Biosynthesis of the 1,3,4,6-Hexanetetracarboxylic Acid Subunit of Methanofuran†

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ABSTRACT: ^{2}H- and ^{13}C-labeled precursors were used to establish the pathway for the biosynthesis of the 1,3,4,6-hexanetetracarboxylic acid (TCA) component of methanofuran, which is found in some methanogenic bacteria. The extent and position of incorporation of label into TCA were measured from the mass spectrum of the tetramethyl ester of TCA that was prepared from methanofuran present in cells grown in the presence of labeled acetate. [2,2,2-$^{2}H_3$]Acetate was found to incorporate deuterium into two separate sites of the TCA molecule, with one on each side of the symmetrical molecule. One site was found to be labeled 37% with deuterium, the same as for the glutamic acid present in the cells; the other site was labeled 77% with deuterium, the same as for the malonate-derived compounds in the cells. An analogue of TCA, 1-hydroxy-1,3,4,6-hexanetetracarboxylic acid, found in methanofuran isolated from *Methanobrevibacter smithii*, was found to incorporate $^{13}C_2$ units from [1,2-$^{13}C_2$]acetate into three positions of the molecule. One of the acetate $^{13}C_2$ units was incorporated into the non-hydroxyl-containing side of the molecule (carbons 4, 5, and 6 and the C-6 carboxylic acid group), and two acetate units were incorporated into the hydroxyl-containing side of the molecule (carbons 1, 2, and 3 and the C-1 carboxylic acid group). On the basis of this and additional information, it is concluded that TCA is biosynthesized by the condensation of α-ketoglutaric acid with malonic acid to form 1,1,2,4-butanetetracarboxylic acid, which is further condensed with a second molecule of malonate, in a series of reactions analogous to those observed during fatty acid biosynthesis, to form TCA.

Methanofuran (Figure 1) is a coenzyme in the first of a series of reactions in methanogenic bacteria that are involved in the reduction of CO_2 to CH_4 (Escalante-Semerena et al., 1984; Leigh et al., 1985). Methanofuran was first isolated by Leigh and Wolfe (1983) from an extract of *Methanobacterium thermoautotrophicum* as a specific, low molecular weight compound that was required along with methanopterin for the production of methane by resolved cell extracts from the same organism. The structure of the methanofuran coenzyme isolated from *M. thermoautotrophicum* was established by Leigh et al. (1984).

As part of my research on coenzyme biosynthesis in archaebacteria, I studied the biosynthesis of the 1,3,4,6-hexanetetracarboxylic acid (TCA) portion of methanofuran. The results of this work are reported in this paper.

Materials and Methods

Bacterial Strains and Growth Conditions. Rumen isolate 10-16B, as described by Lovley et al. (1984), and *Methanobrevibacter smithii* were used in the work described herein. Rumen isolate 10-16B is a methanogenic bacterium with a coccobacillus morphology similar to that of *Methanobrevibacter ruminantium*, but unlike this organism, it is able to grow rapidly (u = 0.24 h^{-1}) in a defined medium at 39 °C. The bacteria were grown in 2-L bottles pressurized to 30 psi with H_2/CO_2 (80/20) on 500 mL of a minimal salt medium containing 2.8 mM cysteine and 2.08 mM sulfide as described by Lovley et al. (1984). The bottles were shaken on their sides at 150 rpm at 39 °C. [2,2,2-$^{2}H_3$]Acetate or [1,2-$^{13}C_2$]acetate was added to the medium at a concentration of 62.5 mM. An ~10% (v/v) inoculum of cells that were grown to $A_{660} > 0.5$ was used to start the 500 mL of grown medium. This inoculum was grown on a medium of the same composition as that used in the experiment. After 48–72 h of growth, the cells were harvested by centrifugation at 5000*g* for 15 min.

