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Biosynthesis of Isoleucine in Methanogenic Bacteria: A ^{13}C NMR Study†

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ABSTRACT: The biosynthesis of isoleucine by seven species of methanogenic bacteria was studied by using ^{13}C NMR, following growth in media containing specifically ^{13}C-labeled compounds. It was found that in these bacteria α-ketobutyrate is not derived from threonine. In all cases, when acetate and CO_2 serve as the total carbon sources, the labeling pattern of isoleucine is consistent with the operation of the pyruvate pathway (condensation of pyruvate with acetyl coenzyme A to form α-ketobutyrate). When propionate or 2-methylbutyrate is added to the medium, alternative pathways contribute to isoleucine biosynthesis. Two additional pathways are postulated: carboxylation of propionate to α-ketobutyrate and conversion of 2-methylbutyrate to isoleucine probably by a carboxylation reaction.

In addition to the standard pathway, in which α-ketobutyrate is derived from threonine, microorganisms have developed several alternative pathways for the synthesis of isoleucine (Figure 1). α-Ketobutyrate can be derived from homoserine (Flavin & Segal, 1964), from glutamate via β-methylaspartate (Phillips et al., 1972), from pyruvate by chain elongation (Vollbrecht, 1974; Charon et al., 1974; Westfall et al., 1983; Kisumi et al., 1977), and from propionate by carboxylation (Sauer et al., 1975; Buchanan, 1969). In some cases, when there is an abundance of volatile fatty acids in the medium, 2-methylbutyrate can be carboxylated directly to isoleucine by some anaerobic bacteria from the rumen (Robinson & Allison, 1969).

Methanogens are members of a recently discovered third kingdom designated the Archaebacteriae (Woese, 1982). Because a number of structural and biochemical features are already known to separate the methanogens from the eubacterial and eukaryotic lines of evolutionary descent, differences in metabolic pathways are likely to occur as well. Metabolic studies have special relevance because methane, a fuel gas, is the major excretion product of metabolism, and a common intermediate may be shared between methanogenic and anabolic pathways (Kenealy & Zeikus, 1982). Metabolic control mechanisms have yet to be explored. In a previous paper (Ekiel et al., 1983), it was concluded from ^{13}C labeling patterns that isoleucine in *Methanospirillum hungatei* (*Msp. hungatei*)[1]

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[1] Abbreviations: *Msp.*, *Methanospirillum*; *Ms.*, *Methanosarcina*; *Mbr.*, *Methanobrevibacter*; *Mc.*, *Methanococcus*; *Mb.*, *Methanobacterium*; CoA, coenzyme A.

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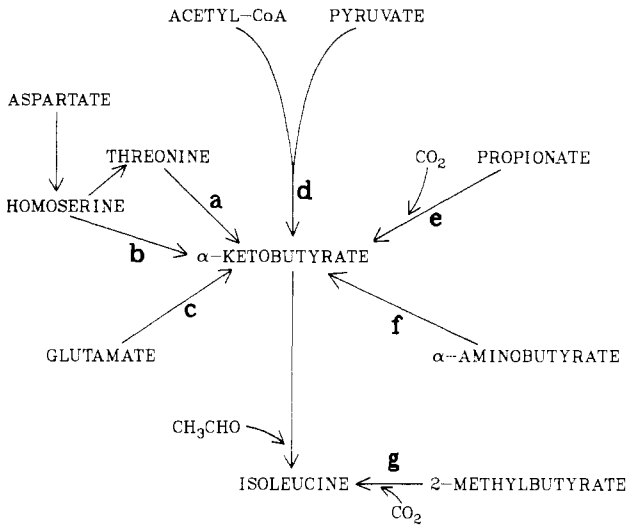

FIGURE 1: Pathways of isoleucine biosynthesis used by microorganisms. This abbreviated scheme does not attempt to illustrate amino transfer reactions.

