate with $^{14}CO_2$ probably via pyruvate synthase. However, the reaction is probably rather limited in this system, as evidenced by the low incorporation of label into these amino acids. The abundance of radioactivity in aspartate suggests that the latter is synthesized from oxalacetate which obtained its label from the carboxylation of pyruvate. The equal distribution of label in aspartate, threonine, and lysine supports the earlier suggestion that the latter two amino acids

Table 13. Distribution of radioactivity in amino acids from protein hydrolysates of C. sporogenes cells labeled for one and two generations with $^{14}CO_2$[a]

Amino Acid[b]	Radioactivity (dpm/mg protein) Labeled for one generation	Labeled for two generations
Aspartate	162,036	153,969
Glutamate	176,438	141,221
Lysine	104,656	134,529
Neutral	265,751	294,682
Basic	126,514	159,924

[a] 50 μCi ^{14}C-$NaHCO_3$ (spec. act. 6.2 μCi/μmole) was added to 20 ml culture in medium B-10 at the cell density of 0.15 O.D. units. Ten ml of the culture was harvested at culture density of 0.3 O.D. units and the remaining ten ml was harvested at cell density of 0.6 O.D. units.

[b] Five- and 10 μl aliquots of protein hydrolysate were separated by paper electrophoresis and the values listed were calculated based on 65.0% recovery of total radioactivity in the samples. Direct counting of the hydrolysate resulted in total radioactivity of 730,146 and 684,355 dpm/mg protein for cultures labeled for one and two generations, respectively.

Table 14. Distribution of radioactivity in amino acids from protein hydrolysates of C. sporogenes cells labeled for one generation with $^{14}CO_2$[a]

Amino Acid[b]	Total nmoles	Radioactivity (dpm)	Specific Activity (dpm/nmole)
Alanine	93	5,282	57
Serine	38	1,484	39
Glycine	51	1,435	28
Aspartate	74	17,364	235
Threonine	35	7,771	222
Lysine	58	10,916	188
Glutamate	84	17,925	213
Isoleucine	51	8,404	165
Valine	61	755	12
	Total recovered	71,336 (97%)[c]	

[a]50 μCi ^{14}C-$NaHCO_3$ (spec. act. 6.2 μCi/μmole) was added to 20 ml culture in medium B-10 at a culture density of 0.15 O.D. units.

[b]Lysine was separated by paper electrophoresis; all other amino acids were resolved by means of an amino acid analyzer (Materials and Methods).

[c]Total radioactivity in the sample of hydrolysate analyzed was 73,848 dpm.

are synthesized from aspartate via the usual pathway as described in E. coli (66). The nearly equal distribution of label in glutamate and aspartate suggest that αKG, which is the precursor of glutamate, is synthesized largely via the TCA cycle. A very significant amount of label was incorporated into isoleucine. The specific activity of the isoleucine formed is reasonably close to that found in aspartate, lysine and threonine. This indicates that there may be one CO_2 incorporated per isoleucine synthesized by C. sporogenes. The low level of radioactivity in valine is consistent with the earlier suggestion that some label in valine and leucine could originate from a $^{14}CO_2$ exchange reaction.

The results obtained from decarboxylation of each of the amino acids labeled with $^{14}CO_2$ are shown in Table 15. Except for isoleucine, the amount of label lost from each of the amino acids examined was consistent with $^{14}CO_2$ exchange reactions and with recognized biosynthetic pathways. Thus, the exchange of $^{14}CO_2$ with pyruvate could account for all the label being lost from alanine and a small percentage of it from threonine and lysine. Similarly, reversible transamination and CO_2 exchange with α-ketoisovalerate could result in the small amount of ^{14}C in the carboxyl group of valine. The 100% loss of label from aspartate is as expected since both carboxyls are removed from this amino acid by the ninhydrin reaction used. Failure to find $^{14}CO_2$ from the C-1 of glutamate indicates that the synthesis of citrate in C. sporogenes involves a re-citrate synthase as in the case of Clostridium kluyveri and few other bacteria (23, 24). This

Table 15. Decarboxylation of amino acids from protein hydrolysate of C. sporogenes cells grown in the presence of $^{14}CO_2$

Amino Acid	Radioactivity (dpm) Initial	Radioactivity (dpm) $^{14}CO_2$	Percent Label Lost
Alanine	704	712	101
Aspartate	580	601	104
Threonine	1,460	297	20
Lysine	1,773	377	21
Glutamate	1,261	0	0
Isoleucine	481	182	38
Valine	112	120	107
Serine[a]	N.D.	N.D.	---
Glycine[a]	N.D.	N.D.	---

[a]Not determined due to insufficient amount of radioactivity in the sample.

Table 16. Distribution of radioactivity in amino acids from protein hydrolysate of C. sporogenes cells labeled for one generation with [3-^{14}C]pyruvate[a]

Amino Acid[b]	Total nmoles	Radioactivity (dpm)	Spec. Act. (dpm/nmole)	Spec. Act./ Spec. Act. alanine
Alanine	63	17,458	277	1.0
Serine	25	10,968	438	1.6
Glycine	35	1,305	37	0.1
Aspartate	50	16,406	328	1.2
Threonine	23	6,830	297	1.1
Lysine	39	21,603	554	2.0
Glutamate	57	35,452	622	2.3
Isoleucine	35	7,713	220	0.8
Valine	41	0	0	
Total recovered		117,735 (94%)[c]		

[a]25 µCi [3-^{14}C]pyruvate (spec. act. 20 µCi/µmole) was added to a 10 ml culture in medium B-10 at the culture density of 0.3 O.D. units.

[b]Lysine was separated by paper electrophoresis; all other amino acids were resolved by means of an amino acid analyzer (Materials and Methods).

[c]The total radioactivity in the sample of hydrolysate analyzed was 125,803 dpm.

results in the synthesis of glutamate with the C-1 derived from the carboxyl carbon of acetate.

Only 38% of the ^{14}C in isoleucine was released by decarboxylation. As in the case of valine part or all of this fraction could have been incorporated by reversible transamination and CO_2 exchange reactions. However, ^{14}C from $^{14}CO_2$ was fixed in at least one additional carbon of isoleucine.

Incorporation of ^{14}C from [3-^{14}C]pyruvate into isoleucine and other amino acids. When C. sporogenes was labeled for one generation with [3-^{14}C]pyruvate, the observed labeling pattern (Table 16) of all of the amino acids except isoleucine was consistent with the biosynthetic pathways outlined in the two earlier sections. The incorporation of large amounts of radioactivity into serine as compared to alanine probably resulted from the relative concentrations of the two amino acids in the medium at the time the labeled pyruvate was added. While the fresh medium (B-10) contained 25 mM serine and no alanine, the serine was mostly depleted and significant amount of alanine accumulated by mid-log phase of growth (Table 5). The data clearly show that the C-3 of pyruvate is incorporated into isoleucine. The specific activity of theisoleucine is close enough to that of aspartate and threonine to suggest that there is one C-3 of pyruvate incorporated per molecule. As expected, no label in the form of $^{14}CO_2$ was released from any of the amino acids following decarboxylation (Table 17).

Table 17. Decarboxylation of amino acids from protein hydrolysate of cells grown in the presence of [3-^{14}C]pyruvate

Amino Acid	Radioactivity (dpm) Initial	$^{14}CO_2$	Percent Label Lost
Alanine	2,301	4	0
Serine	1,557	17	1
Glycine	169	0	0
Aspartate	4,264	16	0
Threonine	788	18	2
Lysine	4,094	18	0
Glutamate	5,188	0	0
Isoleucine	782	0	0

Pulse-labeling with [3-^{14}C]pyruvate. The results of the foregoing experiments showed that label was incorporated into isoleucine in cell proteins when either $^{14}CO_2$ or ^{14}C-pyruvate (uniformly labeled resulting from degradation of L-[U-^{14}C]serine or labeled at C-3) were added to the growth medium. However, when the specific activities of the labeled isoleucine were compared to those of other ^{14}C-amino acids from the same proteins, it was not possible to account for all the carbons in isoleucine. The data indicate that no more than three carbons were derived from pyruvate and a maximum of two carbons from CO_2. One possible explanation for this was that a high percentage of the isoleucine formed was degraded throughout the one generation of growth. This could result in a large dilution of the label incorporated. If such was the case, then one would predict an increased incorporation of radioactivity into isoleucine by cells grown in the presence of label for an abbreviated period of incubation. However, the results of pulse-labeling of C. sporogenes cells with [3-^{14}C] pyruvate (Table 18) show that this was not the case. A considerable amount of radioactivity was incorporated into the cell protein even during a 5 min labeling period. However, when the hydrolysates were chromatographed, only a very small amount of label was found to be associated with the isoleucine fractions.