Extraction, Purification, and Esterification of TCA and Hydroxy-TCA Present in Methanofurans in Whole Bacteria. Cell pellets were suspended in a volume of water equal to their wet weight in grams and heated at 100 °C for 10 min. After centrifugation at 3000*g* for 5 min, the supernate was removed and the pellet reextracted by the same procedure. The resulting supernates were then combined and made 1 M in HCl by the addition of the required amount of 6 M HCl. After the solution was heated for 2 h at 100 °C, the resulting hydrolysate was dried in vacuo, placed in 1–2 mL of water, and passed through a 4 × 30 mm column of Dowex 50W-8X H^+. The eluted material was dried in vacuo, dissolved in methanol, and treated with an excess of CH_2N_2 in ethyl ether for 5 min at room temperature. The resulting precipitate was removed by centrifugation at 3000*g* for 10 min, and after evaporation of the solvents from the resulting clear, yellow-red solution, the TCA tetramethyl ester or the hydroxy-TCA trimethyl ester lactone was purified by preparative thin-layer chromatography (TLC) on precoated silica gel 60 TLC plates (E. Merck, Darmstadt, Germany) with methyl acetate as the solvent. Both the TCA tetramethyl ester and the hydroxy-TCA trimethyl ester lactone had an R_f of 0.62 when methyl acetate was used as the solvent. A known sample of the tetramethyl ester was prepared as described by Hagedorn and Farnum (1977) and was a gift of Dr. Farnum (Department of Chemistry, Michigan State University).

Gas Chromatography–Mass Spectrometry of the Tetramethyl Ester of TCA and the Trimethyl Ester Lactone of Hydroxy-TCA. The tetramethyl ester derivative of TCA had a retention time of about 3.5 min when separated on a 0.3 × 120 cm glass column containing 3% OV-1 on 80/100 Supelcoport (Supelco Inc., Bellefonte, PA) programmed from 100 °C at 10 °C/min. Under the same conditions, the hydroxy-TCA trimethyl ester lactone had a retention time of 4.0 min. Mass spectra were recorded at 70 eV by using a Varian MAT 112 mass spectrometer with a source temperature of

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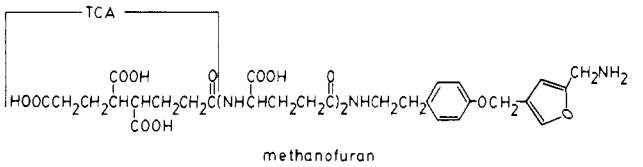

FIGURE 1: Structure of methanofuran isolated from *Methanobacterium thermoautotrophicum*.

200 °C. The method used to convert the measured ion intensities into atom percent distributions has been previously described (White, 1985).

Analysis of Isotopic Distributions of Cellular Constituents. The isotopic distribution of ^{2}H or ^{13}C in the protein-bound amino acids present in the cells was determined by gas chromatography–mass spectrometry (GC–MS) of their *N*-(trifluoroacetyl) and *N,O*-(trifluoroacetyl) *n*-butyl esters and/or their trimethylsilyl derivatives as previously described (White, 1985).

Results and Discussion

The procedure discussed above for the isolation of TCA was established by maximizing the recovery of TCA during its isolation from whole cells. Typically, 1–4 μg of TCA/mg of protein was obtained from those methanogenic bacteria that contained a methanofuran with TCA as part of its structure. This value is in agreement with the amounts reported by Jones et al. (1986) for methanofuran in several different strains of methanogenic bacteria. Gas chromatography of the tetramethyl ester of the final isolated material showed one major peak which gave the mass spectrum shown in Figure 2. The retention time and mass spectrum of this peak was identical with that of a known sample. High-resolution mass measurement of the ions in this mass spectrum was used to support the proposed fragmentation pattern shown in Figure 3. Of particular importance to understanding the biosynthesis of this molecule is the *m/z* 160 fragment ion, which results from the splitting of the molecule into two equal halves. It will be discussed later in the paper.

TCA isolated from strain 10-16B, which was grown with [2,2,2-$^{2}H_3$]acetate, gave a mass spectrum showing molecules with mostly one and two deuteriums. From the intensities of the isotopic ions originating from the $M^+ - OCH_3$ ion at *m/z*

