JOURNAL OF BACTERIOLOGY, June 1974, p. 935-941
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Vol. 118, No. 3
Printed in U.S.A.

Distribution of the Isopropylmalate Pathway to Leucine Among Diverse Bacteria

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Received for publication 21 March 1974

α-Isopropylmalate synthase and β-isopropylmalate dehydrogenase activities were detected in extracts of the following organisms: *Chromatium* D, *Rhodopseudomonas spheroides*, *Hydrogenomonas* H16, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Vibrio extorquens*, *Rhizobium japonicum*, *Alcaligenes viscolactis*, *Escherichia coli* B, *Proteus vulgaris*, *Aerobacter aerogenes*, *Salmonella typhimurium*, *Micrococcus* sp., *Micrococcus lysodeikticus*, *Bacillus polymyxa*, *Bacillus subtilis*, and *Nocardia opaca*. The α-isopropylmalate synthase activity in these extracts was inhibited by low concentrations of L-leucine. Taken together with other data, these results suggest that the isopropylmalate pathway is widespread among organisms that can synthesize leucine.

Leucine is synthesized in *Salmonella typhimurium* and *Neurospora crassa* by a pathway involving substituted isopropylmalates as intermediates (6; Fig. 1). This pathway is controlled, at least in part, by end-product inhibition, i.e., the activity of the initial enzyme in the pathway is inhibited by low concentrations of L-leucine (J. M. Calvo and H. E. Umbarger, Bacteriol. Proc., p. 126, 1963). This study is concerned with two questions relevant to comparative biochemistry. (i) Of those organisms that can synthesize leucine, how many of them utilize the isopropylmalate route. (ii) Is leucine biosynthesis regulated in these organisms and, if so, are the mechanisms different from those which are known to function in *S. typhimurium* and *N. crassa*?

Our studies suggest that the isopropylmalate route to leucine is probably the predominant one in bacteria. This conclusion is based upon the detection of α-isopropylmalate (α-IPM) synthase (α-isopropylmalate α-ketoisovalerate-lyase [coenzyme A acetylating], EC 4.1.3) and β-isopropylmalate (β-IPM) dehydrogenase (β-isopropylmalate:NAD oxidoreductase, EC 1.1.1) activities in extracts prepared from 18 bacteria representing 15 different genera. Moreover, the α-IPM synthase activity in extracts of 17 bacteria that were tested was inhibited by low concentrations of L-leucine.

MATERIALS AND METHODS

Bacteria. The bacteria studied, together with references to the minimal media in which they were cultured, are listed in Table 1. Medium for *Arthrobacter* sp. and *Flavobacterium* sp. contained (per liter of distilled water): KH_2PO_4, 1.2 g; K_2HPO_4, 4.8 g; NH_4NO_3, 500 mg; $MgSO_4$ heptahydrate, 200 mg; $FeCl_3$ hexahydrate, 25 mg; and $CaCl_2$ dihydrate, 25 mg. The pH was adjusted to 7.0, and citrate (0.2%) and glucose (0.1%) were added as carbon sources. Medium for *Nocardia opaca* contained (per liter of distilled water): K_2HPO_4, 2.5 g; KH_2PO_4, 1.0 g (adjusted to pH 7); $MgSO_4$, 100 mg; $CaCl_2$, 40 mg; L-asparagine, 5.0 g; $FeSO_4$ heptahydrate, 2 mg; $ZnSO_4$, 2 mg; $CuCl_2$ dihydrate, 2 mg; $MnCl_2$ tetrahydrate, 2 mg; and glucose to a final concentration of 0.2% *Veillonella alcalescens* cells, grown in a solution of 0.5% yeast extract, 0.5% tryptone, and 1% sodium lactate (pH 7.9), were a gift from E. A. Delwiche. *Clostridium histolyticum* and *Clostridium kluyveri*, both as dried cells, were purchased from Sigma Chemical Co. *Bacteroides ruminocola* cells were a gift from M. J. Allison. In most cases, cultures started