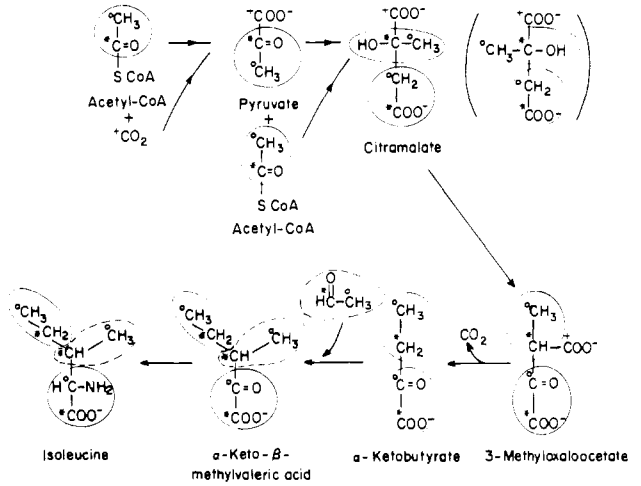

FIGURE 2: Labeling pattern of isoleucine synthesized via the pyruvate pathway. Encircled are molecular fragments derived from acetate units.

GP1 cannot be synthesized from threonine. The observed labeling pattern of isoleucine is shown in Figure 2: C-β, C-γ, and the carboxyl group are labeled from [1-^{13}C]acetate, whereas C-α and both methyl groups are derived from [2-^{13}C]acetate. Such labeling does not correspond to that found in aspartate or glutamate (Ekiel et al., 1983), so pathways b and c (Figure 1) can also be excluded. The only known route that can explain the observed labeling pattern is the pyruvate pathway (Westfall et al., 1983), in which α-ketobutyrate is derived from pyruvate by α-keto acid chain elongation (see Figure 2). In fact, two different pathways are possible, since condensation of pyruvate with acetyl-CoA can give L- or D-citramalate (Charon et al., 1974).

In this study, the labeling pattern of isoleucine in methanogens is explored more closely by using [1,2-$^{13}C_2$]acetate. In addition to *Msp. hungatei*, several other methanogenic bacteria were studied to determine how widespread this unusual pathway is among the methanogenic members of the Archaebacteriae. Six additional methanogens are included: *Ms. barkeri*, *Mbr. smithii*, *Mc. voltae*, *Mbr. arboriphilus*, *Mb. bryantii*, and *Methanobacterium* strain G2R. These bacteria, together with *Msp. hungatei*, represent three major phylogenetic groups (orders) of methanogenic bacteria (Taylor, 1982). All the methanogens studied can grow on media containing acetate and CO_2 as carbon sources, and they incorporate both these compounds into cellular material. However, in anaerobic ecosystems where the nutrient supply is more varied, compounds other than acetate and CO_2 could be used for the biosynthesis of isoleucine. In view of this, some studies were performed on the utilization of propionate and 2-methylbutyrate to test the possibility of the operation of pathways e and g from Figure 1.

Materials and Methods

The preparation of the prereduced basal medium JM and anaerobic manipulations have been described (Jarrell & Sprott, 1982; Ekiel et al., 1983). The gas atmosphere during growth was always H_2/CO_2 (4:1 v/v). In the case of *Mc. voltae*, the complex medium of Whitman et al. (1982) was used. Fatty acids and ^{13}C label additions to the sterile medium were made as filter-sterilized solutions. Additions for each 1-L growth bottle were weighed into 120-mL serum bottles, the bottles were then flushed with CO_2/H_2 (20:80 v/v), and 4–5 mL of prereduced JM medium was injected through the serum stopper by using a disposable plastic syringe gassed previously with CO_2/H_2. This solution was then drawn into a similar syringe, a sterile syringe filter unit which had been preflushed with CO_2/H_2 was attached (Sartorius, 0.2-μm pore size), and the syringe contents were injected into the growth bottle. Final concentrations of reagents are reported under Results and Discussion.

