Madden, K., Kevan, L., Morse, P. D., & Schwartz, R. (1980) *J. Phys. Chem. 84*, 2691–2692.
Mailer, C., Taylor, C. P. S., Schreier-Muccillo, S., & Smith, I. C. P. (1974) *Arch. Biochem. Biophys. 159*, 133–144.
McConnell, H. M. (1976) in *Spin Labeling, Theory and Applications* (Berliner, L. J., Ed.) pp 525–560, Academic, New York.
Meirovitch, E., & Freed, J. H. (1984) *J. Phys. Chem. 88*, 4995–5004.
Merkle, H., Subczynski, W. K., & Kusumi, A. (1987) *Biochim. Biophys. Acta 897*, 238–248.
Müller-Landau, F., & Cadenhead, D. A. (1979a) *Chem. Phys. Lipids 25*, 299–314.
Müller-Landau, F., & Cadenhead, D. A. (1979b) *Chem. Phys. Lipids 25*, 315–328.
Nordio, P. L., & Busolin, P. (1971) *J. Chem. Phys. 55*, 5485–5490.
Pal, B. (1918) *Bull. Calcutta Math. Soc. 9*, 85–95.
Parente, R. A., & Lentz, B. R. (1986) *Biochemistry 25*, 6678–6688.
Pasenkiewicz-Gierula, M., Hyde, J. S., & Pilbrow, J. R. (1983) *J. Magn. Reson. 55*, 255–265.
Polnaszek, C. F., Bruno, G. V., & Freed, J. H. (1973) *J. Chem. Phys. 58*, 3185–3199.
Polnaszek, C. F., Marsh, D., & Smith, I. C. P. (1981) *J. Magn. Reson. 43*, 54–64.
Popp, C. A., & Hyde, J. S. (1981) *J. Magn. Reson. 43*, 249–258.
Presti, F. T., & Chan, S.-I. (1982) *Biochemistry 21*, 3821–3830.
Presti, F. T., Pace, R. J., & Chan, S.-I. (1982) *Biochemistry 21*, 3831–3835.
Rao, K. V. S., Polnaszek, C. F., & Freed, J. H. (1977) *J. Phys. Chem. 81*, 449–456.
Rice, D. M., Meadows, M. D., Scheinman, A. O., Goni, F. M., Gomez-Fernandez, J. C., Schreier, S., Polnaszek, C. F., & Smith, I. C. P. (1978) *Biochim. Biophys. Acta 515*, 375–436.
Schindler, H., & Seelig, J. (1973) *J. Chem. Phys. 59*, 1841–1850.
Schindler, H., & Seelig, J. (1974) *J. Chem. Phys. 61*, 2946–2949.
Shimoyama, Y., Eriksson, L. E. G., & Ehrenberg, A. (1978) *Biochim. Biophys. Acta 508*, 213–235.
Stubbs, C. D., Kouyama, T., Kinosita, K., Jr., & Ikegami, A. (1981) *Biochemistry 20*, 4257–4262.
Subczynski, W. K., Antholine, W. E., Hyde, J. S., & Petering, D. H. (1987) *J. Am. Chem. Soc. 109*, 46–52.
Taylor, M. G., Akiyama, T., Saito, H., & Smith, I. C. P. (1982) *Chem. Phys. Lipids 31*, 359–379.
Wang, C. C., & Pecora, R. (1980) *J. Chem. Phys. 72*, 5333–5340.

Biosynthesis of the 2-(Aminomethyl)-4-(hydroxymethyl)furan Subunit of Methanofuran†

Robert H. White
Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061
Received October 14, 1987; Revised Manuscript Received January 8, 1988