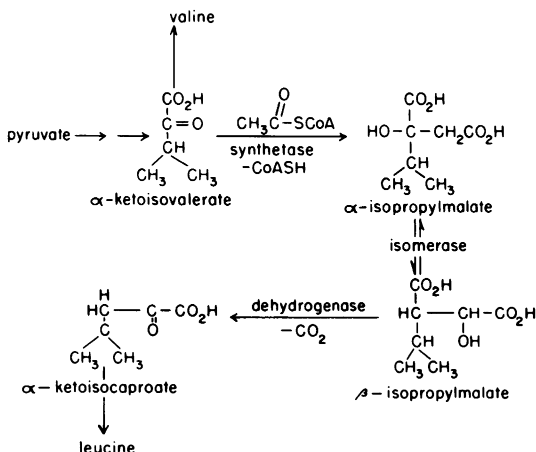

FIG. 1. *Biosynthetic pathway to leucine (isopropylmalate route).*

from a slant were used to inoculate 200 ml of minimal medium, and cells were harvested after growth to stationary phase. The temperature of incubation and the conditions of aeration were those recommended by others (see references in Table 1). *Chromatium* D and *Rhodopseudomonas spheroides* were grown anaerobically in 200-ml, glass-stoppered bottles at 30 C with a light source. *Staphylococcus aureus*, used in a control experiment, was grown in nutrient broth.

Preparation of extracts. Cells were harvested by centrifugation for 15 min at 3,500 × *g* at 6 C, washed once with 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0), and resuspended in 8 ml of the same buffer. *Ferrobacillus ferroxidans* was washed, by the procedure of Silverman et al. (24), to remove precipitated iron. Extracts from most bacteria were prepared by sonic oscillation with a Branson S110 Sonifier for four 15-s periods at 3 A with intermittent cooling, followed by centrifugation at 14,500 × *g* for 20 min to remove cell debris. *Micrococcus* sp. was disrupted in a Braun homogenizer with glass beads, and *Micrococcus lysodeikticus* was disrupted with lysozyme by the procedure of Herbert and Pinsett (12). All extracts were assayed immediately after preparation.

Enzyme assays. α-IPM synthase was assayed by the fluorometric method of Calvo et al. (8), using the acetyl coenzyme A-regenerating system that they describe. In this procedure, α-IPM formed enzymatically is determined as a highly fluorescent umbelliferone derivative. β-IPM dehydrogenase was assayed by determining the keto acid produced in the reaction by a modification of the procedure of Friedemann and Haugen (9). Reaction mixtures contained the following components in a total volume of 1 ml: Tris-hydrochloride (pH 8.0), 300 μmol; KCl, 100 μmol; $MnCl_2$, 1 μmol; nicotinamide adenine dinucleotide (NAD), 2 μmol; and β-IPM, 1 μmol. Reactions were started by the addition of NAD and terminated by the addition of 3 ml of 3.3×10^{-3} M 2,4-dinitrophenylhydrazine in 0.5 M HCl. After standing at room temperature for 15 min, the 2,4-dinitrophenyl hydrazone was extracted with 5 ml of ether. A 4-ml volume of ether extract was shaken with 4 ml of 10% (wt/vol) Na_2CO_3. A