This observation raised the possibility that traces of isoleucine might have been supplied to the cells as contaminants present in one or more of the other amino acids used in preparing medium B-10. Indeed, although the cells might be capable of

Table 18. Incorporation of label into protein by C. sporogenes cells pulsed for varying periods with [3-^{14}C]pyruvate in either fresh or spent medium B-10[a]

Labeling Time (min)	Specific Activity (dpm/mg protein)	
	Fresh Medium	Spent Medium[b]
5	638,763	289,476
10	945,006	433,736
30	1,186,226	659,471

[a]Cells from 160 ml culture were harvested by centrifugation when the culture turbidity was at 0.3 O.D. units. The cells were resuspended in 10 ml of either fresh or spent medium B-10, each containing 50 μCi of [3-^{14}C]pyruvate (spec. act. 20 μCi/μmole), and returned to incubation at 37°C. After the indicated time intervals, aliquots were withdrawn from the culture and the cell pellets harvested and processed as described (Materials and Methods).

[b]The spent medium was the clear supernatant obtained when the culture was harvested at the culture turbidity of 0.3 O.D. units.

synthesizing isoleucine, contaminating traces of the latter could conceivably inhibit its formation. However, no significant incorporation of ^{14}C from [3-^{14}C]pyruvate into isoleucine was observed when the pulse experiments were repeated in "spent medium," i.e., in the supernatant medium of a culture which had been grown to mid-log phase of growth (Table 18).

Effect of isoleucine on incorporation of ^{14}C from [3-^{14}C] pyruvate into isoleucine. From the foregoing, it was not possible to conclude whether or not the incorporation of [3-^{14}C]pyruvate into isoleucine was regulated by isoleucine, and whether or not contaminating isoleucine present in the medium was sufficient to inhibit biosynthesis of this amino acid. In an attempt to resolve this question, a culture of C. sporogenes was labeled with [3-^{14}C]pyruvate in medium B-10 containing 5 mM unlabeled isoleucine and the distribution of label in cellular protein determined (Table 19). The labeling pattern observed among the 6 amino acids quantified was essentially identical with that observed with cells labeled with [3-^{14}C]pyruvate in the absence of added isoleucine (Table 19). Amino acid analysis of the culture medium from this experiment showed that 3.2 mM isoleucine was left in the medium at the time the cells were harvested.

Incorporation of label from a metabolite(s) produced from L-[U-^{14}C]isoleucine into isoleucine. It was subsequently demonstrated that there was a very low level of isoleucine contaminating the basal medium (B-10) used (see below). Therefore, the possi-

Table 19. Effect of unlabeled L-isoleucine on incorporation of Isotopic carbon from [3-^{14}C]pyruvate into amino acids[a]

Amino Acid[b]	Total nmoles	Radioactivity (dpm)	Specific Activity (dpm/nmole)
Alanine	63	17,471	277
Glycine	35	192	6
Aspartate	50	15,273	305
Lysine	39	22,723	583
Glutamate	56	32,747	585
Isoleucine	35	7,111	203
	Total recovered	95,581 (80%)[c]	

[a]25 μCi [3-^{14}C]pyruvate (spec. act. 20 μCi/μmole) and unlabeled L-isoleucine were added when the turbidity of the culture was 0.3 O.D. units. The initial isoleucine concentration was 5 mM.

[b]Aspartate, glutamate, and lysine were isolated by paper electrophoresis; all other amino acids were resolved on an amino acid analyzer.

[c]The total radioactivity for the amount of hydrolysate analyzed is 119,180 dpm.

bility existed that a reaction between some product of isoleucine degradation and pyruvate and CO_2 was responsible for the observed incorporation of label into isoleucine when cells were incubated with L-[U-^{14}C]serine, [3-^{14}C]pyruvate, or $^{14}CO_2$. To test this possibility, a growth medium was designed to contain not only all the ingredients of medium B-10 but also the labeled product of isoleucine degradation (the 2nd medium on Table 20). The latter was obtained from the treated supernatant medium of a culture grown in the presence of L-[U-^{14}C] isoleucine (the 1st medium on Table 20). This was collected when the culture reached a density of 0.3 O.D. unit which is the point at which label was normally added in those cultures incubated with L-[U-^{14}C]serine, [3-^{14}C]pyruvate, or $^{14}CO_2$. Following acidification and cation exchange (AG 50W-X4, H^+ form) treatment of the supernatant from the first culture to remove residual amino acids and CO_2, 4.6% of the original label remained in solution. This constituted the total radioactivity in the second medium.

Ten percent of the total ^{14}C present in the 2nd medium was incorporated into the cell protein. Over 95% of the isotope incorporated was recovered in the isoleucine fraction (9.5% of the initial radioactivity). Upon decarboxylation of the isoleucine fraction, 36% of the label was lost as $^{14}CO_2$. This indicates that some metabolite(s) of isoleucine other than or in addition to 2-methylbutyric acid was incorporated into the isoleucine found in cell protein. While the specific activity of the isoleucine isolated was not high (144 dpm/ mole), it approached the levels found in cells labeled with $^{14}CO_2$, ^{14}C-serine, or ^{14}C-pyruvate.

Table 20. Incorporation of the product of isoleucine degradation into cellular isoleucine

Determination		Radioactivity (dpm)
1st Medium[a]:	Initial	2.05×10^7
	Supernatant of culture at harvest	1.68×10^7
2nd Medium[b]:	Initial	9.51×10^5
	Supernatant of culture at harvest	7.09×10^5
Protein hydrolysate[c]:	Total	9.51×10^4
	Isoleucine fraction	9.09×10^4

[a]Ten μCi of L-[U-^{14}C]isoleucine (spec. act. 360 μCi/μmole) was added to 10 ml culture in medium B-10 as soon as growth was observed; the cells were removed at the culture density of 0.3 O.D. units and the supernatant solution was collected.

[b]This medium contained the supernatant medium from the 1st culture which had been treated to remove $^{14}CO_2$ and amino acids (see Materials and Methods). Non-labeled amino acids, salts, vitamins, and sodium thioglycollate were added to the supernatant solution at the same concentrations as in medium B-10.

[c]Obtained from cells harvested from the second culture at an O.D. of 0.6.

This level of incorporation is very significant considering the low level of total radioactivity present in the 2nd medium (Table 20); less than 0.5 μCi/10 ml of medium.

Metabolites of Isoleucine

Volatile fatty acids. The most likely metabolites of isoleucine produced by C. sporogenes are volatile fatty acids, CO_2, and ammonia. Washed exponential phase cells suspended in a solution containing 100 mM L-isoleucine and 100 mM L-proline produced 2-methylbutyrate as the major volatile fatty acid as expected (Table 21). The low amount of acetate, propionate, and isobutyrate were probably produced from the intracellular pool of alanine, threonine, and valine respectively.

In the isotope labeling experiments using L-[U-^{14}C]serine, [3-^{14}C]pyruvate and $^{14}CO_2$, the label was added to the culture grown in medium B-10 at the turbidity reading of ∿0.3 O.D. units. Therefore, it was of interest to find out the type and the amount of volatile fatty acids present in the culture medium at that particular stage of growth. As shown in Table 21, acetate was the major acid produced. This would be expected because of the high concentration of L-serine in medium B-10 (Table 1). Leucine and valine in the medium must have been fermented to produce the observed isovalerate and isobutyrate respectively. Trace amount of propionate were also observed. While no other volatile fatty acids were detected in these samples, the levels of metabolites necessary for the limited incorporation into isoleucine observed would be below the limits of detection by the method used.

Table 21. Volatile fatty acids obtained from isoleucine fermentation and from a growing culture

Acids	Concentration (mM)	
	Isoleucine Fermentation[a]	Culture Medium[b]
Acetate	4	11
Propionate	1	1
Isobutyrate	traces	2
Isovalerate	---	2
2-Methylbutyrate	65	--

[a]Exponential phase cells grown in medium A were harvested, washed twice with 0.1 M potassium phosphate buffer (pH 7.5), and then resuspended in 20 ml solution containing 100 mM L-isoleucine and L-proline at the rate of 1.35 g of packed cells/ml. After 8 h incubation at 37 °C in the anaerobic chamber, the cells were removed by centrifugation and the clear supernatant was assayed for volatile fatty acids.

