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Biosynthesis of the 7-Mercaptoheptanoic Acid Subunit of Component B [(7-Mercaptoheptanoyl)threonine Phosphate] of Methanogenic Bacteria†

Robert H. White

Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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ABSTRACT: ^{2}H- and ^{13}C-labeled precursors were used to establish the pathway for the biosynthesis of the 7-mercaptoheptanoic acid moiety of component B in methanogenic bacteria. The extent and position of the label incorporated into 7-mercaptoheptanoic acid were measured from the molecular and fragment ions in the mass spectrum of the methyl ester methylthiol derivative of the 7-mercaptoheptanoic acid. Deuterium from [2,2,2-$^{2}H_3$]acetate was found to be incorporated into four separate positions of 7-mercaptoheptanoic acid. One deuterium was equally distributed between the C-2 and the C-3 of the 7-mercaptoheptanoic acid, and the remaining three were at carbons 4–6. The extent of incorporation at the C-2 and C-3 positions was the same as that observed for the incorporation of [2,2,2-$^{2}H_3$]acetate into the α-ketoglutarate produced by the cells. [1,2-$^{13}C_2$]Acetate was incorporated into four separate sites of the 7-mercaptoheptanoic acid molecule. An intact acetate unit was incorporated at C-2 and C-3, and *single* carbons of the acetate were incorporated at C-5, C-6, and C-7. [2,2,3,3-$^{2}H_4$]Succinate was incorporated with the retention of all four deuteriums, and it supplied carbons 1–4. On the basis of this and additional information, it is concluded that 7-mercaptoheptanoic acid is biosynthesized from α-ketosuberate, which arises from α-ketoglutarate by repeated α-keto acid chain elongation. The mechanism for the conversion of α-ketosuberate to a thiol appears to be analogous to that for the conversion of sulfopyruvate to coenzyme M (2-mercaptoethanesulfonic acid).

Component B [(7-mercaptoheptanoyl)threonine phosphate] is a small molecular weight cofactor required for the reduction of methyl coenzyme M to methane in methanogenic bacteria (Noll et al., 1986). The functional portion of the molecule appears to be the thiol group of the 7-mercaptoheptanoic acid, which is known to form a heterodisulfide bond with coenzyme M when methane is produced from methyl coenzyme M in the methylreductase reaction (Bobik et al., 1987). The presence of the 7-mercaptoheptanoic acid portion of component B in archaebacteria is unusual, since, by first inspection, it appears to be a derivative of a fatty acid, a class of compounds that only occurs in very small amounts in methanogenic bacteria (Langworthy, 1985). 7-Mercaptoheptanoic acid is also a homologue of 8-mercaptooctanoic acid, a known intermediate in the biosynthesis of lipoic acid (White, 1980a), a coenzyme known to occur in much lower amounts in archaebacteria than in other cells (Noll & Barber, 1988).

In order to determine if a connection exists between the biosynthesis of lipoic acid and the 7-mercaptoheptanoic acid moiety of component B, the following work was undertaken. The results of this work show that 7-mercaptoheptanoic acid is generated by a biosynthetic pathway completely different from that of lipoic acid. This pathway is novel in that it

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involves a route for the biosynthesis of fatty acids one carbon at a time and it involves the conversion of an α-keto acid into a thiol in a reaction analogous to that recently described in coenzyme M biosynthesis (White, 1985, 1986b; White, 1988b).

Materials and Methods

Labeled Compounds. [$^2H_4$]Acetic acid (99.5 atom % ^2H) and sodium [1,2-$^{13}C_2$]acetate (99 atom % ^{13}C) were purchased from Sigma Chemical Co. [$^2H_6$]Propionic acid (98.8 atom % ^2H) and [2,2,3,3-$^2H_4$]succinic acid (98.3 atom % ^2H) were obtained from Merck Sharp & Dohme of Canada.