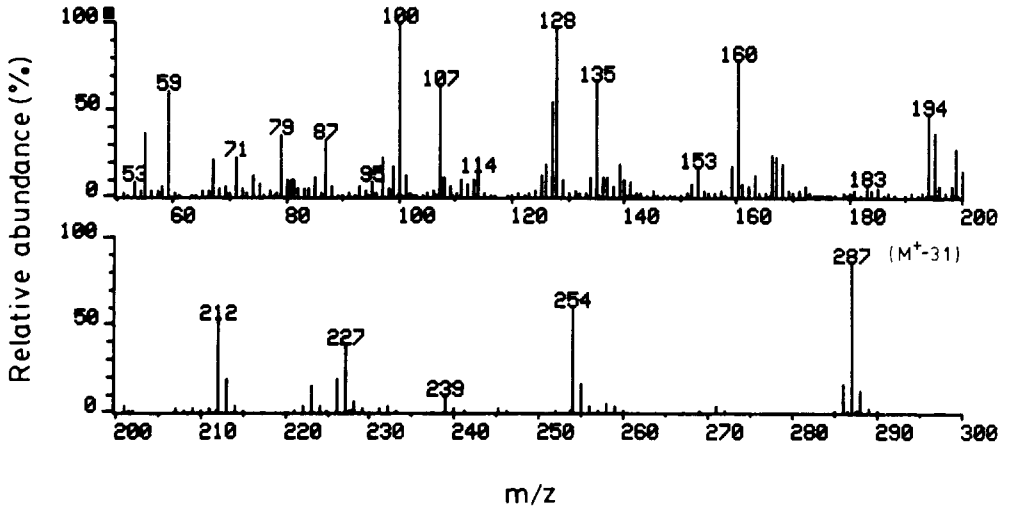

FIGURE 2: Mass spectrum of the tetramethyl ester of 1,3,4,6-hexanetetracarboxylic acid.

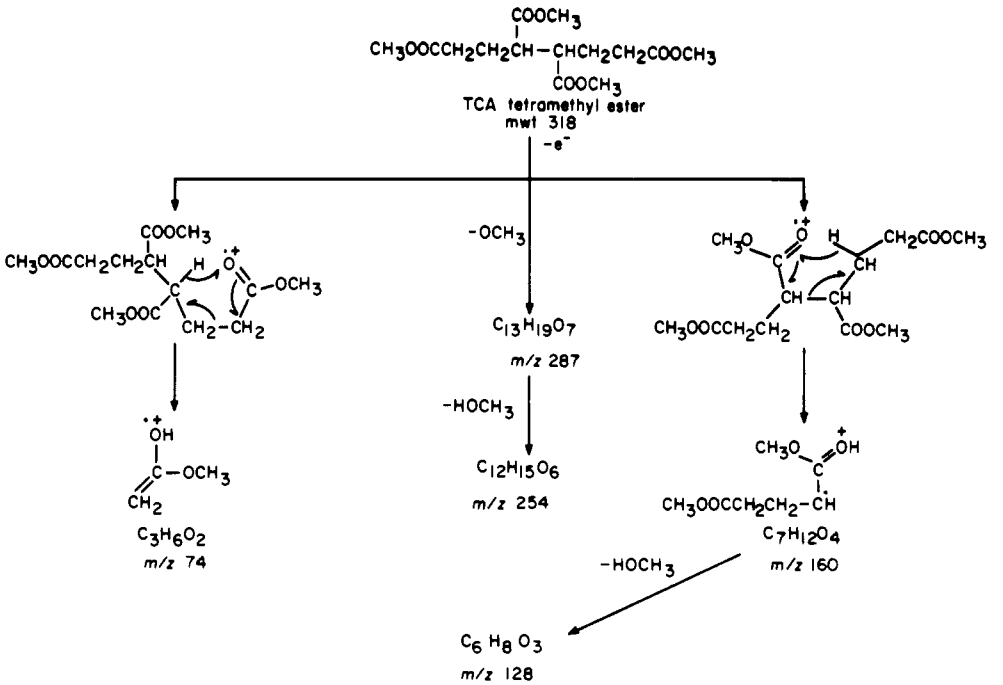

FIGURE 3: Fragmentation of the tetramethyl ester of 1,3,4,6-hexanetetracarboxylic acid.