[b]Cells were grown in 10 ml of medium B-10 and were removed by centrifugation when the culture density reached 0.3 O.D. units; the clear supernatant was assayed for volatile fatty acids.

Growth Requirements

Isoleucine in the medium. When a low dilution of fresh B-10 medium was run through the amino acid analyzer, a very tiny peak was observed in the elution position of isoleucine. The peak was excised from the chart paper, weighed on an analytical balance and the weight so obtained was found (by comparison with those of several amino acids of known concentrations) to correspond to an isoleucine concentration of 0.16 mM. An aliquot of supernatant liquid from a culture grown in this medium until it had reached a turbidity of 0.3 O.D. units (the point of addition of radioactive compounds in the labeling experiments for one generation) was also subjected to amino acid analysis by means of amino acid analyzer. Again, a very tiny peak of isoleucine was found, and by estimation performed in the same manner as described above, the level of isoleucine was 0.08 mM which is roughly 50% of that found in the fresh medium. The protein from a 10 ml culture grown in medium B-10 that was harvested at culture turbidity of 0.6 O.D. units contained about 0.6 μmoles of isoleucine (there were about 518 μmoles isoleucine per mg protein, and there were about 1.2 mg protein in the 10 ml culture). Therefore, there is a possibility that isoleucine is essential for the growth of C. sporogenes. This amount of contaminant in the medium might be sufficient to provide all the isoleucine required for growth.

Growth response to leucine and isoleucine. C. sporogenes exhibits a requirement for high concentrations of leucine for

growth in medium B-10 (Fig. 5) which does not correlate with the amount of this amino acid consumed by the cells (Table 5). Since L-leucine is the most likely source of the contaminating L-isoleucine, it was reasonable to speculate that the requirement for high concentration of L-leucine was only to supply enough L-isoleucine for growth. This was tested by growing C. sporogenes in medium B-10 with 2 mM L-leucine supplemented with varying amounts of L-isoleucine. Growth comparable to control levels (i.e. growth in medium B-10 in which 11.5 mM L-leucine was present) was observed on addition of as low as 0.1 mM L-isoleucine. Particularly surprising, however, was the fact that isoleucine could partially relieve the requirement for leucine. Addition of 10 mM of L-isoleucine to medium B-10 in the absence of leucine supported growth up to 0.42 O.D. units, which is about 50% of the normal growth obtained in this medium.

Growth response to 2-methylbutyrate. Since 2-methylbutyrate is the primary catabolite of isoleucine fermentation, it was the most probable precursor of isoleucine and leucine. Growth responses to increasing concentrations of 2-methylbutyrate in the absence of both leucine and isoleucine was similar to those observed with leucine alone (Fig. 6). At the highest concentration fo 2-methylbutyrate (8 mM) the total growth was equivalent to that normally obtained in medium B-10. These observations indicated that C. sporogenes can synthesize both isoleucine and leucine when 2-methylbutyrate is present.

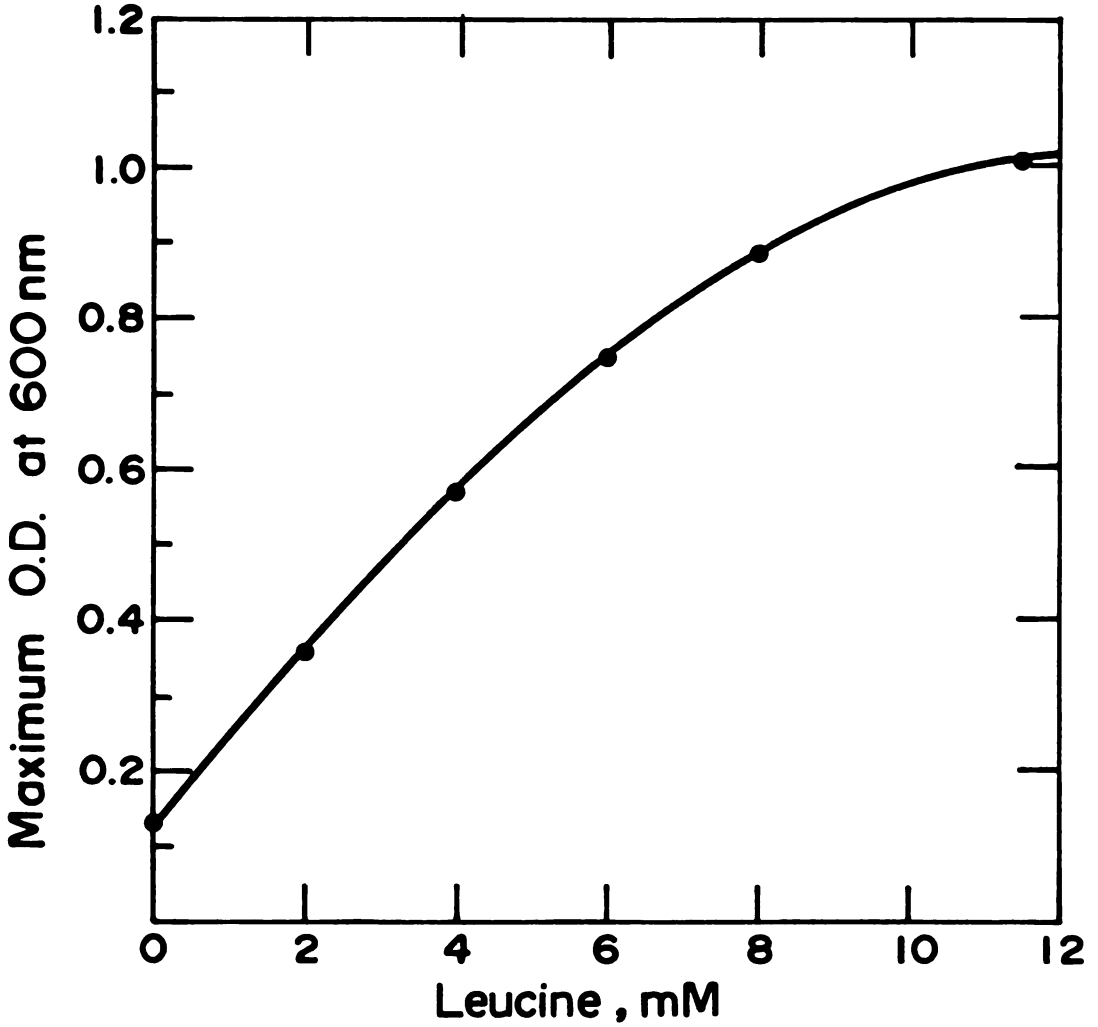


FIG. 5. The effect of leucine on growth of C. sporogenes in medium B-10.

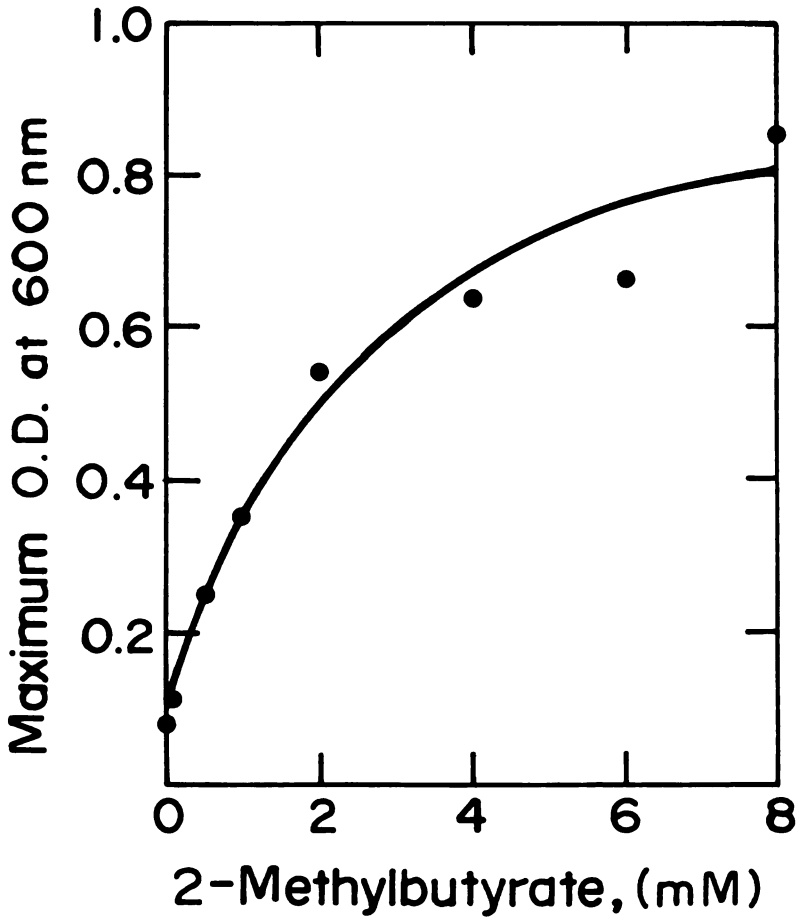


FIG. 6. The effect of 2-methylbutyrate on growth of C. sporogenes in medium B-10 without leucine.