TABLE 1. *Bacteria employed in this study*

Bacteria[a]	Family	Order	Source	Temp of growth (C)	Reference to minimal medium
Chromatium D	*Thiorhodaceae*	*Pseudomonadales*	A. J. Gibson	30	11
Rhodopseudomonas spheroides	*Athiorhodaceae*	*Pseudomonadales*	A. J. Gibson	30	14
Hydrogenomonas H16	*Methanomonadaceae*	*Pseudomonadales*	E. J. Ordal	30	4
Pseudomonas aeruginosa	*Pseudomonadaceae*	*Pseudomonadales*	P. J. VanDemark	37	17
P. fluorescens	*Pseudomonadaceae*	*Pseudomonadales*	P. J. VanDemark	25	17
Ferrobacillus ferrooxidans	*Siderocapsaceae*	*Pseudomonadales*	D. G. Lundgren	30	24
Vibrio extorquens	*Spirillaceae*	*Pseudomonadales*	P. K. Stocks	30	25
Azotobacter chroococcum	*Azotobacteraceae*	*Eubacteriales*	P. J. VanDemark	26	17
Rhizobium japonicum	*Rhizobiaceae*	*Eubacteriales*	M. Alexander	25	10
Alcaligenes viscolactis	*Achromobacteraceae*	*Eubacteriales*	P. J. VanDemark	25	21
Flavobacterium sp.	*Achromobacteraceae*	*Eubacteriales*	M. Alexander	30	
Escherichia coli B	*Enterobacteriaceae*	*Eubacteriales*	R. Alexander	37	17
Proteus vulgaris	*Enterobacteriaceae*	*Eubacteriales*	P. J. VanDemark	37	17[b]
Aerobacter aerogenes	*Enterobacteriaceae*	*Eubacteriales*	P. J. VanDemark	37	17
Salmonella typhimurium LT2	*Enterobacteriaceae*	*Eubacteriales*	P. Margolin	37	17
Bacteroides ruminocola	*Bacteroidaceae*	*Eubacteriales*	M. J. Allison	37	
Micrococcus sp.	*Micrococcaceae*	*Eubacteriales*	I. J. McDonald	25	16
M. lysodeikticus	*Micrococcaceae*	*Eubacteriales*	H. B. Naylor	30	32
Veillonella alcalescens	*Neisseriaceae*	*Eubacteriales*	E. A. Delwiche	37	
Bacillus megaterium	*Bacillaceae*	*Eubacteriales*	P. J. VanDemark	30	13
B. polymyxa	*Bacillaceae*	*Eubacteriales*	P. J. VanDemark	30	13
B. subtilis	*Bacillaceae*	*Eubacteriales*	P. J. VanDemark	37	13
Clostridium histolyticum	*Bacillaceae*	*Eubacteriales*	Commercial		
C. kluyveri	*Bacillaceae*	*Eubacteriales*	Commercial		
Arthrobacter sp.	*Corynebacteriaceae*	*Eubacteriales*	M. Alexander	25	
Mycobacterium phlei	*Mycobacteriaceae*	*Actinomycetales*	P. J. VanDemark	37	28
Nocardia opaca	*Actinomycetaceae*	*Actinomycetales*	R. E. MacDonald	30	
Streptomyces violaceoruber	*Streptomycetaceae*	*Actinomycetales*	G. S. Bradley	30	5

[a] Nomenclature from *Bergey's Manual of Determinative Bacteriology* (7th ed., 1957).
[b] The minimal medium was supplemented with 0.5% nutrient broth.

3-ml volume of the aqueous phase was added to 0.5 ml of 20% (wt/vol) KOH, and the absorbance was read at 510 nm in a Bausch and Lomb Spectronic 20. A standard curve prepared from α-ketoisocaproate was linear to at least 1 μmol of the keto acid.

A product of each reaction was determined after incubation for 15 min, and 1, 2, and 3 h for β-IPM dehydrogenase and 5, 15, 30, and 60 min for α-IPM synthase. The incubation temperature for each assay was the same as the temperature at which the organism was grown (Table 1). Control tubes for each time period lacked one of the substrates: β-IPM and α-ketoisovalerate for the dehydrogenase and synthase assays, respectively. Enzyme activity (micromoles of product formed per hour) was calculated from the initial linear portion of a product-versus-time curve. Protein was determined by the method of McDonald and Chen (15) with lysozyme as a standard.

In assays in which end-product inhibition was studied, reaction mixtures contained L-leucine (10^{-2} to 10^{-5} M) in addition to the standard components. α-IPM formation was determined after incubation for 30 min. The velocity was determined to be linear for at least 30 min for all extracts studied.