Table I: Incorporation of [2,2,2-$^2H_3$]Acetate into Cellular Compounds by Strain 10-16B

compound	distribution of ^2H[b] 0	1	2	3	ion used for measurement	ion intensities in unlabeled sample: ion	ion + 1 m/z	ion + 2 m/z
alanine[a]	58.4 (100)	15.3 (68.2)	14.1 (38.0)	12.2 (31.7)	m/z 140, $M^+ - COOC_4H_9$	100	42.0	2.9
serine	46.4 (100)	42.2 (58.0)	34.0 (83.3)		m/z 132, M^+ – COOTMS	100	15.8	3.5
aspartic acid	67.0 (100)	25.7 (66.6)	7.2 (36.6)		m/z 334, $M^+ - 15$	100	28.2	15.0
glutamic acid	61.8 (100)	35.9 (79.6)	2.3 (26.2)		m/z 246, M^+ – COOTMS	100	21.5	9.9
	63.4 (100)	33.6 (82.2)	3.0 (35.3)		m/z 348, $M^+ - 15$	100	29.2	15.1
proline	59.9 (100)	39.5 (73.9)	0.7 (8.0)		m/z 166, $M^+ - COOC_4H_9$	100	8.0	1.6
	60.0 (100)	39.1 (83.7)	0.9 (15.1)		m/z 267, M^+	100	18.5	1.5
TCA	4.0 (29.4)	57.4 (100)	33.0 (74.3)	5.6 (22.0)	m/z 287, $M^+ - 31$	100	16.8	2.5
	39.0 (100)	55.9 (154.3)	5.1 (36.4)		m/z 160, $M^+ - (CH_3OOCCH_2)_2CH_2$	100	11.0	7.3

[a] The alanine data were collected from a separate experiment. [b] The numbers in parentheses are the observed normalized ion intensities.

287 (Table I), it was calculated that 57.4% of the molecules contained one deuterium and 33.0% of the molecules contained two deuteriums. This distribution is derived from the measured ion intensities that are reported in Table I. The next step was to determine where these deuteriums were distributed within the TCA molecule. Since the deuterium distribution in the m/z 160 ion (Figure 3 and Table I) shows molecules with only one deuterium, all of the TCA molecules with two deuteriums must come from TCA molecules having only one deuterium on each side of the m/z 160 fragment. (If one side of the TCA had had two deuteriums incorporated into it, then one of the two symmetrical m/z 160 fragments would also have had two deuteriums.)

Measuring the deuterium in the m/z 160 ion gives only the average deuterium incorporated into both sides of the molecule. We can, however, use the measured distribution of deuterium in the whole molecule to determine the relative amount of deuterium in each side of the molecule. If we let a and b represent the mole fraction of half of the molecule with no and one deuterium, respectively, and c and d represent the mole fraction of the other half of the molecule with no and one deuterium, respectively, then $(a + b)(c + d)$ will define the fractional distribution of deuterium in the whole molecule, where a^2 = mole fraction of the molecules with no deuterium, $ac + bd$ = mole fraction of the molecules with one deuterium, and b^2 = mole fraction of the molecules with two deuteriums. Since the mole distribution in the whole molecule is known (Table I), the values of a, b, c, and d can be calculated.

Calculation revealed that one side of the molecule contained one site with 39% deuterium and the other side contained one site with 77% deuterium. This unequal distribution of deuterium clearly eliminates the possibility that TCA is biosynthesized by the condensation of two groups of similar biochemical origin such as two α-ketoglutaric acids or an α-ketoglutaric acid and a glutaric acid derived from the α-ketoglutaric acid. It is possible, however, that half of the molecule comes from α-ketoglutaric acid and the other half from a non α-ketoglutaric acid derived carbon chain. If this is the case, then one of the deuterium distributions calculated above should be the same as that found in the α-ketoglutaric acid present in the cells. The distribution of deuterium in the α-ketoglutaric acid can be determined by measuring the deuterium distribution in one of its principal biosynthetic products, glutamic acid. Analysis of the deuterium in the glutamic acid (Table I) showed that 36% of the molecules contained one deuterium, which indicates that one of the halves of the TCA could have arisen from α-ketoglutaric acid. The discovery that the glutamic acid that was produced by the cells incorporates only one deuterium from [2,2,2-$^2H_3$]acetate is completely consistent with the proposed biosynthesis of α-ketoglutaric acid from acetate outlined in Figure 4. This pathway for the biosynthesis of α-ketoglutaric acid in methanogenic bacteria has been confirmed by carbon magnetic resonance spectroscopy (Ekiel et al., 1983, 1984) and is consistent with the known metabolism of methanogenic bacteria (Fuchs & Stupperich, 1984; Whitman, 1985). Since the γ protons of the glutamic acid exchange during acid hydrolysis (Cohen & Putter, 1970; White, 1980), the actual abundance of deuterium in the cellular glutamic acid should be slightly higher. This was confirmed in that proline, which is derived from α-ketoglutaric acid, was found to contain 39–40% monodeuterated molecules (Table I). However, since succinate is a symmetrical intermediate, the deuterium incorporated into the α-ketoglutaric acid will be equally distributed between the C-3 and C-4 of the α-ketoglutaric acid and, therefore, the C-5 and C-6 of the resulting TCA (Figure 4).