Incorporation of ^{14}C from [U-^{14}C]2-Methylbutyric Acid into Isoleucine

A total of 4.33 μCi of [U-^{14}C]2-methylbutyric acid (1.6 ml of 0.967 μCi/μmole) was added to a growing culture in medium B-10 without added leucine but containing 4 mM unlabeled 2-methylbutyrate at a culture density of 0.3 O.D. units; the final volume was 14.2 ml. The labeling was allowed to occur for one generation. Label was found to be incorporated into the cell protein; the specific activity was 58,584 dpm/mg protein. Amino acid analysis of the protein hydrolysate showed that all of the radioactivity was in the isoleucine fraction; the calculated specific activity was 113 dpm/nmole. Unfortunately, since the concentration of 2-methylbutyric acid in the culture at the time the label was added was not examined, the quantitative aspect of this labeling experiment may only be roughly estimated as follows:

Total volume of culture = 14.2 ml.

Total unlabeled 2-methylbutyrate added to the culture = 40 μmoles.

Total [U-^{14}C]2-methylbutyrate added = 4.48 μmoles.

Total initial radioactivity in the culture = 1.08×10^7 dpm.

Sp. act. of ^{14}C.-isoleucine in the cell protein = 113 dpm/nmole.

A. If all the original unlabeled 2-methylbutyrate was completely consumed prior to the addition of label:

sp. act. of ^{14}C-2-methylbutyrate = 1.08×10^7 dpm/4.48 μmoles.

= 2,411 dpm/nmole.

= 482 dpm/nmole C.

This corresponds to only 25% of a single carbon of 2-methylbutyrate being incorporated into each molecule of isoleucine.

B. If none of the added unlabeled 2-methylbutyrate was consumed prior to the addition of label:

sp. act. of ^{14}C-2-methylbutyrate = 1.08×10^7 dpm/44.48 μmoles.

= 243 dpm/nmole.

= 49 dpm/nmole C.

This corresponds to two carbons of 2-methylbutyrate incorporated into each molecule of isoleucine.

The above estimations suggest that no more than two carbons from 2-methylbutyric acid could have been incorporated into isoleucine. Therefore, if C. sporogenes synthesizes isoleucine via carboxylation of 2-methylbutyrate as occurs in some rumen bacteria (55), the label incorporated into isoleucine must have been diluted in the culture. However, if this was the only way in which carbons from 2-methylbutyrate were incorporated, the C-1 of isoleucine should not be labeled. One third of the total radioactivity of the isoleucine fraction was removed by decarboxylation. Therefore, the reductive carboxylation pathway must not be the only manner of isoleucine synthesis from 2-methylbutyric acid.

The absence of label incorporation into leucine fraction was totally unexpected and there is no ready explanation for this.

DISCUSSION

The specific activity of threonine dehydratase in C. sporogenes was found to be comparable to that in E. coli (49) but higher than that in C. tetanomorphum cells (70). However, a biosynthetic, isoleucine sensitive form of threonine dehydratase could not be demonstrated in C. sporogenes. Although it is possible that this form of the enzyme might have been masked by the very active biodegradative threonine dehydratase, it is unlikely, since, in all microorganisms examined thus far except for R. rubrum (28), the biosynthetic threonine dehydratase is extremely sensitive to isoleucine inhibition. For example, the biosynthetic threonine dehydratase of Corynebacterium sp (6) and R. capsulata (28) is 100% inhibited by isoleucine concentrations of 1.0 and 0.1 mM, respectively, in the presence of 10 mM threonine; while in Pseudomonas sp, 0.15 mM L-isoleucine produces a 50% inhibition of the enzyme (39). The biosynthetic threonine dehydratase of R. rubrum is inhibited by isoleucine but only at low substrate concentrations (28). In E. coli (14) and P. multivorans (39), the inhibitory effect of isoleucine on threonine dehydratase can be reversed at higher pH values. However, no inhibition by isoleucine was observed when the enzyme in extracts of C. sporogenes was assayed over a wide range of pH values and concentrations. These data and results of subsequent experiments all indicate that threonine is not a precursor of isoleucine in this organism.

The threonine dehydratase of C. sporogenes is similar to that of C. tetanomorphum in several respects (27, 70); viz., (a) the enzyme is activated by ADP and not by AMP, (b) the activation by ADP occurs only at low threonine concentrations, (c) in the presence of ADP, the Km value of the enzyme from both sources is lowered by a factor of ∿13, and (d) the enzyme is constitutive. Two forms of threonine dehydratase were resolved from extracts of C. tetanomorphum by diethylaminoethyl (DEAE) cellulose chromatography (27). However, neither form of the enzyme was subject to inhibition by isoleucine. It is probable that the function of the threonine dehydratase in both of these clostridia is catabolic. The latter has been described by Tokushige et al (62) as the breakdown of threonine to propionic acid and ammonia accompanied by ATP production.

Multivalent repression of biosynthetic threonine dehydratase is a common phenomenon in E. coli and S. typhimurium (66) and results from the simultaneous presence of excess amounts of valine, leucine, and isoleucine in the growth medium (65). C. sporogenes required substantial amounts of valine (17 mM) and leucine (11.5 mM) in medium B-10 (Table 1). This medium which supposedly "lacked" isoleucine, was subsequently found to contain traces of this amino acid as a contaminant; however, the amount of isoleucine present (∿0.16 mM) was far below the level required for multivalent repression as reported in other bacteria (65). Thus, although it was not possible to grow C. sporogenes without valine and leucine, the level of isoleucine present should have allowed detection of a biosynthetic threonine dehydratase if it were truly present. Some bacteria (Corynebacterium

sp (6), and P. multivorans (39) produce biosynthetic forms of threonine dehydratase which are not sensitive to multivalent repression by valine, leucine and isoleucine. However, these enzymes are sensitive to feedback inhibition by isoleucine. All the data accumulated in this study strongly suggest that C. sporogenes only contains the biodegradative form of threonine dehydratase which is constitutively synthesized. It is not repressed by glucose, and its primary role in vivo is for energy generation from threonine degradation presumably at a low energy charge.

Attempts to demonstrate threonine aldolase (EC 2.1.2.1) and threonine dehydrogenase (EC 1.1.1.103) activities in extracts of C. sporogenes cells were unsuccessful. Either enzyme could lead to the production of glycine from threonine. Threonine aldolase cleaves threonine to acetaldehyde and glycine in some organisms (6, 19, 31, 76); whereas threonine dehydrogenase catalyzes the oxidation of threonine to α-amino-β-ketobutyrate, which in turn could be degraded via two possible pathways; i.e., the aminoacetone route and the glycine route. In the first route, α-amino-β-ketobutyrate is decarboxylated non-enzymatically into aminoacetone (38). In the glycine route, α-amino-β-ketobutyrate is cleaved to acetyl coenzyme A and glycine by the action of an enzyme variously known as aminoacetone synthase (44) or glycine coenzyme A ligase (73). Threonine dehydrogenase of S. marcescens and of E. coli K-12 are controlled by catabolite repression and by leucine-mediated induction (37, 47). No threonine dehydrogenase activity was detected in extracts of C. sporogenes cells grown in the absence of glucose and in the presence of excess leucine.

Although neither of the two enzyme activities was detected, glycine production from threonine was found to occur to a minor extent in C. sporogenes cells (Table 9). It is not known which enzyme is responsible for that conversion. Auxotrophic mutants lacking serine transhydroxymethylase enzyme would be very useful in such investigation.