ABSTRACT: ^{2}H- and ^{13}C-labeled precursors were used to establish the pathway for the biosynthesis of the 2-(aminomethyl)-4-(hydroxymethyl)furan (F1) component of methanofuran in methanogenic archaebacteria. The extent and position of the label incorporated into F1 were measured from the mass spectrum of the diacetyl derivative of F1. [1,2-$^{13}C_2$]Acetate was found to be incorporated into two separate positions of the F1 molecule as a unit. The extent of incorporation of $^{13}C_2$ into each of these positions was the same as that observed for the incorporation of acetate into the alanine and proline produced by the cells. From [2,2,2-$^{2}H_3$]acetate, deuterium was incorporated into two separate sites of the F1 molecule, one containing up to two deuteriums and the other only one. On the basis of the fragmentation pattern of the F1 diacetyl derivative, it was determined that two deuteriums were incorporated into the hydroxymethyl group at C-4 and one was incorporated at C-3 of the furan ring. The extent and distribution of the incorporated deuterium at the C-4 methylene were the same as that observed for C-6 of the glucose produced by the cells. On the basis of this and additional information presented in this paper, it is concluded that F1 is generated by the condensation of dihydroxyacetone phosphate with pyruvate. The resulting dihydroxy-substituted tetrahydrofuran after elimination of 2 mol of water would produce the phosphate ester of 2-carboxy-4-(hydroxymethyl)furan. Reduction of the carboxylic acid to an aldehyde and subsequent transamination would produce the phosphate ester of F1.

Natural products containing a furan ring have been isolated from a large number of different organisms. These furans can be classified into three major structural types: the furanoterpenoids, which can contain mono-, di-, and trisubstituted furan ring(s) (Hikino & Konno, 1976; Sullivan et al., 1983); the furanocoumarins, in which the C-2 and C-3 carbons of the furan are shared by an aromatic ring (Floss & Mothes, 1966; Games & James, 1972); and the fatty acid derived furans, which are generally 2,5-disubstituted furans (Morris et al., 1966; Rahn et al. 1979; Sand et al., 1983; Rau et al., 1984). On the basis of the structures of these furans and a limited number of labeling experiments (Hikino & Konno, 1976), it is concluded that the first two classes of furans are terpene derived and that the latter is fatty acid derived. None of these

†This work was funded by National Science Foundation Grant PCM-8217072 and is the second in a series of papers on the biosynthesis of the subunits of methanofuran.

0006-2960/88/0427-4415$01.50/0

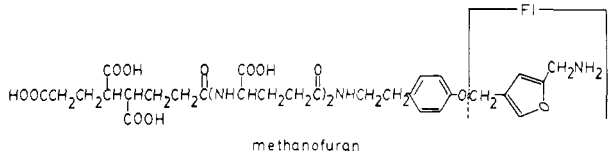

FIGURE 1: Structure of methanofuran from *Methanobacterium formicicum* and strain 10-16B.

structures, however, contain a 2,4-disubstituted furan ring like that found in methanofuran, a recently characterized coenzyme involved in the reduction of CO_2 to methane in methanogenic archaebacteria (Figure 1) (Leigh et al., 1983–1985). This can be taken as evidence that the biosynthesis of the furan in methanofuran occurs by a new biosynthetic pathway. In this paper, I report on a series of stable isotope labeling experiments that demonstrate that the furan ring of methanofuran is biosynthesized by an alternate route that involves the condensation of two three-carbon units.

Materials and Methods

Labeled Compounds. [$^2H_6$]Acetic anhydride (99+ atom % ^2H), [$^2H_4$]acetic acid (99.5 atom % ^2H), and sodium [1,2-$^{13}C_2$]acetate (99 atom % ^{13}C) were purchased from Sigma Chemical Co. [$^2H_2$]Paraformaldehyde (99 atom % $^2H_2$) was obtained from Cambridge Isotope Laboratories, Inc., Woburn, MA.

Bacterial Strains and Growth Conditions. Rumen isolate 10-16B, as described by Lovley et al. (1984), was used in the work described herein. It is a methanogenic bacterium with a coccobacillus morphology similar to that of *Methanobrevibacter ruminatium*, 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 the minimal salts medium containing 2 g/L yeast extract and 2 g/L trypticase as described by Lovley et al. (1984). 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 (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 the medium at a concentration of 62.5 mM. 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 with the same composition as that used in the experiment but containing unlabeled acetate. After 48–72 h of growth, the cells were harvested by centrifugation at 5000g for 15 min.