RESULTS

The results of the β-IPM dehydrogenase assays are shown in Table 2. Extracts of 20 organisms that were studied catalyzed the formation of a keto acid in a reaction that was dependent upon the presence of β-IPM. We assume that the keto acid formed was α-ketoisocaproate; however, this has not been established except in experiments in which *S. typhimurium* extracts were used (6). A consideration of some importance to this study is the specificity of the catalyst in these extracts. For example, some extracts might contain a nonspecific dehydrogenase capable of converting β-IPM to a keto acid. It is difficult to disprove this hypothesis in individual cases, but control experiments at least indicate that such a hypothetical catalyst is not widely distributed. Fresh extracts of guinea pig kidney, *S. aureus*, and *S. typhimurium leuBCD39* did not catalyze the formation of a keto acid from β-IPM. The latter strain has a multisite mutation that covers the cistron known to specify β-IPM dehydrogenase (17). The other two organisms are known to require leucine for growth and thus were not expected to have leucine biosynthetic enzymes.

TABLE 2. *β-IPM dehydrogenase activity in diverse bacteria*

Organism	Sp act of β-IPM dehydrogenase[a]
Chromatium D (organic medium)	2.08
Chromatium D (inorganic medium)	0.76
Rhodopseudomonas spheroides (anaerobic)	4.93
R. spheroides (aerobic)	3.65
Hydrogenomonas H16	2.71
Pseudomonas aeruginosa	5.27
P. fluorescens	6.02
Vibrio extorquens	2.19
Azotobacter chroococcum	1.72
Rhizobium japonicum	2.16
Alcaligenes viscolactis	3.45
Escherichia coli B	11.56
Proteus vulgaris	19.00
Aerobacter aerogenes	6.36
Salmonella typhimurium LT2	7.39
Micrococcus sp.	2.80
M. lysodeikticus	3.33
Bacillus megaterium	1.54
B. polymyxa	0.55
B. subtilis	0.37
Arthrobacter sp.	1.06
Nocardia opaca	3.77

[a] Micromoles of product per hour per milligram of protein.

In addition to the organisms listed in Table 2, β-IPM dehydrogenase activity was found in *F. ferroxidans*, *Flavobacterium* sp., *Mycobacterium phlei*, and *Streptomyces violaceoruber* (specific activity: 1.50, 1.53, 1.33, 3.20 μmol per h per mg of protein, respectively). No further work was done with these strains.

Extracts prepared from the anaerobic organisms *C. histolyticum*, *C. kluyveri*, *V. alcalescens*, and *B. ruminocola* had no detectable dehydrogenase activity when assayed by the standard procedure. Furthermore, there was no indication of the presence of inhibitors in these extracts because, when mixed with a *Salmonella* extract, no depression of activity was observed. However, slight activity was detected in extracts of *B. ruminocola* when the assay was modified in the following way. Sonic oscillation and assays were carried out under argon; the buffer contained 1.6 mM dithiothreitol; and frozen cells were resuspended and sonically treated directly in the reaction mixture. Under these conditions, β-IPM dehydrogenase activities of 0.33 and 0.44 μmol per h per mg of protein were observed for cells grown with and without isovalerate, respectively.

The results of α-IPM synthase assays are shown in Table 3. Under the conditions described above (Tris buffer, disruption by sonic oscillation), α-IPM synthase activity was detected in extracts of the following 13 strains: *R. spheroides*, *Hydrogenomas* H16, *P. aeruginosa*, *P. fluorescens*, *Rhizobium japonicum*, *Alcaligenes viscolactis*, *Escherichia coli B*, *Proteus vulgaris*, *Aerobacter aerogenes*, *S. typhimurium* LT2, *Micrococcus* sp., *Bacillus subtilis*, and *N. opaca*. The other strains in

Table 3 showed negligible activity when assayed under the same conditions. Extracts of *Chromatium* D and *M. lysodeikticus*, however, had good α-IPM synthase activity when potassium phosphate was used as buffer and breakage was effected by X-press (Biotech. Inc., New York) and lysozyme treatment, respectively. Extracts of *V. extorquens*, *Arthrobacter* sp., and *B. polymyxa* had low but detectable α-IPM synthase activity when Tris was replaced by phosphate buffer and, for the first two, when cells were disrupted by an X-press. α-IPM synthase activity was at the borderline of detectability in extracts of *A. chroococcum* or *B. megaterium* under several conditions of assay (Table 3). These same extracts did possess β-IPM dehydrogenase activity, however.