Each amino acid preparation required 100 mL of cells grown in a 1-L storage bottle with CO_2/H_2 (1:4 v/v) as the gas phase. Cells in the late exponential growth phase (2–3 days with 10% inoculum) were harvested aerobically with yields of 40–50 mg, dry weight. The cells were broken and fractionated as described in detail previously (Ekiel et al., 1983). Cytoplasmic proteins, precipitated with 60% ethanol at 4 °C, were used as a source of amino acids. Proteins were hydrolyzed for 30–48 h in 6 M HCl under vacuum at 110 °C. In some cases, the amino acids were partially separated by thin-layer chromatography on cellulose plates. All details have been published elsewhere (Ekiel et al., 1983).

NMR spectra were run on a Bruker CXP-300 spectrometer (75.47 MHz for ^{13}C), using quadrature detection, 50° pulses, 2-s recycle times, and gated decoupling. Under these conditions, quaternary carbons were still partially saturated. Usually 1000–10 000 scans were accumulated. All spectra were run in 0.1 M HCl solution in D_2O, at room temperature. A sealed capillary containing tetramethylsilane was used as a reference.

The contributions of various volatile fatty acids to the biosynthesis of isoleucine were quantitated as follows. A spectrum of the protein hydrolysate of the culture from medium containing [2-^{13}C]acetate (A) and a spectrum for the culture from medium containing [2-^{13}C]acetate and volatile fatty acid (A^+) were acquired with 80° pulses and a long (10-s) delay. Ratios of the signal intensities of A and A^+ were determined for each peak and found to be the same for all except those of isoleucine. Thus, the two spectra A and A^+ could be normalized to each other by using the average ratio of signal intensities (excluding those of isoleucine). Following normalization, the ratio of the peak intensities (A and A^+) for isoleucine carbons gives the contribution to isoleucine biosynthesis of the pyruvate pathway (in the case of C-α and C-δ) or the pyruvate pathway plus that starting from propionate (in the case of the methyl carbon C-γ'). Signal intensities were measured by computer integration using a curve-fitting program from the standard Nicolet software. ^{13}C-Labeled sodium acetate and sodium propionate were from Merck Sharp &

Dohme with an enrichment level of 90%.

Results and Discussion

Biosynthesis of Isoleucine from Acetate. Among the seven methanogenic bacteria included in this study, six were grown on acetate (12.0 mM) and CO_2 as the sole carbon sources: *Ms. barkeri*, *Mbr. arboriphilus*, *Mb. bryantii* strain *M.o.H.*, *Msp. hungatei* GP1, *Mb.* strain G2R, and *Mbr. smithii*. The sources of these cultures have been described (Sprott & Jarrell, 1982). *Mc. voltae* was grown on undefined medium supplemented with acetate. It has been reported (Whitman et al., 1982) that isoleucine (and leucine) is required in the medium to support growth of *Mc. voltae*. In agreement with this report, we have not observed any incorporation of acetate into isoleucine or leucine in this methanogen. Since 2-methylbutyrate substituted for isoleucine in supporting growth, and since most of its label was incorporated into protein, it is likely that *Mc. voltae* can synthesize isoleucine from 2-methylbutyrate (Whitman et al., 1982). All six other methanogens synthesized isoleucine from acetate, and the observed labeling patterns were identical with those previously found in *Msp. hungatei* (see Figure 2); both methyl groups and C-α are labeled from the C-2 of acetate, whereas C-β and the methylene C-γ (and in part the carboxyl group, as later discussed) are from the C-1 of acetate. This shows that they all use the same pathway, which is different from the standard route via threonine.

The α-carbon of isoleucine, which gives a resonance at 57.4 ppm in the ^{13}C NMR spectra, is particularly useful in detecting any contribution of the threonine pathway—it does not overlap with other signals, so that any label in this position can be detected without separation of amino acids in the protein hydrolysate. This carbon should be labeled from [1-^{13}C]-acetate if the threonine pathway operates (Ekiel et al., 1983); in all seven methanogens studied, there was no specific labeling of this carbon from C-1 of acetate. However, in all methanogens except *Msp. hungatei* and *Mb. bryantii*, some overall scrambling of label was observed. This scrambling results in a partial labeling of the positions directly derived from C-1 by the C-2 of acetate and vice versa (unpublished results). Importantly, the labeling of C-α of isoleucine from [1-^{13}C]-acetate was not higher than expected from such scrambling. These results suggest either that methanogenic bacteria do not have an active threonine dehydratase or that the threonine pathway contributes only negligibly (<5%) to the biosynthesis of isoleucine.