Extraction and Purification of Methanofuran from Whole Bacteria and Subsequent Isolation of Its F1 Fragment as the Diacetyl Derivative. Cell pellets (1.4–2 g) were suspended in a volume of water equal to 3 times their wet weight in grams and heated at 100 °C for 20 min. After centrifugation at 25000g for 15 min, the supernate was removed and the pellet reextracted by the same procedure. The resulting supernates were combined and applied to a QAE-Sephadex column, which was equilibrated in 0.2 M ammonium bicarbonate, and the methanofuran was eluted with a linear gradient consisting of 200 mL of 0.2 M ammonium bicarbonate and 200 mL of 1.5 M ammonium bicarbonate. Five-milliliter fractions were collected, and the methanofuran was identified by reacting 0.3 mL of each fraction with 0.5 mL of Ehrlich reagent [prepared by dissolving 0.1 g of 4-(dimethylamino)benzaldehyde in 5 mL of concentrated hydrochloric acid]. The fractions containing methanofuran, identified by the generation of a pink color, were combined and evaporated at 80 °C on a rotary evaporator. Water was added, and the evaporation and water additions were repeated 3 times more in order to remove the ammonium bicarbonate. The resulting residue was mixed with 0.5 mL of 1 M HCl, heated under N_2 for 15 min at 100 °C, and dried under vacuum at room temperature. The resulting F1 was converted into the diacetyl derivative when reacted overnight with 0.3 mL of an equal mixture of acetic anhydride/pyridine (v/v). After evaporation of the solvents, the F1 diacetyl derivative 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. Under these conditions, the F1 diacetyl derivative had an R_f of 0.34.

Gas Chromatography–Mass Spectrometry of the Diacetyl Derivative of F1. The diacetyl derivative of F1 had a retention time of ~5.7 min when separated on a 0.3 × 120 cm glass column containing 3% SP2340 on 80/100 Supelcoport (Supelco Inc., Bellefonte, PA) programmed from 115 °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).

Chemical Synthesis of the Diacetyl Derivative of 2-(Aminomethyl)-4-(hydroxymethyl)furan. 3-(Hydroxymethyl)furan (Aldrich Chemical Co.) was converted to the acetate ester by reaction with an excess of an equal mixture of acetic anhydride/pyridine (v/v) at room temperature for 12 h. After evaporation of the solvents, the resulting acetate was purified by column chromatography on silica gel with diethyl ether as the eluting solvent. The acetate was coupled with α-methoxyacetamide, under acidic conditions, in a reaction patterned after that described by Shono et al. (1981). Thus, to 1 g (7.1 mmol) of the acetate were added 0.45 g (4.3 mmol) of *N*-(methoxymethylene)acetamide [prepared as described by Chwala (1948) from acetamide and paraformaldehyde] and 30 mg (0.4 mmol) of 4-toluenesulfonic acid. (An excess of the acetate was used in the reaction in order to reduce the formation of the difunctionalized product.) The resulting homogeneous solution was kept at room temperature for 3 days, after which time an excess of solid sodium bicarbonate was added to neutralize the 4-toluenesulfonic acid and the products were isolated by extraction with methyl acetate. Chromatographic resolution of these reaction products on silica gel, with methyl acetate as the eluting solvent, gave, in addition to the starting product, two, more polar, chromatographically pure (TLC) products, designated as fraction 1 (114 mg) and fraction 2 (132 mg).

Fraction 1 had the same R_f by TLC as an authentic F1 diacetyl sample prepared from methanofuran. GC–MS of this fraction, however, showed that it consists of two compounds in the ratio of 2:1, which gave similar mass spectra. The second and less abundant peak to elute had the same retention time and mass spectrum as the sample of the diacetyl derivative of F1 prepared from methanofuran. Thus, the second peak is the diacetyl derivative of 2-(aminomethyl)-4-(hydroxymethyl)furan, and the first is the diacetyl derivative of 2-(aminomethyl)-3-(hydroxymethyl)furan. This peak assignment was confirmed by the NMR of fraction 1 in $CDCl_3$,

which consisted of two parts of the diacetyl derivative of 2-(aminomethyl)-3-(hydroxymethyl)furan [δ 7.31 (1 H, C-5, d, J = 1.9 Hz), 6.37 (1 H, C-4, d, J = 1.9 Hz), 4.99 (2 H, CH_2O, s), 4.68 (2 H, CH_2N, d, J = 5.6 Hz), 2.05 (3 H, CH_3CONH, s), 2.00 (3 H, CH_3COO, s)] and one part of the diacetyl derivative of 2-(aminomethyl)-4-(hydroxymethyl)furan [δ 7.37 (1 H, C-5, s), 6.27 (1 H, C-3, s), 4.90 (2 H, CH_2O, s), 4.38 (2 H, CH_2N, d, J = 5.6 Hz), 2.05 (3 H, CH_3CONH, s), 1.98 (3 H, CH_3COO, s)]. NMR data for the less abundant compound were identical with those reported by Leigh et al. (1984) for the diacetyl derivative of 2-(aminomethyl)-4-(hydroxymethyl)furan.