Several attempts were made to detect α-IPM synthase activity in extracts of *B. ruminicola*. No activity was detected, even when sonic treatment was carried out under argon in the presence of substrates (Tris or phosphate buffers). Extracts prepared from *B. ruminicola* did not inhibit synthase activity within *Salmonella* extracts.

In summary, extracts of 18 strains possessed activity presumed to be due to α-IPM synthase. α-Isopropylmalate was not actually identified as a product in any of the reaction mixtures. However, it is very likely that the product measured was indeed α-IPM because the assay employed is relatively specific for substituted malic acid derivatives (8) and the activity demonstrated in each case was dependent upon the presence of α-ketoisovalerate. In addition, the leucine inhibition studies described below suggest that the activity in question is catalyzed by α-IPM synthase.

α-IPM synthase from *S. typhimurium* and *N. crassa* is known to be inhibited by low concentrations of L-leucine (Calvo and Umbarger, Bacteriol. Proc., p. 126, 1963; 31). The activity of α-IPM synthase in crude extracts of the strains listed in Table 3 was determined as a function of L-leucine concentration (Table 3, Fig. 2). In all cases, the addition of L-leucine caused a significant decrease in activity. That this decrease was not caused by α-amino acids in general was demonstrated by parallel experiments in which L-alanine (10^{-3} M) replaced

TABLE 3. *Specific activity of, and effect of, leucine upon α-IPM synthase from various bacteria*

Organism	Sp act[a] α-IPM synthase	Concn (M) of leucine for:		
		10% Inhibition	50% Inhibition	90% Inhibition
Chromatium D (inorganic medium)	1.03[bc]	2.5×10^{-4}	6.0×10^{-4}	$>10^{-2}$ [d]
Rhodopseudomonas spheroides (aerobic)	1.28	2.5×10^{-4}	7.0×10^{-4}	$>10^{-2}$ [d]
R. spheroides (anaerobic)	0.80	2.5×10^{-4}	7.0×10^{-4}	$>10^{-2}$ [d]
Hydrogenomonas H16	0.26	5.0×10^{-5}	4.6×10^{-4}	1.4×10^{-3}
Pseudomonas aeruginosa	0.69	3.5×10^{-4}	6.0×10^{-4}	1.2×10^{-3}
P. fluorescens	0.65	5.0×10^{-5}	3.3×10^{-4}	9.3×10^{-4}
Vibrio extorquens	0.05[bc]	2.0×10^{-5}	7.0×10^{-5}	$>10^{-2}$ [d]
Azotobacter chroococcum	<0.01[e]			
Rhizobium japonicum	1.72	1.5×10^{-4}	5.5×10^{-4}	$>10^{-2}$ [d]
Alcaligenes viscolactis	6.52	1.2×10^{-4}	2.7×10^{-4}	5.3×10^{-4}
Escherichia coli B	0.85	8.0×10^{-5}	2.8×10^{-4}	9.0×10^{-4}
Proteus vulgaris	0.96	2.0×10^{-5}	1.4×10^{-4}	6.0×10^{-4}
Aerobacter aerogenes	1.22	8.0×10^{-5}	2.1×10^{-4}	4.7×10^{-4}
Salmonella typhimurium LT2	0.43	4.0×10^{-5}	1.2×10^{-4}	2.0×10^{-4}
Micrococcus sp.	0.53	5.0×10^{-6}	8.0×10^{-5}	5.8×10^{-4}
Micrococcus lysodeikticus	0.27[b]	2.0×10^{-6}	3.3×10^{-5}	8.5×10^{-4}
Bacillus megaterium	0.013[f]			
Bacillus polymyxa	0.04	1.0×10^{-5}	5.0×10^{-5}	7.0×10^{-4}
B. subtilis	0.28	2.5×10^{-5}	5.0×10^{-4}	1.8×10^{-3}
Arthrobacter sp.	0.06[bc]			
Nocardia opaca	1.64	6.0×10^{-5}	1.0×10^{-4}	2.5×10^{-4}