Bacterial Strains and Growth Conditions. Rumen isolate 10-16B, as described by Lovley et al. (1984), and *Methanococcus volta* strain P.S. (DSM 1537) (Whitman et al., 1982) 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. Each bacterial strain was grown in a 2-L bottle pressurized to 30 psi with H_2/CO_2 (80/20) on 500 mL of medium. The minimal salts medium described by Lovley et al. (1984), supplemented with 2 g/L yeast extract and 2 g/L trypticase, was used for the growth of strain 10-16B. *M. volta* cells were grown on the medium described by Whitman et al. (1982). The bottles were shaken on their sides at 150 rpm at 39 °C. [2,2,2-$^2H_3$]Acetate (99 atom % ^2H) or [1,2-$^{13}C_2$]acetate (10 mol % $^{13}C_2$) [prepared by mixing 9 parts of unlabeled sodium acetate with 1 part of sodium [1,2-$^{13}C_2$]acetate (99 atom % ^{13}C)] was added to each medium at a concentration of 62.5 mM. [2,2,3,3-$^2H_4$]Succinate (98.3 atom % ^2H) was added at a concentration of 10 mM to media containing 62.5 mM unlabeled acetate. An ~10% (v/v) inoculum of cells grown to $A_{660} > 0.5$ was used to start the 500 mL of growth medium. This inoculum was grown on a medium of the same composition as that used in each experiment but containing unlabeled acetate. After 48–72 h of growth, the cells were harvested by centrifugation at 5000g for 15 min.

Extraction, Purification, and Derivatization of 7-Mercaptoheptanoic Acid from Whole Bacteria. Cell pellets (0.5–2 g) were suspended in a volume of water equal to three times their wet weight in grams and heated at 100 °C for 20 min. After centrifugation at 39000g for 15 min, the supernate was removed and the pellet reextracted by the same procedure. The resulting supernates were combined, adjusted to pH 3.0 by the addition of 1 M HCl, passed through a column of Dowex 50W-8X H^+ (0.5 × 2 cm), and heated under a stream of nitrogen until concentrated to a volume of 2 mL. The sample was then brought to room temperature, and 2 mL of methanol, 0.4 mL of 7 M NH_4OH, and 50 mg of $NaBH_4$ were added. After 5 min, the sample was cooled to 0 °C, and 20 μL of methyl iodide was added with shaking. After 15 min, an additional 20 μL of methyl iodide was added with shaking, and the sample was allowed to come to room temperature over the next 15 min. The sample was then concentrated to 1 mL by evaporation with a stream of nitrogen to remove the methanol, ammonia, and any unreacted methyl iodide. After the addition of 1 mL of concentrated HCl, the sample was heated at 110 °C for 4 h, cooled, and extracted three times with 1 mL of CH_2Cl_2. The combined methylene chloride extracts were dried with Na_2SO_4, concentrated by evaporation, reacted with an excess of diazomethane in ether, and evaporated to dryness. The resulting methyl 7-(methylthio)heptanoate was purified by preparative thin-layer chromatography (TLC) on precoated silica gel 60 TLC plates (E Merck, Darmstadt, West Germany) with 20% diethyl ether in hexane as the solvent. Under these conditions, the methyl 7-(methylthio)heptanoate had an R_f of 0.37.

Gas Chromatography–Mass Spectrometry of the Dimethyl Derivative of 7-Mercaptoheptanoic Acid. The methyl 7-(methylthio)heptanoate had a retention time of ~6.8 min when separated on a 0.3 × 120 cm glass column containing 3% OV-1 in 80/100 Supelcoport (Supelco Inc., Bellefonte, PA) programmed from 50 °C at 10 °C/min. Mass spectra were recorded at 70 eV on a VG70-70E-HF mass spectrometer with a source temperature of 200 °C. The method used to convert the measured ion abundances into atom percent distributions has been previously described (White, 1985).

Analysis of Isotopic Distributions of Cellular Constituents. The isotopic distribution of ^2H 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*-bis(trifluoroacetyl) *n*-butyl esters and/or their trimethylsilyl derivatives as previously described (White, 1985).

Synthesis of Labeled Compounds. [7,7-$^2H_2$]Mercaptoheptanoic acid was synthesized as previously described for the synthesis of [8,8-$^2H_2$]mercaptooctanoic acid but starting with monomethylsuberic acid (White, 1980a). [2,2-$^2H_2$]-7-Mercaptoheptanoic acid was prepared by the base-catalyzed exchange of 7-mercaptoheptanoic acid with sodium methoxide in CH_3OD. These compounds were then converted into their dimethyl derivatives as described above. Labeled DL-glutamine was prepared by the deuteriation of 6-carboxy-3(2*H*)-pyridazinone (Evans & Wiselogle, 1945) with deuterium gas in ^2H_2O with 5% Pd/C as described by Stogniew et al. (1981). The final product, assayed as the tris(trimethylsilyl) derivative (M^+ 362), had an isotopic distribution of carbon-bound deuterium of 9.6% $^2H_2$, 43.3% $^2H_3$, and 48.0% $^2H_4$.