This conclusion is supported by several reports on the atypical biosynthesis of isoleucine in methanogenic bacteria: *Mbr. ruminantium* strain M1 requires 2-methylbutyrate, which is probably carboxylated directly to isoleucine (Robinson & Allison, 1969). An atypical pathway was also suggested for the mixed culture *Methanobacterium omelianskii* (Knight et al., 1966), although in this case it could represent metabolism of the nonmethanogenic "S" organism (Taylor, 1982).

Pathways using volatile fatty acids could be used (e or g in Figure 1) in those cases where rumen fluid is included in the growth medium, but when CO_2 and acetate are the only carbon sources in the media, the pyruvate pathway seems to be dominant. For other microorganisms, it has been suggested that the pyruvate pathway for isoleucine biosynthesis shares, in part, the same system for enzymes used for the synthesis of leucine (Charon et al., 1974; Kisumi et al., 1977). The same could also be true for methanogenic bacteria, where a defect in this shared enzyme system could explain the auxotrophic nature of *Mc. voltae* for *both* isoleucine and leucine.

Incorporation of Doubly Labeled Acetate. Growth on doubly labeled acetate can give information about the incorporation of whole acetate units, as required to provide strong support of the pathway shown in Figure 2. Such studies were performed for *Mb.* strain G2R, *Ms. barkeri*, and *Msp. hungatei* GP1. For these last bacteria, dilution of [1,2-$^{13}C_2$]acetate with unlabeled acetate in the growth media was necessary to avoid complex multiplets in the ^{13}C NMR spectra. In Figure 3, a spectrum is shown of the branched-chain amino acids and of phenylalanine from *Msp. hungatei* GP1 grown on [1,2-$^{13}C_2$]acetate. Carbon atoms originating from one acetate molecule should give a pair of doublets, but if the acetate unit is broken during biosynthesis, single lines are expected (because of dilution with unlabeled acetate). It can be seen that both methyl groups of isoleucine give signals which are doublets and that more than half of the intensity of C-α is in a doublet (the origin of the rest of the C-α intensity will be discussed below). The coupling constant to C-α is 57 Hz, typical of one-bond C–C coupling to a carboxyl group (Sogn et al., 1974). Spectra similar to that in Figure 3 were obtained for *Mb.* strain G2R and *Ms. barkeri*, with the difference that more scrambling was observed and that the central signal was stronger. Double labeling experiments clearly show that the isoleucine molecule is essentially composed from three acetate units, which are encircled in Figure 2. This further supports the operation of the pyruvate pathway.

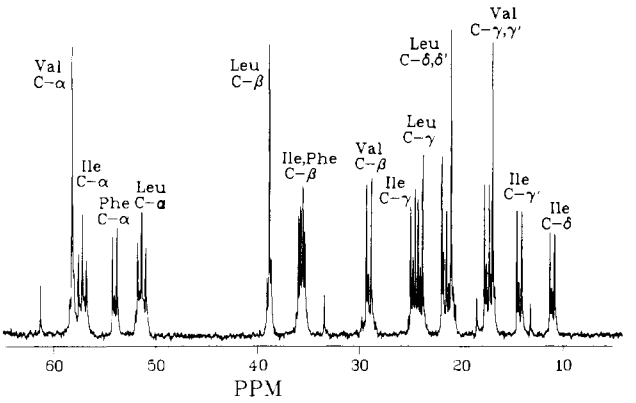

FIGURE 3: ^{13}C NMR spectrum of an isoleucine-containing amino acid fraction separated by thin-layer chromatography from a protein hydrolysate of *Msp. hungatei* GP1 grown on [1,2-$^{13}C_2$]acetate. Cells were grown in JM medium containing 2.0 mM [1,2-$^{13}C_2$]acetate diluted with 7.7 mM sodium acetate.