The second fraction was converted into the same products as the first fraction after acetylation with acetic anhydride/pyridine. This, as well as the NMR data (not shown), confirmed that this fraction consisted of the O-deacetylated derivatives of the furans isolated in fraction 1. The two isomeric diacetyl derivatives could be separated by preparative gas chromatography; however, since all mass spectrometric measurements were recorded from samples separated by gas chromatography, no attempt was made to separate these samples on a preparative scale.

Chemical Synthesis of Selectively Deuteriated F1 Diacetyl Derivatives. The diacetyl derivative of 2-(aminomethyl)-4-(hydroxymethyl)furan, specifically labeled with deuterium at the methylene of the 2-aminomethyl group, was prepared as described above by using deuteriated *N*-(methoxymethylene)acetamide prepared from acetamide and [$^2H_2$]-paraformaldehyde.

Saponification of the diacetyl derivative of 2-(aminomethyl)-4-(hydroxymethyl)furan in 0.1 M NaOH in methanol for 5 min at 65 °C resulted in complete cleavage of the acetate ester. Reacetylation with [2,2,2-$^2H_3$]acetic anhydride/pyridine generated the *O*-[2,2,2-$^2H_3$]acetyl *N*-acetyl derivative of 2-(aminomethyl)-4-(hydroxymethyl)furan.

The diacetyl derivative of [C-5-^{2}H]-2-(aminomethyl)-4-(hydroxymethyl)furan was prepared by the acid-catalyzed exchange of the unlabeled diacetyl derivative with ^2H_2O (Salomaa et al., 1973) as follows. The diacetyl derivative of 2-(aminomethyl)-4-(hydroxymethyl)furan (10 mg) and hydroquinone (1 mg) were dissolved in 0.5 mL of dimethyl sulfoxide and 0.5 mL of ^2H_2O, and the resulting solution was made 0.2 M in acid by the addition of 70% perchloric acid. After the solution was heated at 100 °C for 1 h, solid sodium bicarbonate was added, the reaction mixture evaporated with a stream of nitrogen, and the residue extracted with methyl acetate. After evaporation of the methyl acetate, the resulting deuteriated 2-(acetamidomethyl)-4-(hydroxymethyl)furan was reacetylated with acetic anhydride/pyridine. The final product was labeled 90% with a single deuterium at the C-5, as measured by mass spectrometry.

Results and Discussion

The F1 diacetyl derivative, which was derived from the methanofuran present in methanogenic bacteria by the extraction, purification, hydrolysis, and acetylation procedure described above, essentially gave only one gas chromatographic peak. The critical step in recovering enough of the F1 fragment for mass spectral analysis was the acid cleavage of the ether linkage between the F1 and the tyramine at the position indicated in Figure 1. Cleavage of this linkage without hydrolytic destruction of the furan ring is possible only because of its extreme lability, as demonstrated by a study of the hydrolysis of 3-(phenoxymethyl)furan (R. H. White, unpublished results). The ether linkage between the phenol and the 3-methylene group of this compound was found to have a