Support for the pathway shown in Figure 4 comes from the observation that the pyruvate-derived alanine and serine present in the cells were both observed to have deuterium distributions which indicated that more than 50% of the C-3 of these molecules had come from [2,2,2-$^2H_3$]acetate via [*methyl*-$^2H_3$]pyruvate with the retention of one or more deuteriums (Table I). Carboxylation of the cellular pyruvate should then produce oxalacetate with a deuterium distribution similar to that observed in serine. This distribution can be measured by determining the deuterium distribution in aspartic acid which is, in turn, derived from oxalacetate. When this was done (Table I), aspartic acid was found to contain molecules with up to two deuteriums, but the amount recorded was significantly less than that predicted based on the serine data. This decreased labeling is the result of the exchange of deuterium from aspartic acid during acid hydrolysis (Cohen & Putter, 1970; White, 1980). The resulting oxalacetate is reduced to malic acid, which subsequently eliminates DHO to produce fumaric acid with only one deuterium. (On the basis of the serine data, the abundance of this deuterium will be less than that expected because of H–D exchange that occurs from oxalacetate.) Reduction of fumaric acid gives succinate that contains only one deuterium. Since succinate is a symmetrical intermediate, its carboxylation to α-ketoglutaric acid gives a final product with half of the deuterium residing on carbon 3 and the other half on carbon 4. This is depicted in Figure 4 as $D_{1/2}$.

In addition to being consistent with α-ketoglutaric acid being a precursor of one side of the TCA molecule, the above labeling results also indicate that none of the pyruvate-derived metabolic intermediates could serve as an intermediate for the biosynthesis of the other side of the molecule since none of them are labeled with enough deuterium at a single site to account for the 77% labeled site in the other half of the TCA molecule. The only compounds in the cells found to be labeled with enough deuterium to be a source of the non-α-ketoglutaric