The presence of the single line in the center of the C-α signal of isoleucine is not consistent with the unique occurrence of the scheme shown in Figure 2 and suggests either that a second mechanism contributes to isoleucine biosynthesis or that there is an exchange of the carboxyl group with CO_2. This second possibility seems to be more probable. Note in Figure 3 that the same structure is present for the C-α signal of leucine, which is labeled as expected for the typical biosynthetic pathway from α-acetolactate (Ekiel et al., 1983). Carboxyl group exchange reactions are known to exist in the case of α-keto acids (e.g., pyruvate and α-ketoglutarate) (Zeikus et al., 1977) so they could be active also in the case of 2-keto-isocaproate and α-ketobutyrate (or 2-keto-3-methylvalerate). Such reactions could be catalyzed by enzymes carboxylating volatile fatty acids. In the case of *Msp. hungatei*, although we have not studied the possibility of carboxylation of isovalerate, both propionate and 2-methylbutyrate are easily converted to α-ketobutyrate and isoleucine, respectively (see below).

Biosynthesis of Isoleucine from Propionate and 2-Methylbutyrate. Since there are suggestions in the literature that propionate and 2-methylbutyrate can be used by bacteria as the precursors of isoleucine (pathways e and g in Figure

Table I: Percent Contribution from Various Volatile Fatty Acids to the Biosynthesis of Isoleucine[a]

contributing fatty acid	supplement in the medium: none	propionate[b] *Mb.*[f]	propionate[b] *Msp.*	propionate[b] *Mbr.*	butyrate[c] *Mb.*	2-methylbutyrate[d] *Mb.*	2-methylbutyrate[d] *Msp.*	2-methylbutyrate[d] *Mbr.*	propionate, butyrate, and 2-methylbutyrate[e] *Mb.*
acetate	100	27	35	25	100	14	10	25	13
propionate		73	65	75					15
2-methylbutyrate						86	90	75	72

[a] Percent contributions were calculated from intensities of ^{13}C NMR signals as described under Materials and Methods. All media contained 12.2 mM sodium acetate (enriched 90% in ^{13}C). [b] Propionate concentration was 2.5 mM. [c] Butyrate concentration was 2.5 mM. [d] 2-Methylbutyrate concentration was 2.1 mM. [e] The following concentrations were used: propionate, 6.8 mM; butyrate, 7.2 mM; 2-methylbutyrate, 0.6 mM. [f] *Mb. bryantii*, *Msp. hungatei*, and *Mbr. smithii*.

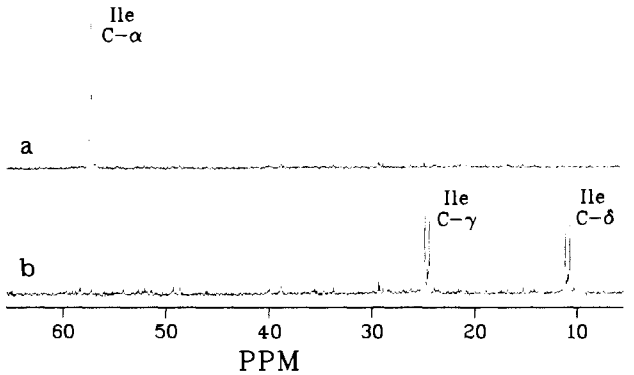

FIGURE 4: ^{13}C NMR spectra of hydrolyzed cytoplasmic proteins from *Msp. hungatei*: (a) 2.2 mM [1-^{13}C]propionate present in the growth medium containing 2.0 mM sodium acetate; (b) 2.0 mM [2,3-$^{13}C_2$]propionate added to the medium containing 5.0 mM sodium acetate.

1), we decided to determine if such pathways can be utilized by methanogenic bacteria. Methanogens are exposed to volatile fatty acids, produced by part of the microbial flora during the anaerobic digestion of complex organic substrates (Taylor, 1982). Especially in the rumen, because of the presence of high concentrations of volatile fatty acids (Annison, 1954), the operation of pathways e and g (Figure 1) might be expected to occur. For these studies, the following three species have been chosen: *Msp. hungatei* GP1, *Mb. bryantii*, and *Mbr. smithii*.