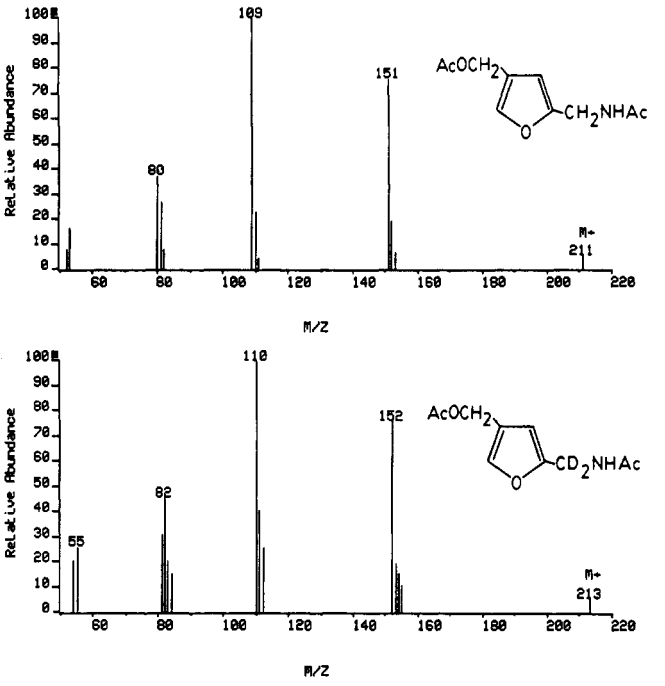

FIGURE 2: Mass spectra of the diacetyl derivative of 2-(aminomethyl)-4-(hydroxymethyl)furan without and with deuterium at the methylene of the 2-aminomethyl group.

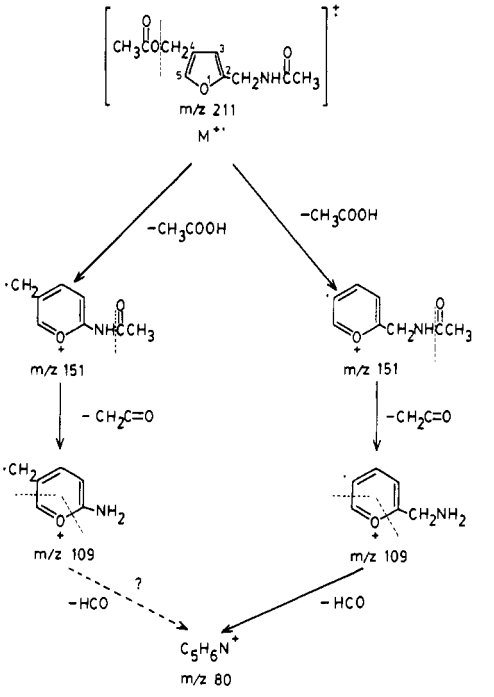

FIGURE 3: Proposed fragmentation of the 2-(aminomethyl)-4-(hydroxymethyl)furan diacetyl derivative.

half-life of 3 min at 100 °C in 1 M HCl. This rapid cleavage of an ether is consistent with the known susceptibility of 2- and 3-halomethylenefurans to substitution reactions (Cariou, 1978; Dunlop & Peters, 1953; Divald et al., 1976).

The mass spectrum of the F1 diacetyl derivative isolated from the cells (Figure 2) and its gas chromatographic retention time were identical with those of the synthetic diacetyl derivative of 2-(aminomethyl)-4-(hydroxymethyl)furan. Important mass spectral fragmentations observed for this molecule, which are critical to the evaluation of the positions at which labeled acetates are incorporated into the molecule, are shown in Figure 3. The loss of acetic acid from the molecular ion is the first major fragmentation observed and leads to the

Table I: Incorporation of [1,2-$^{13}C_2$]Acetate into Cellular Compounds by Strain 10-16B

compound	distribution of ^{13}C[a]					ion used for measurement	ion abundances in unlabeled samples[a] (m/z)			
	0	1	2	3	4		ion	ion + 1	ion + 2	ion + 3
F1 diacetyl derivative	87.6 (100)	0 (12.1)	12.4 (15.5)	0 (13.0)	ND[b]	m/z 211, $M^{\cdot+}$	(100)	(13.0)	(1.4)	
	87.8 (100)	0 (23.2)	11.8 (17.3)	0 (2.6)	0.4 (0.7)	m/z 151, $M^+ - CH_3COOH$	(100)	(24.3)	(8.5)	(1.2)
	89.0 (100)	0 (23.4)	10.9 (21.9)	0 (4.34)	0.2 (1.5)	m/z 109, $M^+ - CH_3COOH - CH_2CO$	(100)	(25.1)	(5.1)	(1.2)
	89.3 (100)	0 (55.1)	10.7 (31.4)	ND[b]		m/z 80, C_5H_6N	(100)	(55.4)	(19.4)	
alanine	93.4 (100)	0.8 (34.5)	5.8 (8.4)			m/z 140, $M^+ - COOC_4H_9$	(100)	(34.5)	(1.9)	
proline	93.8 (100)	0.1 (8.2)	6.1 (6.9)			m/z 166, $M^+ - COOC_4H_9$	(100)	(8.0)	(0.45)	
phenylalanine	88.3 (100)	4.9 (14.1)	6.4 (8.8)			m/z 91, C_7H_7	(100)	(9.6)	(0.6)	
	90.4 (100)	0 (13.4)	9.6 (11.6)			m/z 204, $M^+ - CF_3CONH_2$	(100)	(14.1)	(1.0)	