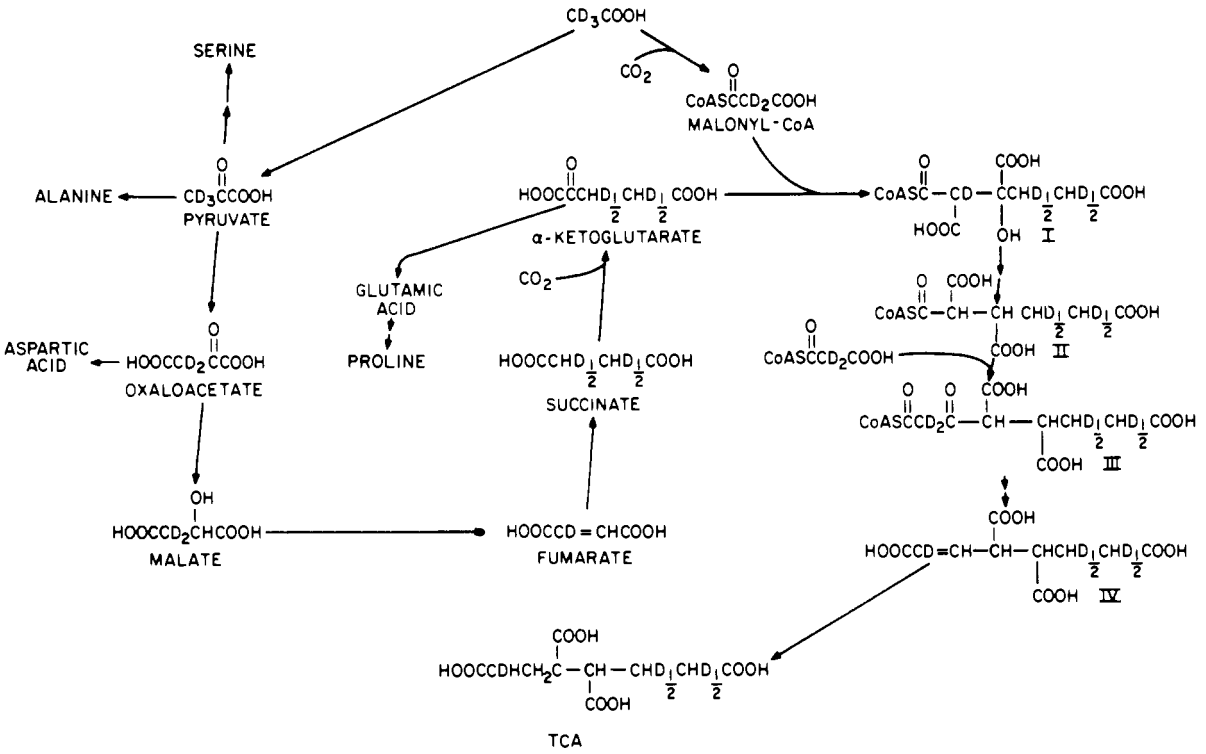

FIGURE 4: Proposed biosynthesis of 1,3,4,6-hexanetetracarboxylic acid from [2,2,2-$^2H_3$]acetate.

acid side of the TCA molecule were those molecules that were derived from acetate without the intermediacy of pyruvate, i.e., the methyl groups of squalene and the isoprenoid-derived lipids (Ekiel et al., 1983; White, 1985). Thus, only a compound more closely related to acetate, i.e., malonate, could be the source of the single deuterium.

A proposed mechanism for the incorporation of 77% deuterium at a single site during the biosynthesis of the other half of the TCA molecule would begin with the condensation of α-ketoglutaric acid with malonyl-CoA to produce compound I (Figure 4). Elimination of DHO from this molecule and reduction of the resulting 4–5 double bond would produce compound II with deuterium only in the α-ketoglutaric acid derived portion of the molecule. After decarboxylation, condensation of compound II with a second molecule of deuterated malonyl-CoA would produce compound III (Figure 4) with three deuterated positions. Compound III, after reduction of the 3-ketone, elimination of water, and reduction of the resulting double bond, would produce TCA with a high percentage of its molecules having a single deuterium on C-2 of the malonate-derived side of the molecule. These last steps are analogous to those in fatty acid biosynthesis where a single deuterium is retained on the even-carbon positions of the fatty acids produced from [2,2,2-$^2H_3$]acetate via [2,2-$^2H_2$]-malonyl-CoA (Bressler & Wakil, 1961; Arnstadt et al., 1975; White, 1980a).

That C-2 of the TCA is very enriched (>70%) at a single site with deuterium is supported by the observation that the m/z 74 ion in the mass spectrum of the tetramethyl ester of the deuterated TCA, which contains the C-2 carbon as well as both of its hydrogens (Dinh-Nguyen et al., 1961), was found to contain about 48% deuterium. Since the m/z 74 ion can originate from both ends of the molecule, its deuterium abundance should be the average of that for the two ends; i.e., $(37\%/2 + 77\%)/2 \sim 48\%$.

Additional support for the proposed condensation of malonyl-CoA, or some other thio ester of malonic acid, with α-ketoglutaric acid to form compound II comes from the observation that hot-water extracts of whole cells of *Methanobacterium formicicum* were found to contain the free acid of compound II in addition to α-ketoglutaric acid. These compounds were identified by GC–MS of suitable derivatives. The $(TMS)_4$ derivative was used for the identification of the free acid of compound II. The mass spectrum of this derivative showed principal ions at $M^+ - 15$, m/z 507 (2.0%); $M^+ - 15 - 90$, m/z 417 (2.0%); $M^+ - 15 - 90 - 28$, m/z 389, (6.1%); m/z 315, (12.0%); m/z 147 (31.0%); m/z 93 (45.0%); and m/z 75 (100.0%). The *O*-methoxylamine $(TMS)_2$ derivative was used for the identification of α-ketoglutaric acid (Horning et al., 1968).