The data shown in Figure 4 illustrate how amino acids are labeled from C-1 (a) and C-2 and C-3 (b) of propionate in *Msp. hungatei*. The C-1 of propionate is selectively incorporated into C-α of isoleucine (δ 57.66) and C-2 and C-3 into C-γ and C-δ (24.76 and 11.00 ppm, respectively). This is exactly the labeling expected if propionate were directly carboxylated to α-ketobutyrate. No significant amount of label is incorporated into other amino acids; their signals have intensities of about 1.5%–3% of that of labeled isoleucine, which is close to the ratio expected for natural abundance. This shows that, except for direct conversion to α-ketobutyrate, propionate is not utilized by other pathways (e.g., through part of the tricarboxylic acid pathway). Incorporation of [1-^{13}C]propionate into amino acids was also studied for *Mb. bryantii*, with the same results as for *Msp. hungatei* GP1: the C-1 of propionate was specifically incorporated into the C-α position of isoleucine.

Carboxylation of propionic acid to α-ketobutyrate was found in certain nonmethanogenic rumen bacteria (Sauer et al., 1975), which encounter large amounts of propionate in their medium. Thus, it would not be surprising to find this reaction in rumen methanogens. However, neither *Msp. hungatei* nor *Mb. bryantii* are rumen inhabitants, and their synthetic media do not normally contain propionate. The results above suggest that carboxylation of propionate, if it is available in the medium, is a common reaction among methanogens.

Msp. hungatei and *Mb. bryantii* were grown also on [2-^{13}C]acetate with addition of unlabeled propionate to check if both of these compounds can be used simultaneously for biosynthesis of isoleucine. The results for *Mb. bryantii* are shown in Figure 5. Spectrum a for growth with no propionate in the medium serves as a reference; spectrum b for amino acids obtained from growth with propionate included in the growth medium shows decreased intensities of C-α and C-δ of isoleucine but no change in the intensity of C-γ'. As both C-α and C-δ originate from α-ketobutyrate, the spectrum shows explicitly that α-ketobutyrate is partially made from propionate. Integration of the signals gives ~27% of α-ketobutyrate derived from acetate and 73% from propionate (Table I). The signal intensities from all other amino acids are unchanged upon comparison with spectrum a, as expected, since no other amino acids are synthesized from propionate (Figure 4). Spectrum c in Figure 5 shows labeling of amino acids from [2-^{13}C]acetate in the presence of 2-methylbutyrate in the medium. All isoleucine signals are reduced to ~14% of the intensity of the corresponding signals in Figure 5 a—there is nearly no incorporation of acetate into isoleucine, and no specificity as in Figure 5b. This suggests that 2-methylbutyrate is incorporated as a whole molecule, probably by reductive carboxylation as postulated for *Mbr. ruminantium* (Robinson & Allison, 1969). Since only 14% of the isoleucine was made from acetate in cultures supplemented with 2-methylbutyrate in comparison with 27% for cultures containing extra propionate, it appears that 2-methylbutyrate is a preferred source of carbon for isoleucine biosynthesis in *Mb. bryantii*, when present in the medium.

Similar experiments, conducted by adding propionate or 2-methylbutyrate to the growth medium containing [2-^{13}C]-acetate, were also performed for *Msp. hungatei* and *Mbr. smithii*, with results very similar to those presented for *Mb. bryantii* (see Table I). It is noteworthy that these concentrations of volatile fatty acids did not retard growth.