[*methyl*,4,4-$^2H_5$]-L-Methionine was prepared by the base-catalyzed exchange of methionine sulfoxide with ^2H_2O as follows. L-Methionine sulfoxide (0.33 g) (Sigma Chemical Co.) was dissolved in 4 mL of ^2H_2O and 0.5 mL of 40% NaOD in ^2H_2O and heated under N_2 for 20 h at 110 °C. After the addition of 0.3 mL of acetic acid, the solution was concentrated almost to dryness and heated at 120 °C for 1.5 h with 4.2 mL of acetic acid, 0.66 g of red phosphorus, and 1.66 mL of 40% HI. After removal of the phosphorus by centrifugation and evaporation of the acetic acid, the resulting residue was dissolved in water and applied to a Dowex 50W-8X H^+ column (0.5 × 5 cm) to absorb the methionine. Elution of the column with aqueous ammonia (7 M) and crystallization of the eluted material from water/ethanol gave 260 mg of [*methyl*,4,4-$^2H_5$]-L-methionine. Mass spectral analysis of the material as the *n*-butyl trifluoroacetyl derivative gave a label distribution of 1.9% $^2H_3$, 18.4% $^2H_4$, 76.2% $^2H_5$, and 3.4% $^2H_6$ measured from the molecular ion at m/z 301. (The small percentage of molecules with six deuteriums indicates that only a small percentage of racemization of the methionine occurred during the synthesis.)

Results and Discussion

Five possible biosynthetic pathways for the generation of the 7-mercaptoheptanoic acid moiety of component B are outlined in Figure 1. Each proposed pathway is based on current knowledge of biosynthetic pathways both within and outside the archaebacteria.

Pathway I is based on the remarkable structural similarity between 7-mercaptoheptanoic acid and 8-mercaptooctanoic acid, an intermediate in the biosynthesis of lipoic acid (White, 1980a). Lipoic acid is known to be biosynthesized by the direct, sequential insertion of sulfurs into the C-6 and C-8