If the biosynthetic pathway is as shown in Figure 4, then three intact acetate units should be incorporated into the TCA molecule, one coming from acetate via α-ketoglutaric acid and the other two coming from acetate via malonate. Thus, six of the ten carbons of the TCA molecule would come from acetate, and the remaining carbon atoms in the molecule would come from CO_2. This can be tested experimentally by growing cells with [1,2-$^{13}C_2$]acetate and determining the extent of incorporation of $^{13}C_2$ units into the tetramethyl ester derivative of TCA. The total number of $^{13}C_2$ units incorporated can be measured easily from the isotopic ions of the m/z 287 ion. The problem with this experiment, however, is that the m/z 160 fragment ion of this derivative of TCA can come from either the α-ketoglutaric acid or malonate derived portion of the molecule. Thus, the measurement of [1,2-$^{13}C_2$]acetate incorporation into this ion will only give an average of that incorporated into both halves of the molecule and will not allow one to determine the extent of incorporation of $^{13}C_2$ units into each half of the molecule.

This problem was best solved by measuring the incorporation of [1,2-$^{13}C_2$]acetate into 1-hydroxy-TCA. ([1,2-$^{13}C_2$]Acetate was chosen over [2,2,2-$^2H_3$]acetate because only an experiment that measures the incorporation of [1,2-$^{13}C_2$]acetate will prove that three acetate units are incorporated.) This compound has recently been shown to replace the TCA component of the methanofuran isolated from *M. smithii* (R. H. White, un-

published experiments). This compound readily forms a five-member lactone, which involves the 1-hydroxyl group and the C-4 carboxylic acid, during its isolation from intact cells of *M. smithii* by the procedures described above for the isolation of TCA. The resulting lactone, as the trimethyl ester, produces the m/z 159 fragment $CH_3OOCCHCH_2CH_2$-$COOCH_3^+$, which originates exclusively from the non-hydroxyl-containing side of the molecule. It is proposed that the hydroxyl group in the 1-hydroxy-TCA is introduced on the malonate-derived side of the TCA by the reverse addition of water to intermediate IV, which is shown in Figure 4 (R. H. White, unpublished experiments). This fragment can then be used to determine the distribution of the $^{13}C_2$ units that are incorporated into the α-ketoglutaric acid derived side of the TCA molecule.

Thus, when acetate with 15.25% of the molecules containing [1,2-$^{13}C_2$]acetate was fed to *M. smithii*, the mass spectra of the isolated 1-hydroxy-TCA showed an $M^+ - 31$ ion at m/z 271 (10%) with 66.7% $^{13}C_0$, 27.5% $^{13}C_2$, 5.8% $^{13}C_4$, and ~0.2% $^{13}C_6$; an $M^+ - 59$ ion at m/z 243 (20%) with 66.1% $^{13}C_0$, 28.2% $^{13}C_2$, 5.7% $^{13}C_4$, and ~0.2% $^{13}C_6$; and an ion at m/z 159 (50%) with 88.4% $^{13}C_0$ and 11.6% $^{13}C_2$. The base peak ion at m/z 211 ($M^+ - 59 - 32$) had interfering fragment ions that obscured the exact measurement of the isotopic incorporation but did show molecules with one, two, and three $^{13}C_2$ acetate units. Since molecules with up to three $^{13}C_2$ units are observed, three acetate units must have been incorporated into the whole molecule. The m/z 159 ion, analogous to the m/z 160 ion in the TCA mass spectrum minus a proton, was found to contain 11.7 atom % $^{13}C_2$, the same as found in the glutamic acid present in the cells, which supports α-ketoglutarate as the source of this part of the molecule. If we assume that the other two acetate units were each incorporated to the same extent from malonate, we can then determine the atom percent of the $^{13}C_2$ units of the malonate that must be combined with the m/z 159 fragment abundance in order to generate the distribution found in the whole molecule. This value was found to be 12.4 atom % $^{13}C_2$. The fact that this value is slightly higher than that found in glutamic acid simply means that the amount of acetate incorporated into the α-ketoglutaric acid is less than that incorporated into the malonate. This reduced acetate incorporation results from the infusion of a small amount of non-acetate-derived carbon into α-ketoglutaric acid. A likely source of this non-acetate-derived carbon would be the cysteine present in the growth medium, the carbon of which is known to be incorporated into the amino acids of strain 10-16B (White, 1985).

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