The absence of a biosynthetic threonine dehydratase raised a question as to how isoleucine is synthesized in C. sporogenes. In preparation for isotopic labeling experiments employing ^{14}C-substrate to find the source of carbon skeleton for isoleucine, determinations were made of the amino acid composition of cell proteins, free intracellular pools, and of the growth medium. Of particular interest were the high levels of glutamic acid, alanine and proline observed in the intracellular pool during the exponential phase of growth. None of these three amino acids were added to the growth medium, so they must be synthesized. C. sporogenes has been shown to accumulate proline, glutamic acid, and γ-aminobutyric acid in the amino acid pool in response to increased environmental sodium chloride (45). Measures (45) reported that growth of non-halophilic bacteria at low water activities (high solute concentrations) depends on the ability of the cell to balance the environmental osmotic pressure by intracellular accumulation of amino acids, and on the types of amino acid which accumulate. The synthetic medium employed to grow C. sporogenes cells (medium B-10) may have a sufficiently high salt content to influence the pool.

Of the 8 amino acids that comprise medium B-10 (Table 1), serine is probably the main if not the sole source of pyruvate. C. botulinum, which is closely related to C. sporogenes (30, 75), was reported to produce alanine, acetate, CO_2 and ammonia from argine, which would involve pyruvate as an intermediate. However, this fermentation occurred only to a minor extent (46). Serine appeared to be consumed very rapidly during the early exponential phase, accompanied by the concomittant accumulation of a substantial amount of alanine. Therefore, it is reasonable to assume that at a certain point during the growth the cells must have switched to using alanine as the source of pyruvate. This assumption is supported by the labeling pattern of the amino acids obtained from cells incubated in the presence of L-[U-^{14}C]serine or [3-^{14}C]pyruvate (Table 11 and 16).

A variety of enzymes are known that are capable of deaminating L-serine to produce pyruvate and ammonia (65); viz., (a) dehydratases specific for L-serine, e.g. the enzyme from E. coli described by Alfodi et al (2); (b) dehydratases that act on other substrates, most frequently L-threonine, e.g. the biosynthetic and biodegradative L-threonine dehydratases of E. coli and S. typhimurium (65); and (c) enzymes primarily catalyzing a different type of reaction, e.g. the B protein of tryptophan synthetase (EC 4.2.1.20) of E. coli (18). Of those enzymes capable of degrading serine as reviewed by Umbarger (65), none were activated by AMP. It is possible that there is more than one enzyme in C. sporogenes cells that could act on serine, and that such activity is not regulated, at least under the conditions of growth in medium B-10 employed.

The synthesis of alanine from pyruvate in E. coli occurs via the glutamate-alanine transaminase and valine-alanine transaminase (transaminase C); however, the latter serves only a minor role (66). Except for the repression control over transaminase C, alanine biosynthesis does not seem to be regulated (66). While it is not known which transaminase catalyzes the conversion between pyruvate and alanine in C. sporogenes, the assumption that the enzyme is not regulated is indicated by the accumulation of alanine both intra- and extracellularly.

There was considerable incorporation of ^{14}C into cell protein when cells were grown to stationary phase in the presence of the following ^{14}C-labeled compounds: threonine, valine, leucine, arginine, ornithine, proline, glucose, histidine, acetate, methionine, phenylalanine, and tyrosine. However, there was no significant incorporation of label into the cellular isoleucine. Improved experimental conditions (labeling for only one generation instead of for the entire growth period, and harvesting the cells during exponential phase) gave similar results. The absence of significant incorporation of label from L-[U-^{14}C]aspartate or L-[U-^{14}C]threonine into cellular isoleucine (Table 8) is consistent with the failure to detect the presence of biosynthetic threonine dehydratase in C. sporogenes extracts. It is very unlikely that isoleucine is synthesized from threonine in this organism. However, there were other potential sources of α-ketobutyrate in the medium used which might dilute out the α-ketobutyrate produced from threonine. C. sporogenes was found to convert L-methionine to α-ketobutyrate, ammonia and methylmercaptan (72).

A mutant of E. coli Crookes strain deficient in threonine dehydratase was found to synthesize isoleucine from glutamate which in turn serves as the source of α-ketobutyrate (50). However, no labeled isoleucine was obtained when C. sporogenes cells were grown in the presence of L-[U-^{14}C]methionine (Table 7) or L-[U-^{14}C]glutamate (Table 8). The initial specific activities of the labeled substrates (aspartate, threonine, methionine or glutamate) were sufficiently high, that even if dilution of labeled α-ketobutyrate occurred labeled isoleucine should still have been detected if the classical pathway of isoleucine biosynthesis was operative.

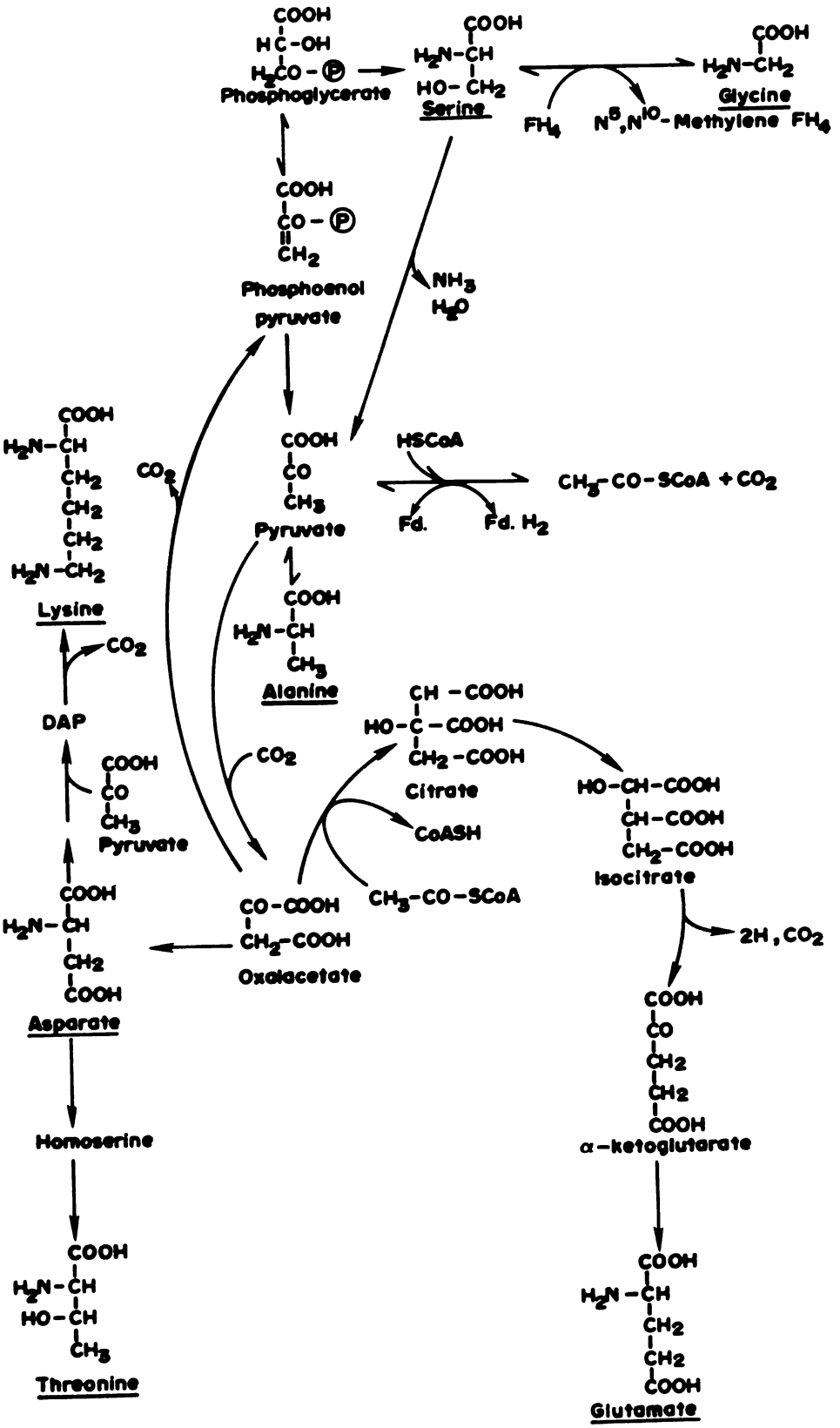
The total amount of label incorporated into the cellular protein was much higher when the culture was labeled with L-[U-^{14}C] threonine than when labeled with L-[U-^{14}C]aspartate or with L-[U-^{14}C] glutamate even though the initial specific activities of all three substrates were nearly equal (Table 8). This suggests that threonine biosynthesis is very tightly regulated and that the biosynthesis of aspartate and glutamate are either not regulated or only weakly controlled. The small amount of threonine present in the added radioactive threonine must have been sufficient to shut off its synthesis during most of the labeling period. In E. coli, two enzyme activities in the pathway of threonine biosynthesis, aspartokinase I and homoserine dehydrogenase I are sensitive to inhibition by threonine (66). Aspartate is formed by transamination of oxalacetate via a glutamate-aspartate transaminase activity; in E. coli, it is the transaminase A which is primarily responsible (66). While aspartate in the growth medium was reported to repress the bulk of glutamate-aspartate

transaminase activity (68), there is no evidence for a regulatory site for any effector molecule that would modulate the activity of the enzymes involved. The reaction is freely reversible (66). While further studies would be required to establish the regulation of threonine, aspartate, and glutamate biosynthesis in C. sporogenes, the lack of strict regulation of glutamate biosynthesis should be anticipated because of the high intracellular pool of glutamate found in this organism (Table 5).