[a] Specific activity: Micromoles of product per hour per milligram of protein. Those values lacking a footnote notation were obtained under conditions described in Materials and Methods.

[b] Extracts were prepared and assayed in 0.05 M phosphate buffer (pH 6.8) instead of Tris buffer.

[c] Cells were broken by means of an X-press.

[d] Ninety percent inhibition was not reached at the highest concentration of leucine tested (10^{-2} M).

[e] Extracts were prepared (twice by sonic oscillation and once with an X-press) and assayed in Tris buffer.

[f] Extracts were prepared (sonic oscillation) and assayed in both Tris and phosphate buffers.

L-leucine; in no instance did L-alanine inhibit activity. The curves in Fig. 2 represent examples of three types of inhibition patterns that were observed. The first included those curves that approached 100% inhibition at high leucine concentrations and which rose very steeply over a narrow leucine concentration range (inhibition increased from 10% to 90% with a less than 10-fold increase in leucine concentration). Such curves were observed for *P. aeruginosa*, *A. viscolactis*, *E. coli*, *A. aerogenes*, *S. typhimurium*, and *N. opaca*. The second included those curves that approached 100% inhibition at high leucine concentrations but that rose from 10% to 90% inhibition over a wider (25- to 300-fold) leucine concentration range. Such curves were observed for *Hydrogenomonas* H16, *P. fluorescens*, *P. vulgaris*, *Micrococcus* sp., *M. lysodeikticus*, *B. polymyxa*, and *B. subtilis*. The third included those curves (four examples) that did not approach 100% inhibition at a high (10^{-2} M) leucine concentration. The curve for one such case, *R. japonicum*, is shown in Fig. 2. The curves for *Chromatium* D and *R. spheroides* were almost identical and reached 55% inhibition at 10^{-2} M leucine. The activity in extracts of *V. extorquens* was inhibited 70% at 10^{-2} M leucine.

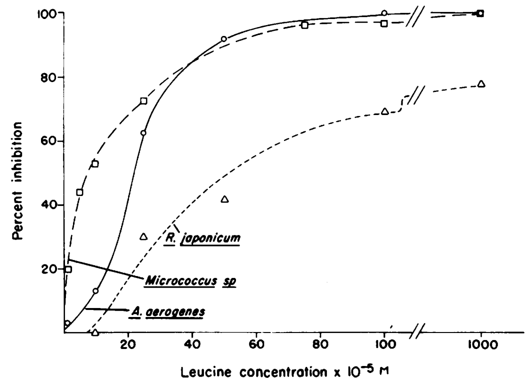

FIG. 2. *Inhibition of α-IPM synthase from crude extracts of several strains as a function of leucine concentration.*

DISCUSSION

Studies of amino acid biosynthesis have generally supported the notion of a "unity of biochemical reactions" throughout the biological world (19, 29). Lysine biosynthesis represents the most notable exception. The comparative study of the distribution of this pathway has contributed valuable information concerning the evolutionary relationships between groups of organisms (30). In higher fungi and those phycomycetes possessing posteriorly uniflagellate spores, lysine synthesis occurs by a pathway involving α-aminoadipic acid as an intermediate. In almost all other organisms studied, including bacteria, blue-green and green algae, and higher plants, a completely different pathway operates involving diaminopimelic acid (30). Thus far, no organism has been found that possesses both the diaminopimelate and the aminoadipate pathways.