Because various volatile fatty acids are simultaneously available for metabolism by methanogens in anaerobic digestion systems, an experiment was performed with *Mb. bryantii* where a mixture of acetate, propionate, butyrate, and 2-methylbutyrate was supplied in the growth medium (Figure 5d). Proportions in the mixture were chosen so that they approximated those found in anaerobic digesters, realizing that the proportions of the volatile fatty acids of digesters have been shown to vary widely with growth rates and other conditions (Hobson et al., 1974). Comparison of signal intensities in Figure 5a shows that there is a definite preference for 2-methylbutyrate as an isoleucine precursor (Table I). Butyrate

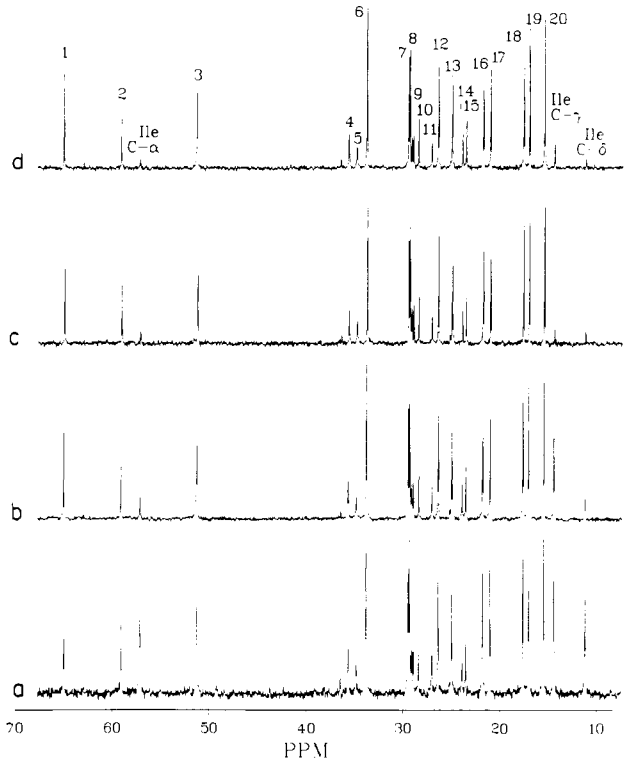

FIGURE 5: ^{13}C NMR spectra of hydrolyzed cytoplasmic protein from *Mb. bryantii* labeled with [2-^{13}C]acetate (12.2 mM). Bacteria were grown by using CO_2 and labeled acetate as carbon sources. (a) No addition. Identification of the signals: (1) Thr, C-β; (2) Ser, C-β; (3) Leu, C-α; (4) Phe, C-β; (5) Tyr, C-β; (6) Asp, C-β; (7) Glu, C-γ; (8) Lys, C-δ; (9) Met, C-γ; (10) Pro, C-β; (11) Arg, C-β; (12) Lys, C-β; (13) Glu, C-β; (14) Arg, C-γ; (15) Pro, C-γ; (16, 17) Leu, C-δ; (18, 19) Val, C-γ; (20) Ala, C-β. Eighty degree pulses were used with a recycling time of 10 s; the decoupler was on only during an acquisition time. The medium was supplemented with (b) 2.2 mM sodium propionate, (c) 2.1 mM 2-methylbutyrate, and (d) 6.8 mM sodium propionate, 7.2 mM butyrate, and 0.6 mM 2-methylbutyrate.

was not specifically incorporated into isoleucine.

The above results show that, in addition to the reductive carboxylation of acetyl-CoA to pyruvate, and of succinyl-CoA to α-ketoglutarate, methanogens are able to perform two other carboxylation reactions, starting from propionate and 2-methylbutyrate. During anaerobic digestion, where volatile fatty acids are available, it is very possible that one or both of these reactions are used as a main pathway to isoleucine biosynthesis.

Alternative pathways for the biosynthesis of isoleucine may have developed to direct the flow of carbon from acetyl-CoA into other competing pathways. On the other hand, the pyruvate pathway, which is dependent on a central metabolic intermediate (acetyl-CoA), would ensure the cell a supply of isoleucine in media devoid of 2-methylbutyrate, propionate, and isoleucine.

Added in Proof

While this paper was in press, Eikmanns et al. (1983) also reported the specific incorporation of [1-^{14}C]propionate into the C-α of isoleucine in *Mb. thermoautotrophicum*, *Mbr. arboriphilus*, and *Ms. barkeri*. Two possible pathways were presented by these authors, one of which is consistent with our ^{13}C NMR data (Figure 2).

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