Subsequent labeling experiments showed that labeled isoleucine was obtained when cultures were incubated with L-[U-^{14}C]serine, [3-^{14}C]pyruvate or $^{14}CO_2$ (Table 11, 16 and 14). The distribution of the label found in alanine, aspartate, threonine, lysine, glutamate, serine, and glycine was consistent with well established biosynthetic pathways (Fig. 7). Comparisons of the specific activities of these amino acids with that of the ^{14}C-isoleucine formed provide a basis for estimates of the number of carbon atoms from each labeled substrate contributing to isoleucine biosynthesis. Therefore, the labeling patterns resulting from each ^{14}C-substrate will be discussed individually.

Serine degradation in a culture labeled with L-[U-^{14}C]serine would result in pyruvate with all of its three carbons labeled, which in turn could be directly involved in several different reactions. Those that will be specifically discussed here are: (a) the pyruvate-CO_2 exchange reaction; (b) transamination to alanine; and (c) carboxylation into oxalacetate. A pyruvate-CO_2 exchange reaction in C. sporogenes probably occurs via pyruvate synthase, an enzyme which is

FIG. 7. Interpretative scheme of the amino acid labeling pattern for all radioactive experiments with C. sporogenes.


Phosphoglycerate
Serine
Glycine
FH4
N5,N10-Methylene FH4
Phosphoenol pyruvate
NH3
H2O
HSCoA
CH3-CO-SCoA + CO2
Fd.
Fd. H2
CO2
Pyruvate
Alanine
Lysine
DAP
Citrate
CoASH
CH3-CO-SCoA
Isocitrate
2H, CO2
Oxalacetate
Asparate
Homoserine
α-ketoglutarate
Threonine
Glutamate

widely distributed in anaerobic organisms (12). Consequently, the label of C-1 of pyruvate would likely be diluted by any unlabeled CO_2 present in the medium. However, studies with $^{14}CO_2$ indicate that this exchange reaction in C. sporogenes only occurs to a very limited extent under the growth condition employed (see below). Thus, pyruvate synthase in this organism probably operates mainly in the oxidation of pyruvate to acetyl coenzyme A and CO_2, a property reported for the same enzyme from C. acidi-urici and C. pasteurianum (12).

Transamination of pyruvate derived from L-[U-^{14}C]serine would result in alanine with all of its three carbons labeled. As pointed out earlier, alanine was excreted into the medium very actively during the early phase of growth. Although this process might continue to occur only shortly after the addition of the label, the high specific activity of the added label (sp. act. 162 μCi/ mole; 25 μCi total) would be sufficient to establish a pool of alanine in the medium with a relatively high specific activity. However, because of the relative concentrations of unlabeled alanine and serine at the time the ^{14}C-serine was added to the medium, there would be more dilution of the labeled alanine formed and then incorporated into protein than of the ^{14}C-serine incorporated directly into cells. This was reflected by the specific activity per carbon atom of the serine from protein; it was about twice that of the activity per carbon in alanine. Therefore, it is more appropriate to compare the specific activities per carbon atom for other amino acids with alanine than with serine. Such comparisons indicate that alanine, glycine, aspartate, threonine, lysine, and glutamate were uniformly labeled (Table 11), although the

specific activity per carbon of threonine was low. Decarboxylation of each of these amino acids also indicated that they were uniformly labeled. In contrast, the radioactivity per carbon of isoleucine was only about 50% that in alanine, and decarboxylation resulted in the release of about 25% of the total label present. These data incicate that only 3 or 4 of the 6 carbons in isoleucine were synthesized from pyruvate.

Oxalacetate is probably synthesized from pyruvate via pyruvate carboxylase reaction which is known to occur in many organisms (58). Oxalacetate is an important intermediate; it serves as a precursor of not only the biosynthesis of several amino acids, but also of gluconeogenesis. The first reaction in gluconeogenesis is probably the conversion of oxalacetate into phosphoenolpyruvate (PEP) catalyzed by PEP-carboxykinase, an enzyme which is widely distributed in many organisms including a number of bacteria (69). These suggested pathways are supported by the labeling pattern of the amino acids in proteins of cells grown in the presence of labeled serine, pyruvate, or CO_2 (Table 11, 16 and 14), and each will be discussed in detail accordingly.

Uniformly labeled oxalacetate is required for the synthesis of uniformly labeled aspartate, threonine, lysine, and glutamate, respectively. Therefore, the CO_2 molecule that was incorporated into oxalacetate must have been radioactive and contained approximately the same specific activity as that of pyruvate or alanine. This would be possible if a significant amount of CO_2 is derived from the oxidation of [U-^{14}C]pyruvate via the pyruvate synthase reaction. This

proposed pathway is consistent with the earlier suggestion on the role of the latter enzyme in C. sporogenes. Had the C-1 of pyruvate been greatly diluted by CO_2 in the medium, the results obtained on decarboxylation of the labeled amino acids (Table 12) would not have been expected. The data all indicate that C. sporogenes utilizes the same biosynthetic pathways for biosynthesis of aspartate, threonine and lysine as known to occur in other bacteria (66).

Cooper and Costilow (16) suggested glutamate synthesis might occur from proline. In such a case, glutamate from cells incubated with L-[U-^{14}C]serine should not have been so heavily labeled because proline in this organism is produced from arginine (which is essential for growth) via ornithine (17, 46). The specific activity per carbon of glutamate formed from ^{14}C-serine was the same as that per carbon of alanine. Therefore, glutamate must be synthesized from pyruvate (Fig. 8). In most organisms, α-ketoglutarate is synthesized from pyruvate via the forward TCA cycle (66), and these data are consistent with this pathway. However, some anaerobes use a reductive pathway for glutamate synthesis (4, 5) and this is not excluded by these results.

The results of decarboxylation of ^{14}C-isoleucine formed from L-[U-^{14}C]serine (26.4% of label loss) suggest that a total of three to four carbons in that amino acid were labeled. Since the total specific activity of isoleucine was 120 dpm/nmole (Table 11), if only three carbons were labeled, the specific activity of each labeled carbon would be 40 dpm. This value is similar to that for alanine. However, this information is not sufficient to give any indication