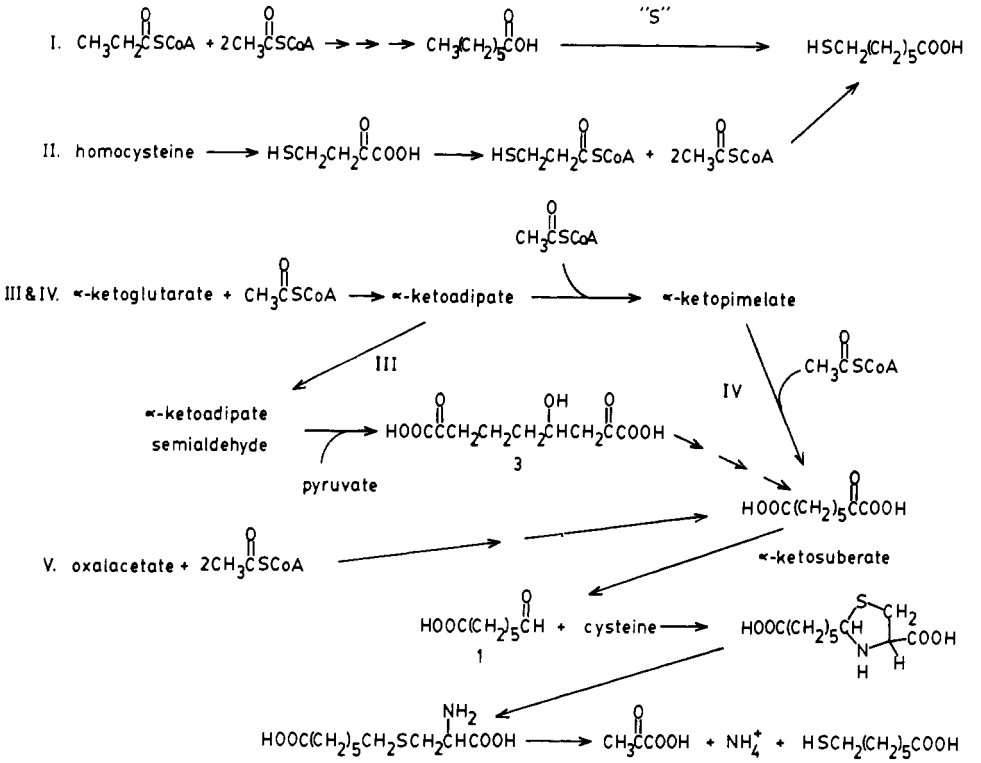

FIGURE 1: Possible pathways for the biosynthesis of 7-mercaptoheptanoic acid.

saturated positions of octanoic acid (Parry, 1977; White, 1980b). A similar reaction occurring on only the C-7 of heptanoic acid would lead directly to the production of 7-mercaptoheptanoic acid. The heptanoic acid required for the reaction could, in turn, be produced by the condensation of two acetyl-CoA with propionyl-CoA by a fatty acid biosynthetic scheme. [The biosynthesis of small amounts of fatty acids in archaebacteria is well documentated despite the fact that their lipid molecules are largely isoprenoids (Langworthy, 1985).]

Pathway II begins with the transamination of homocysteine to its α-keto acid, which then undergoes an oxidative decarboxylation to 3-mercaptopropionyl-CoA. This compound could then serve as an initiator of fatty acid biosynthesis, which, after the addition of two acetates, would lead directly to 7-mercaptoheptanoic acid.

Pathways III and IV begin with the α-keto acid chain elongation of α-ketoglutaric acid to α-ketoadipic acid by a series of reactions known to occur during the biosynthesis of lysine by the aminoadipate pathway (Rodwell, 1969). In pathway III, the resulting α-ketoadipate is subsequently reduced to the semialdehyde and condensated with the C-3 of pyruvate to generate intermediate **3**. Elimination of water from this acid, followed by reduction of the resulting double bond, would remove the alcohol group from the C-4 position of the molecule. Oxidative decarboxylation of one of the α-keto acid groups of this product would produce α-ketosuberic acid. The α-ketosuberic acid could also be produced by pathway IV, in which the α-ketoadipate would undergo two additional sequential chain elongation cycles with the production of α-ketoadipic acid, α-ketopimelic acid, and α-ketosuberic acid at the end of each cycle. Two sequential condensations of oxalacetate with acetate in a fatty acid type biosynthesis could also generate α-ketosuberic acid by pathway V.

The α-ketosuberic acid generated by any of these last three pathways could then be converted into a thiol by a series of reactions (Figure 1) which are based on those known to occur

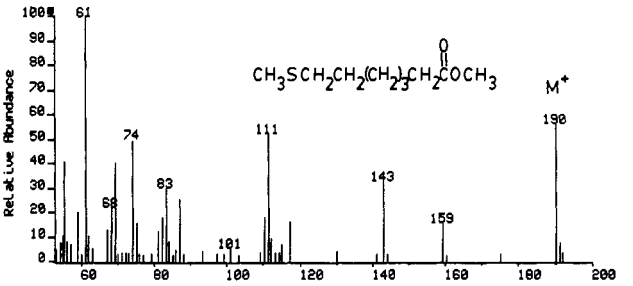

FIGURE 2: Mass spectrum of the methyl ester of 7-(methylthio)-heptanoic acid.

in the biosynthesis of the thiol group of coenzyme M (White, 1985, 1986b, 1988b).

The operative pathway can be established by measuring the extent and position at which ^2H- and ^{13}C-labeled precursors are incorporated into the 7-mercaptoheptanoic acid by mass spectrometry. This approach has been used in establishing the biosyntheses of several coenzymes and has been discussed previously (White, 1978, 1980b, 1985, 1987).