However, in many instances, studies of amino acid biosynthesis have been limited to relatively few organisms, attention generally being focused on *E. coli*, *N. crassa*, and several mammals. One of the purposes of this study was to survey a number of microorganisms to determine the distribution of the isopropylmalate pathway. The results reported here support the hypothesis that the majority of bacteria that can synthesize leucine do so by the isopropylmalate route. Extracts of 18 strains of bacteria had β-IPM dehydrogenase and α-IPM synthase activities, the latter being inhibited by low concentrations of leucine. Although this observation is not direct evidence that the isopropylmalate route is functioning in each case, the indication that these organisms have both the capacity to produce leucine and the ability to control that production make it likely that such is the case.

Besides the organisms mentioned above, the isopropylmalate pathway has been firmly established in *Saccharomyces cerevisiae* (23, 26), *N. crassa* (6), *Hydrogenomonas H16* (F. F. Hill, Ph.D. thesis, Univ. of Göttingen, Germany, 1968), and maize (20). It is the only pathway to leucine in *S. cerevisiae*, *N. crassa*, *Hydrogenomonas* H16, *S. typhimurium*, and *E. coli* because leucine auxotrophs derived from these organisms have been shown to lack one or more of the leucine-forming enzymes. In addition, there is relatively good evidence for the isopropylmalate pathway in *Torulopsis utilis* (27), *Aspergillus fumigatus* (18), *Streptomyces erythreus* (18), *Nocardia lurida* (18), *P. aeruginosa* (22), and sorghum and flax (7).

Thus, it seems clear that the isopropylmalate pathway is widely distributed in nature. There is some evidence that leucine can be formed in certain rumen bacteria from isovaleric acid. Allison et al. determined the labeling pattern in leucine isolated from *Ruminococcus flavefaciens* after the organism was incubated with [^{14}C]isovaleric acid. The fact that most of the label in leucine was in carbon 2 when [1-^{14}C]isovalerate was administered is not easily reconciled with a synthetic route through isopropylmalate (1). *Bacteroides ruminicola* and *Peptostreptococcus elsdenii* are also able to

convert isovalerate to leucine (2). These organisms are adapted to an environment that contains relatively high concentrations of branched-chain acids, including isovalerate, and for this reason may have evolved the capacity to synthesize leucine by reductive carboxylation of isovalerate to α-ketoisocaproate followed by transamination (3). It is not yet clear whether these organisms use the isopropylmalate route when growing in the absence of isovalerate.

The conclusions which can be drawn from the magnitude of the specific activities in Tables 2 and 3 are limited because the optimal conditions of temperature, pH, and ionic strength for the assay of each extract are not known. However, under the conditions that are optimal for the assay of α-IPM synthase and β-IPM dehydrogenase from *S. typhimurium*, the specific activities of these enzymes in extracts of other organisms seem sufficiently high in most cases to account for leucine biosynthesis. For *S. typhimurium*, which has a generation time of 46 min in our minimal medium, the specific activity of α-IPM synthase and β-IPM dehydrogenase was 0.43 and 7.3 μmol per h per mg, respectively. Other strains with similar growth rates had comparable specific activities. Two of the *Bacillus* species, however, had much lower dehydrogenase specific activities than might be predicted from their growth rates. Whether this was the result of sub-optimal assay conditions or unstable enzymes, or whether this portends a second pathway is not known.

ACKNOWLEDGMENTS

This investigation was supported by grant GB-7636 from the National Science Foundation and by Public Health Service Training Grant 5 T01 GM00824-07 from the National Institutes of General Medical Sciences.

We thank those investigators who supplied strains and, in particular, R. Z. Korman, M. J. Allison, B. A. Hart, and J. D. Nelson for cells and extracts of several strains. We are indebted to Susan Jones for technical assistance and to R. E. MacDonald and M. J. Allison for helpful suggestions.

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