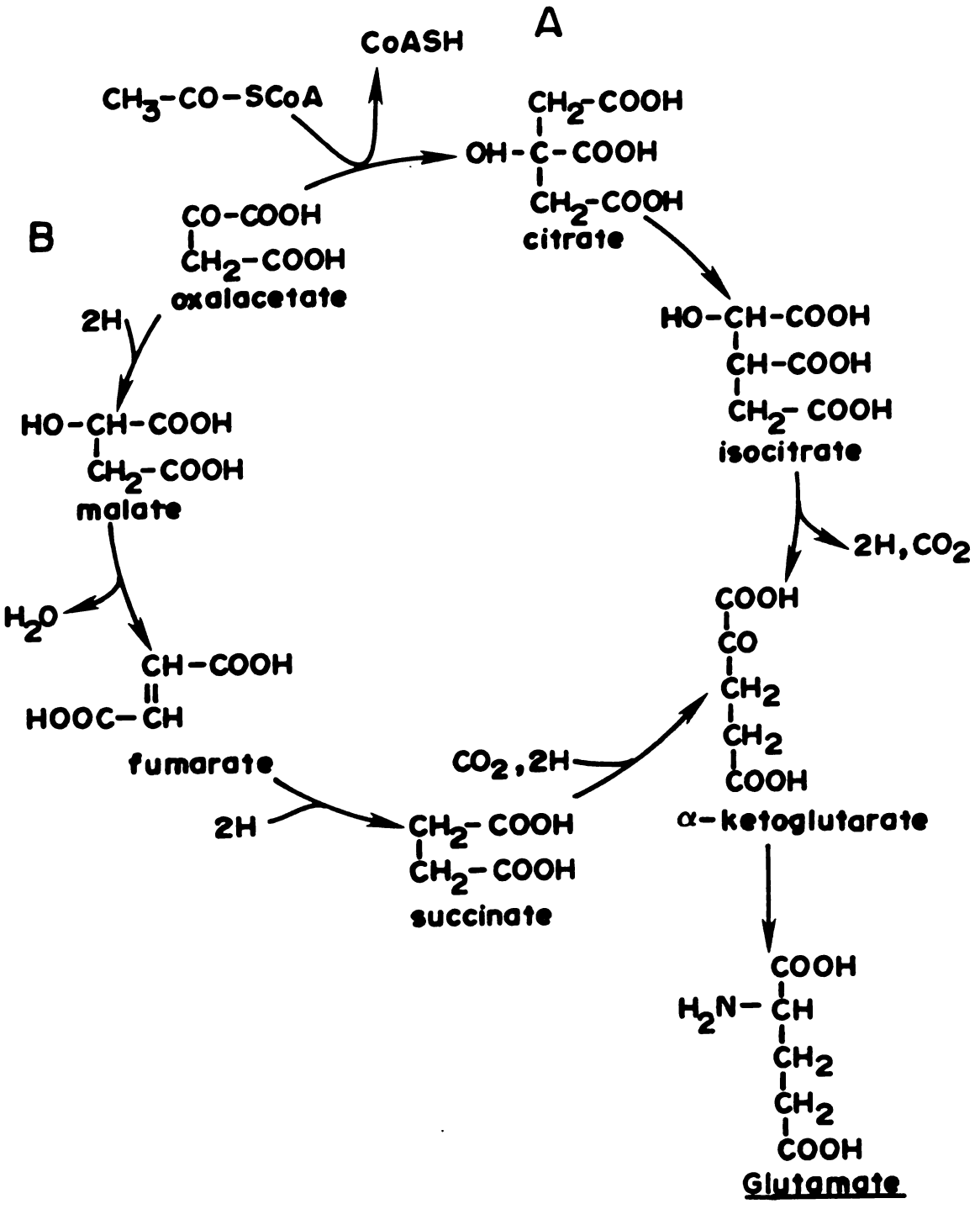


FIG. 8. Glutamate synthesis from oxalacetate. A, via the forward TCA cycle (66); B, via the reductive pathway (4, 5).

on which carbons of pyruvate were incorporated into isoleucine or how the process occurred.

Cultures labeled with $^{14}CO_2$ resulted in the incorporation of relatively high levels of radioactivity into the cellular protein (Table 13). The amount of radioactivity incorporated per mg protein in the culture labeled for two generations was only 6% lower than that labeled for one generation. This suggests taht there was little dilution of the label by unlabeled CO_2 resulting from doubling the incubation period. Following is an interpretation of the labeling pattern of the amino acids obtained from cells incubated with $^{14}CO_2$ for one generation (see Fig. 7).

Pyruvate would acquire label at C-1 due to the pyruvate-$^{14}CO_2$ exchange reaction, thus transamination of pyruvate would result in alanine also labeled at C-1. This suggestion is supported by the complete loss of label upon decarboxylation of the alanine isolated from cell protein (Table 15).

Oxalacetate synthesized by carboxylation of pyruvate would be labeled from $^{14}CO_2$ at C-4; some label would be expected at C-1 depending on the extent of pyruvate-CO_2 exchange reaction. During the formation of PEP from oxalacetate, C-4 of the latter would be lost and thus the PEP would be labeled only at C-1. Accordingly, serine would also acquire label at C-1 via the phosphoglycerate pathway. Glycine from serine would also be labeled at C-1. As shown in Table 14, alanine, serine, and glycine showed relatively low specific activities. This suggests that pyruvate-CO_2 exchange reaction in C. sporogenes only occurred to a limited extent. The specific activity of glycine is

only slightly less than that of serine, which is consistent with the earlier suggestion that the majority of glycine is synthesized from serine.

Biosynthesis of aspartate, threonine, and lysine from oxalacetate which was labeled at C-1 and C-4 would result in all three amino acids being labeled also at C-1 and C-4. As expected, aspartate lost all its label upon decarboxylation by ninhydrin (Table 15); this treatment removes both carboxyl carbons (26). On the other hand, the chloramine-T treatment should remove only C-1 of threonine and lysine respectively (33). As shown (Table 15), both amino acids lost only ∿20% of their label. Since, by the classical pathways, C-1 for both amino acids would be derived from C-1 of pyruvate, these results are consistent with the earlier suggestion that the pyruvate-CO_2 exchange reaction occurred to a limited extent. The specific activities per nmole of alanine, serine, and glycine were only ∿20% of that of aspartate.

The specific activity of glutamate labeled from $^{14}CO_2$ was to be nearly equal to that of aspartate. There are two ways glutamate is known to be synthesized from oxalacetate; (a) via the forward TCA cycle as in E. coli and other bacteria (66, Fig. 8A); (b) via the reduction of oxalacetate to succinate and the carboxylation of succinate to α-ketoglutarate as found in some rumen bacteria (4, 5, Fig. 8B). During the synthesis of glutamate from C-4 labeled oxalacetate there would be one molecule of CO_2 lost in the first pathway, whereas one molecule of CO_2 would be incorporated in the second one. Therefore, if glutamate in C. sporogenes was synthesized via the

reverse TCA cycle in the presence of $^{14}CO_2$, one would expect the specific activity of glutamate to be about 2X that of aspartate. However, the data show that the two amino acids had about the same specific activity. Because of the high intracellular glutamate pool at the time $^{14}CO_2$ was added to the culture, these data alone are not conclusive. However, failure to release any $^{14}CO_2$ on decarboxylation of the labeled glutamate excludes the possibility of synthesis via the reverse TCA cycle. Glutamate in this organism must be synthesized via the forward TCA cycle.

The distribution of label from $^{14}CO_2$ among the 5 carbons of glutamate would be different depending on the stereospecificity of the citrate synthase enzyme (23, Fig. 9), Most organisms contain the si-type synthase and the glutamate synthesized from oxalacetate labeled at C-1 and C-4 is only labeled at C-1 due to the removal of one molecule of CO_2 in the isocitrate dehydrogenase step. On the other hand, under the same conditions re-citrate synthase which is found in a small group of anaerobic bacteria, gives rise to glutamate being labeled only at C-5 derived from C-4 of oxalacetate. The C-1 of oxalacetate is lost in the isocitrate dehydrogenase step. No label was lost upon decarboxylation of glutamate (Table 15). Only C-1 of glutamate is removed by the decarboxylation method used (33). Therefore, it is likely that C. sporogenes possesses citrate synthase of the re-type stereospecificity.

The amount of radioactivity incorporated into isoleucine during one generation of growth in the presence of $^{14}CO_2$ was quite significant. The specific activity of the isoleucine was not

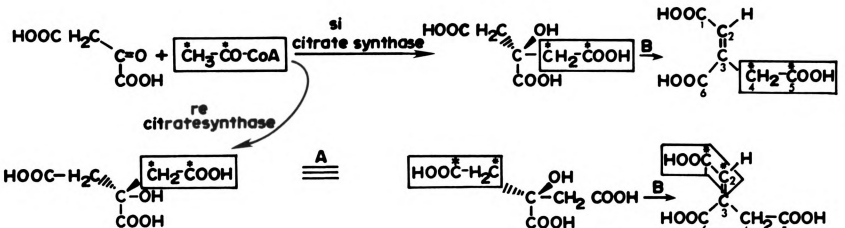


FIG. 9. Formation of citrate by si-citrate synthase and by re-citrate synthase. A, molecule turned 120°; B, sterospecific removal of water by cis-aconitase (25).

significantly less than that of lysine which should be equivalent to that of aspartate (Table 14). Upon decarboxylation, isoleucine lost 38% of its label while threonine and lysine lost 20%. If classical pathways were operative in the formation of the latter 2 amino acids, the label lost was probably derived from pyruvate-CO_2 exchange reactions. The $^{14}CO_2$ fixed into oxalacetate via pyruvate carboxylase would be in the C-4 of threonine and lysine. Simple calculations indicate that for each carbon fixed, the dpm/μmole should be near 160-170. This is the level of radioactivity found in isoleucine. However, since 38% of the label was lost on decarboxylation, radioactivity from $^{14}CO_2$ must have been incorporated into more than one carbon of isoleucine.

The low specific activity of valine in cell proteins produced in the presence of $^{14}CO_2$ and the complete loss of the label upon its decarboxylation is consistent with the possibility suggested above that valine could acquire label from a low level of valine-CO_2 exchange reaction.