The first step in this approach is to choose a suitable derivative of 7-mercaptoheptanoic acid. Three considerations are important in determining the chemical nature of this derivative. The derivative must be chemically stable, i.e., not prone to disulfide formation which would lead to multiple products. It must be easily prepared and isolated from the component B present in the cells, and the derivative must have an intense molecular ion and well-defined fragment ions which allow for the measurement of the extent and position at which the stable isotopically labeled precursors are incorporated.

The derivative chosen for this work was the methyl ester of the *S*-methyl ether of the 7-mercaptoheptanoic acid. As can be seen in Figure 2, this derivative has an intense molecular ion (M^+ m/z 190) from which the total amount of label incorporated into the molecule can be measured. In addition, as outlined in Figure 3, this derivative has fragments at m/z 61, 74, and 87 which allow for the specific measurement of

Table I: Incorporation of Labeled Compounds into 7-Mercaptoheptanoic Acid by Methanogenic Bacteria

		distribution of ^2H or ^{13}C[a,b]							
		m/z 61 [$CH_3SCH_2^{+}$][c]			m/z 190 M^{+}[c]				
compound fed	cell used	0	1	2	0	1	2	3	4
[2,2,2-$^2H_3$]acetate	10-16B	100 (100)	0.0 (11.1)	0.0 (~5.1)	47.6 (100)	34.5 (84.0)	15.0 (45.5)	2.6 (13.2)	0.3 (3.5)
	M. volta	100 (100)	0.0 (10.9)	~0.0 (5.7)	63.8 (100)	33.0 (63.2)	3.2 (16.6)	0.0 (4.0)	
[1,2-$^{13}C_2$]acetate[d]	10-16B	92.9 (100)	7.6 (18.7)	~0.0 (6.2)	73.4 (100)	18.4 (36.7)	6.4 (17.2)	0.7 (4.0)	1.1 (2.2)
	M. volta	92.5 (100)	6.5 (18.0)	0.0 (5.2)	64.7 (100)	23.5 (47.9)	8.7 (23.2)	0.8 (8.6)	~2.3 (~4.8)
[2,2,3,3-$^2H_4$]succinate	10-16B	100 (100)	0.0 (10.6)	0.0 (5.2)	97.2 (100)	0.0 (11.7)	0.0 (5.6)	1.4 (1.9)	1.4 (1.6)
	M. volta	98.0 (100)	0.9 (12.1)	1.1 (6.0)	44.4 (100)	0.0 (11.0)	0.2 (6.1)	4.4 (10.5)	50.9 (115.8)
[7,7-$^2H_2$]-7-mercapto-heptanoic acid[e]	10-16B	85.1 (100)	4.1 (16.0)	10.8 (18.0)	85.3 (100)	~4.4 (~16.8)	10.6 (18.3)	0.0 (4.4)	

[a] Expressed as the percent of the total molecules containing from 0 to 4 ^2H or ^{13}C. [b] The numbers in parentheses are the observed normalized ion abundances. [c] The ion abundances for the m/z 61 and 190 for an unlabeled sample were 100, 11.1, 4.8 and 100, 11.6, 5.6, 0.51, respectively. [d] Only 10% of the total molecules contained [1,2-$^{13}C_2$]acetate. [e] The label distribution in the fed molecule was 2.9% $^2H_0$, 19.6% $^2H_1$, and 77.5% $^2H_2$.

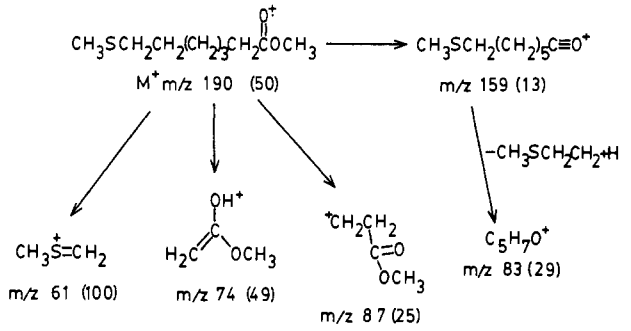

FIGURE 3: Fragmentation of the methyl ester of 7-(methylthio)-heptanoic acid. The numbers in parentheses are the relative ion intensities of each ion normalized to the base peak m/z 61 ion.

the position of label incorporation. The fragmentation outlined in Figure 3 was supported by the nominal masses of the fragments, the presence or absence of the sulfur-34 isotope ion in the fragment, the shift in the mass of the fragment in specifically deuteriated molecules, and specific knowledge of the fragmentation of fatty acid methyl esters. Thus, the mass spectrum of the derivative produced from [7,7-$^2H_2$]-7-mercaptoheptanoic acid showed a 2-m/z increase in only the M^+ m/z 190, 159, and 61 ions in agreement with the proposed fragmentation patterns. The derivative formed from [2,2-$^2H_2$]-7-mercaptoheptanoic acid, however, showed an increase of 2 m/z in all of the fragments listed in Figure 3 except the m/z 61 fragment ion. Thus, the measurement of label incorporated into the m/z 61 ion specifically measures the extent of label incorporated at C-7. The incorporation of label into the m/z 74 ion can measure either carbon incorporation at C-1 and/or C-2 or the sum of the deuterium incorporated at C-2 plus half of that incorporated at C-4. [This ion arises by a site-specific McLafferty rearrangement involving the specific abstraction of one of the γ-hydrogens in its generation (Dinh-Nguyen et al., 1961). The exact measurement of the deuterium incorporated at C-4 is complicated by the known preference, although slight, for the transfer of a γ-hydrogen over a γ-deuterium (MacLeod & Djerassi, 1966, 1967; Williams et al., 1964). On the basis of the known magnitude of this isotope effect, the error should not exceed 10 %.] The m/z 87 ion will measure total deuterium incorporation at C-3 and about half of that incorporation at C-2 on the basis of the fragmentation of specifically deuteriated fatty acid methyl esters (Dinh-Nguyen et al., 1961).

Growth of strain 10-16B in the presence of 15 mM [$^2H_5$]propionate or 1.7 mM [*methyl*,4,4-$^2H_5$]methionine, followed by the analysis of the deuterium incorporated into the biosynthesized 7-mercaptoheptanoic acid, showed less than 0.5% of the molecules to contain deuterium. Since propionate is known to be incorporated by methanogenic bacteria (Robbins, 1987) and since methionine is known to be incorporated into the protein of 10-16B cells under these growth conditions to an extent of 10% (White, 1986a), this would indicate that pathways I and II are not involved in the biosynthesis of the 7-mercaptoheptanoic acid.

When grown with [2,2,2-$^2H_3$]acetate, strain 10-16B and *M. volta*, both of which are known to readily take up acetate (Whitman et al., 1982; White, 1985, 1987), produced 7-mercaptoheptanoic acid that contained up to four deuteriums (Table I). However, the incorporation of label at each site was low; strain 10-16B, which incorporated the largest amount of deuterium, contained ~15% deuterium in each of the four labeled sites. (The 15% deuterium in each labeled site was calculated from the experimental data by assuming that each of the four labeled sites was labeled to the same extent.) Since the extent of [1,2-$^{13}C_2$]acetate incorporation into the other cellular components, i.e., alanine and proline, is ~60% (White, 1988a), the four deuteriated methyls of the acetate must have been incorporated into the final product through a sequence of reactions whereby each undergoes an extensive exchange.

The samples of deuteriated 7-mercaptoheptanoic acid isolated from both of the methanogenic bacteria showed no deuterium in the m/z 61 ion, indicating that no label was incorporated at C-7. For the sample derived from strain 10-16B, which had the highest incorporation of deuterium and was, therefore, the sample best suited for analysis, the m/z 74 ion showed 24% with a single deuterium and a few percent with two deuteriums. [Accurate measurement of the percentage of the m/z 74 fragments with two deuteriums was made impossible because of interference from an unknown compound(s) with a m/z 76 ion eluting close to the 7-mercaptoheptanoic acid derivative.] The m/z 87 ion, on the other hand, showed 38.3% of the molecules with only a *single* deuterium. Since the m/z 74 contains deuterium incorporated at C-2 and half of that at C-4 and the m/z 87 contains deuterium incorporated at C-2 and C-3, the same amount of deuterium must have been incorporated at C-2 and C-3 but never in the same molecule. The extent of incorporation of deuterium at C-2 and C-3 was approximately the same as for the glutamic acid and proline in the cells (36–40%), suggesting that an intermediate such as α-ketoglutarate, which is derived from the symmetrical intermediate succinate, is involved in the biosynthesis of the carboxylic acid terminal of the 7-mercaptoheptanoic acid.