Growth in the presence of [3-^{14}C]pyruvate should give rise to alanine labeled at C-3 via transamination and acetyl coenzyme A labeled at C-2 via oxidation catalyzed by pyruvate synthase. Carboxylation of pyruvate would result in oxalacetate labeled at C-4 and upon its decarboxylation the resulting PEP would be labeled at C-3, therefore, phosphoglycerate synthesized from PEP would also be labeled at C-3.

The incorporation of significant radioactivity into serine suggests the presence of serine synthesis via the phosphoglycerate pathway in C. sporogenes as occurs in other organisms (66). This was

rather unexpected since the growth medium employed (medium B-10) contained 25 mM L-serine. However, analysis of the growth medium at the time that labeled substrate was added showed that the residual serine concentrations were usually less than 1 mM. As noted above, considerable alanine had accumulated in the medium. Therefore, one would expect more dilution of the labeled pyruvate by alanine than serine. The results agreed with this. Therefore, the specific activities of other amino acids labeled with [3-^{14}C]pyruvate are most appropriately compared to that of alanine.

If glycine is synthesized from serine, C-3 of serine would be removed during the serine hydroxymethylase reaction, and glycine would not be labeled. The very low amount of ^{14}C found in glycine could not have been due to glycine biosynthesis from threonine via established pathways. Threonine in this experiment should be labeled at C-3, and glycine synthesized from threonine should retain only the C-1 and C-2 of threonine. It is of interest, however, that results obtained earlier did show incorporation of ^{14}C into cellular glycine when L-[U-^{14}C]threonine was used as substrate (Table 9).

Oxalacetate labeled at C-3 would give rise to aspartate and threonine labeled at their C-3 positions. However, since one molecule of pyruvate is incorporated into lysine during its synthesis from aspartate, lysine would be expected to be labeled at C-3 and C-5 (label at C-5 is derived from pyruvate incorporation). If C. sporogenes contains re-citrate synthase as suggested earlier, the synthesized glutamate would be labeled at C-2 and C-4 (the label at C-2 is derived from C-3 of oxalacetate). The ratios of the specific activities of

alanine, aspartate, threonine, lysine, and glutamate synthesized from [3-^{14}C]pyruvate were very close to values expected from these organisms (Table 16).

Although the exact location of the label in each of the foregoing amino acids cannot be proved conclusively at this point, the results of their decarboxylation also supports the suggested pattern of carbon flow during their syntheses. There was no label lost upon decarboxylation of all the amino acids examined (Table 17); therefore, the label resided at a carbon atom(s) other than the carboxyl carbon.

Isoleucine isolated from cell proteins produced in the presence of [3-^{14}C]pyruvate had a specific activity not significantly different from alanine. In addition, none of this ^{14}C was lost from the labeled isoleucine upon decarboxylation. This is very direct evidence that C. sporogenes is able to synthesize at least some isoleucine from rather simple precursors. While the data suggest that no more than one C-3 of pyruvate is incorporated per isoleucine formed, this is quite speculative because of the possible dilution by the contaminating isoleucine (see above).

The fact that there was a low concentration of isoleucine contaminating the growth medium greatly complicated the interpretation of the data. It was not possible to accurately estimate the number of labeled carbons in isoleucine by comparison of the specific activity of the isoleucine with other amino acids. However, the extent to which ^{14}C from [3-^{14}C]pyruvate was incorporated into isoleucine was not influenced by the addition of excess isoleucine to the growth medium. Indeed it appears probable that degradation products of

isoleucine are necessary for incorporation of carbons from CO_2 and pyruvate. This is one possible explanation for failure to find any significant label in the isoleucine of cell protein when washed cells were incubated for very short periods of time in the presence of [3-^{14}C]pyruvate.

The accumulated evidence at this point suggested that isoleucine or a catabolite of isoleucine was essential for growth of C. sporogenes and that the observed incorporation of label from pyruvate and CO_2 depended on the presence of low amounts of isoleucine in the medium. The ∿0.16 mM isoleucine found contaminating medium B-10 is more than would be required for protein synthesis during the entire growth period. Approximately 2 mg cell protein were obtained from a 10 ml culture (harvested at the end of log phase) in medium B-10 and there are ∿518 nmoles isoleucine per mg protein (Table 4), so 0.1 mM isoleucine would be sufficient for protein synthesis. Furthermore, the requirement of a high concentration of leucine (11.5 mM) for maximal growth in medium B-10 did not correlate with leucine consumption (Table 6), suggesting that leucine might be the source of the contamination isoleucine. This was supported by the ability of C. sporogenes to grow in the presence of a low concentration of leucine provided that a small amount of isoleucine (0.1 mM) was added to the medium.

A very significant amount of label was incorporated into the isoleucine of the cell protein during growth in medium B-10 containing the non-cationic degradation products of L-[U-^{14}C]isoleucine (Table 20). The specific activity of the isoleucine fraction (144 dpm/nmole)

approached that obtained in cells labeled with $^{14}CO_2$, ^{14}C-serine, or ^{14}C-pyruvate (Table 14, 11, 16). However, the efficiency of label incorporation from the degradation product(s) based on the initial amount of label added to the culture was very high (9.5%) as compared to the efficiencies observed with labeled CO_2, serine or pyruvate (0.15-0.22%). The latter three substrates were subject to much more dilution in the growth medium than the catabolite(s) of labeled isoleucine.

An attempt was made to identify the degradation product of isoleucine by extraction and separation of the volatile fatty acid fraction obtained from isoleucine fermentation by C. sporogenes. As expected, the major fraction was found to be 2-methylbutyric acid (Table 21). Although the latter was not detected in the mid-log phase culture grown in medium B-10, this fact does not necessarily indicate its non-involvement in isoleucine synthesis. In fact, growth experiments showed that 2-methylbutyrate eliminated the requirement for both isoleucine and leucine during growth in medium B-10.

This finding suggested the synthesis of both isoleucine and leucine from 2-methylbutyrate, and C. sporogenes cells labeled for one generation with [U-^{14}C]2-methylbutyrate showed considerable incorporation of label into the cell protein. However, all of the label was recovered in the isoleucine fraction; none in leucine. It was estimated that no more than 3 carbons from 2-methylbutyrate could have been incorporated into isoleucine, and decarboxylation of the ^{14}C-isoleucine isolated released one third of the radioactivity. While this does not rule out isoleucine synthesis via reductive carboxylation of

2-methylbutyrate, obviously other reactions are involved. It is possible that some of the 2-methylbutyrate in the culture was degraded to release $^{14}CO_2$ and this would have been greatly diluted by the CO_2 in the medium. The culture was incubated in a 5% CO_2 atmosphere.

The accumulated evidence thus far suggests that C. sporogenes is able to synthesize isoleucine via an unusual pathway and the synthesis is not regulated by isoleucine. Pyruvate, CO_2, or 2-methylbutyrate alone probably cannot serve as the sole precursor of the carbon skeleton of isoleucine. This is supported by the finding that growth in medium B-10 can only be supported at low leucine concentrations when a small amount of isoleucine or a significant level of 2-methylbutyrate is present since both pyruvate and CO_2 are abundant under the growth conditions used. The incorporation of significant levels of ^{14}C from both [3-^{14}C]pyruvate and $^{14}CO_2$ into carbons other than the carboxyl group of isoleucine represents conclusive evidence that isoleucine synthesis in C. sporogenes can occur by mechanisms other than simple reductive carboxylation of 2-methylbutyrate. Obviously, further studies will be required to resolve this question. Studies of the extent of ^{14}C incorporation from ^{14}C-pyruvate and $^{14}CO_2$ in the presence and absence of 2-methylbutyrate should be helpful. Also, labeling of cellular isoleucine with specifically labeled substrates followed by stepwise degradation of the isoleucine formed should provide information regarding possible intermediates and reactions involved.

Growth data indicated that C. sporogenes could synthesize leucine when 2-methylbutyrate was added to the medium. Addition of this volatile fatty acid eliminated the requirement for leucine while substitution of isoleucine for leucine resulted in only an intermediate level of growth. However, a labeling experiment with ^{14}C-2-methylbutyrate showed that the latter volatile fatty acid is not a precursor of leucine. There is no ready explanation at this point as to how leucine is synthesized by C. sporogenes, but it would be of interest to determine the extent of ^{14}C incorporation from both ^{14}C-pyruvate and $^{14}CO_2$ into leucine in leucine-free medium supplemented with 2-methylbutyrate.

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