The involvement of α-ketoglutarate could be confirmed by measuring the incorporation of labeled L-glutamic acid or α-ketoglutarate into the 7-mercaptoheptanoic acid by cells grown with these substances. As previously reported for *Methanobacterium thermoautotrophicum* (Gilles et al., 1983),

attempts to demonstrate the incorporation of significant amounts (>5%) of labeled glutamic acid into cells of strain 10-16B were unsuccessful.

Glutamine was considered as a promising precursor as reports indicated that glutamine may be incorporated as a nitrogen source by methanogenic bacteria grown on a nitrogen-deficient medium (Bhatnagar et al., 1984, 1986). Growth of strain 10-16B in a nitrogen-deficient medium could be stimulated by the addition of glutamine, but the incorporation of [2,2,3,3-$^2H_4$]glutamine at a concentration of 13.7 mM was so low (<3%) that it was impossible to obtain accurate data on the extent and position of incorporation.

Succinate, a precursor to α-ketoglutaric acid in methanogenic bacteria (Fuchs & Stupperich, 1984; Jones et al., 1987), was, however, found to be readily incorporated into 7-mercaptoheptanoic acid. This was confirmed by the measurement of the incorporation of [2,2,3,3-$^2H_4$]succinate into 7-mercaptoheptanoic acid (Table I). The incorporation was the greatest for *M. volta*, with more than half of the 7-mercaptoheptanoic acid molecules containing four deuteriums. Analysis of the amino acids Gly, Ala, Thr, Ser, Val, Leu/Ilu, Asp, Phe, Trp, Met, Glu, and Pro in these cells showed only proline and glutamate to contain deuterium. For strain 10-16B, the cellular proline contained 0.8 and 0.7 mol % of the molecules with $^2H_3$ and $^2H_4$, respectively, whereas the proline of *M. volta* contained 2.3 and 35.4 mol % of the molecules with $^2H_3$ and $^2H_4$, respectively. The ratio of the number of prolines with three deuteriums to the number of prolines with four deuteriums for strain 10-16B and *M. volta* is 1.14 and 0.065, respectively, which is about the same as that found in the 7-mercaptoheptanoic acid isolated from each cell type. This indicates that both proline and 7-mercaptoheptanoic acid are derived from succinate via an intermediate that undergoes the same extent of exchange. The difference in the ratios between the different organisms simply reflects different exchange rates. (Since proline is derived from α-ketoglutarate and since the deuteriums of glutamic acid exchange during acid hydrolysis, the incorporation of deuterium into the proline was used to measure the incorporation into α-ketoglutarate.) The reason for the incorporation of less label into proline than into 7-mercaptoheptanoic acid is not clear.

Since succinate is derived from acetate via pyruvate and oxalacetate, [1,2-$^{13}C_2$]acetate should be incorporated into the C-2 and C-3 of the 7-mercaptoheptanoic acid as a unit. Incorporation of an intact acetate unit is confirmed by the M^+ m/z 190 data (Table I) which show that cells grown with acetate containing 10% of the molecules with [1,2-$^{13}C_2$]acetate incorporated from 6.4% to 8.7% of the molecules with an intact C_2 unit. The most important finding from the data, however, is that the remaining carbon atoms in the structure are derived from a *single* carbon of the acetate. This was determined both from the observation that the $M^+ + 2$ ion is not higher than 6.4% and 8.7% (the incorporation of two acetate units would have required an $M^+ + 2$ of >12%) and from the very high $M^+ + 1$ ion (18.4% and 23.5%) which measures the number of molecules with a single ^{13}C. Since [1,2-$^{13}C_2$]acetate is incorporated as a unit into α-ketoglutarate and succinate to an extent of 60% with little scrambling (White, 1988a), three single carbon atoms of the acetate, diluted to an extent of 40% with unlabeled carbon, would be required to account for the labeling pattern observed.

Since the m/z 61 ion from the 7-mercaptoheptanoic acid isolated from both strains shows an incorporation of ~7% ^{13}C (Table I) and since the $^{13}C_2$ unit is at C-2 and C-3, one of these single acetate incorporations must be at C-7. If the first four carbons of 7-mercaptoheptanoic acid arise from succinate or α-ketoglutarate and if the seventh comes from a single carbon of the acetate, then the remaining carbons, 5 and 6, must also come from acetate one carbon at a time. In addition, as no deuterium is incorporated from [2,2,2-$^2H_3$]acetate at C-7, this methylene must be derived from acetate by a mechanism by which none of the acetate methyl protons are incorporated. Since a total of four deuteriums are incorporated from [2,2,2-$^2H_3$]acetate and since one of these comes via succinate, one deuterium from the [2,2,2-$^2H_3$]acetate must be incorporated when each of the additional single carbons is incorporated.

A biochemically plausible explanation for these observations would be the conversion of α-ketoglutaric acid, which is derived from acetate via succinate, to α-ketosuberic acid by repeated α-keto acid chain elongations. The first set of reactions in this process would produce α-ketoadipic acid from α-ketoglutarate via homocitric, homoaconitic, homoisocitric, and oxaloglutaric acids, intermediates that are used in the biosynthesis of lysine by the aminoadipic acid pathway (Rodwell, 1969). The second set of reactions would generate α-ketopimelate from α-ketoadipic acid, and a subsequent chain elongation of the α-ketopimelate would yield α-ketosuberate. The α-keto group of the α-ketosuberate would then be converted into an alkyl thiol by a series of reactions known to occur in the conversion of sulfopyruvate to coenzyme M (White, 1985, 1986b, 1988b). These reactions involve the nonoxidative decarboxylation of α-ketosuberate to 7-oxoheptanoic acid, reaction of the aldehyde with cysteine to form the thioazolidine adduct, and reductive cleavage of the C–N bond in the adduct to form 7-(*S*-cysteinyl)heptanoic acid, followed by elimination of pyruvate and ammonia to form the final product.

The last two α-keto dicarboxylic acids postulated to be involved in the biosynthesis of 7-mercaptoheptanoic acid intermediates have never been identified as natural products. A recent survey, using GC–MS, on the occurrence of these compounds in a wide range of bacteria has shown that α-ketoadipic acid occurs in all bacteria and that α-ketopimelate and α-ketosuberate occur only in the methanogenic bacteria. Neither α-ketoazelaic acid nor higher homologues of α-keto dicarboxylic acids were found in any of the wide variety of bacteria examined. In addition, biosynthetic experiments using ^{13}C-labeled precursors have demonstrated that all of the α-keto dicarboxylic acids are biosynthesized as discussed above (R. H. White, unpublished results). Additional support for pathway IV comes from the ability of cell-free extracts of methanogenic bacteria to convert α-ketosuberate into 7-mercaptoheptanoic acid (R. H. White, unpublished results).

One last point to be considered is how well this mechanism explains the observed incorporation of deuterium from [2,2,2-$^2H_3$]acetate into the 7-mercaptoheptanoic acid. The condensation of the deuteriated acetate with α-ketoglutarate will generate homocitrate containing two deuteriums at C-2. Hydration and rehydration of the homocitrate to homoisocitrate would be expected to result in the loss of one of these C-2 deuteriums, and the remaining C-2 deuterium would be lost during the conversion of homoisocitrate to oxaloglutarate, resulting in no protons of the acetate being incorporated. However, if we assume that the enzyme can reintroduce the deuterium removed at C-2 during the dehydration of homocitrate back at C-3 of homoisocitrate before it undergoes complete exchange with the solvent, the deuterium from the acetate can then be incorporated at the carbonyl carbon of the acceptor α-keto acid. This partial retention of label is documented in the conversion of citrate to isocitrate by aconitase

where one of the protons from the C-2 of the citrate is transferred to the C-3 of the isocitrate (Speyer & Dickman, 1956; Rose, & O'Connell, 1967). This mechanism does, therefore, provide a clear explanation for the incorporation of three of the acetate deuteriums and for the absence of deuterium on the C-7 of the 7-mercaptoheptanoic acid.

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Registry No. Component B, 104302-77-4; 7-mercaptoheptanoic acid, 52000-32-5; acetic acid, 64-19-7; succinic acid, 110-15-6; α-ketosuberate, 96406-05-2; α-ketoglutarate, 328-50-7.

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