[a] The numbers in parentheses are the observed normalized ion abundances. [b] Ion abundance was too small for accurate measurement.

production of the $M^+ - 60$ ion at m/z 151. In the mass spectra of the diacetyl derivative of 2-(amino[$^2H_2$]methyl)-4-(hydroxymethyl)furan and the *O*-[2,2,2-$^2H_3$]acetyl *N*-acetyl derivative of 2-(aminomethyl)-4-(hydroxymethyl)furan, this fragmentation corresponds to the loss of CH_3COOD (m/z 61) and CD_3COOH (m/z 63) from the molecular ion, respectively. These observations establish that the acetate radical of the acetic acid comes largely from the ester acetate and that the proton radical comes only from the C-2 methylene carbon. This fragmentation is outlined in the left-hand side of Figure 3. This rather unexpected fragmentation appears to proceed by a concerted mechanism despite the fact that C-2-substituted furans are known to yield abundant $M^+ - 1$ ions (Heyns et al., 1966). The concerted loss of acetic acid is supported in that neither *N*-acetylfurfurylamine (Heyns et al., 1966) nor the F1 diacetyl derivative showed any detectable $M^+ - 1$ ion. Rearrangement of the furan to the pyran (Figure 3) could occur either before, during, or after the loss of acetic acid and is consistent with the expected increased stability of the pyran ring over the furan ring (Porter, 1985). As a result of the specific loss of a 2-methylene proton during the generation of the m/z 151 ion, the difference between the isotopic abundance of the M^+ ion at m/z 211 and the $M^+ - CH_3COOH(D)$ ion at m/z 151 can be used to establish the extent of deuterium incorporation specifically at the C-2 methylene.

The next step in the fragmentation is the loss of ketene (CH_2CO) from the m/z 151 ion to produce the m/z 109 ion. This ion is shifted to m/z 110 in the C-2 methylene dideuteriated F1 diacetyl derivative, indicating that no additional proton loss from the C-2 methylene of the furan is involved in its formation. The loss of m/z 29 (HCO) from the m/z 109 ion would then be expected to generate the m/z 80 ion as indicated by the dashed arrow in the left-hand side of Figure 3. However, the m/z 80 ion is found at m/z 82 in the mass spectrum of the C-2 methylene dideuteriated F1 diacetyl derivative, indicating that it must be generated by an alternate pathway. A possible pathway is depicted in the right-hand side of Figure 3. The abundances of the m/z 153 and 111 ions in the mass spectra of the C-2 methylene dideuteriated F1 diacetyl derivative, which must contain both of the deuteriums in the original molecule, were only 1 and 14% of those of the m/z 152 and 110 ions, respectively.

The mass spectrum of the C-5 monodeuteriated F1 diacetyl derivative showed the expected 1 m/z shift in the m/z 211, 151, and 109 ions. The m/z 80 ion, arising from the loss of CHO from the m/z 109 ion, should have contained no deuterium but was labeled with about 50% deuterium, indicating that some scrambling of deuterium occurred during its generation. This ion is important because it can be used to determine if ^{13}C and, to a lesser extent, ^2H are incorporated into the C-5 position of the furan. One problem, however, with using the m/z 80 ion for measuring isotope incorporation is that the abundances of the (ion + 1) m/z at m/z 81 and the (ion + 2) m/z at m/z 82 have a significant contribution from ions with the composition C_5H_7N and C_5H_8N. Accurate measurement of the ion abundances of the desired ions was easily accomplished simply by recording the required ion abundances under high-resolution conditions, which allowed resolution of the nitrogen-containing ion from the oxygen-containing ion at each of the masses.

Data on the incorporation of [1,2-$^{13}C_2$]acetate into F1 by strain 10-16B are shown in Table I. From the measured ^{13}C incorporations, it is clear that the labeled acetate is incorporated into the F1 molecule only as an intact unit. This follows from the fact that only molecules with $^{13}C_2$ units are observed. In addition, since the original acetate contained only 9.7% of the molecules with $^{13}C_2$, the finding of more than 11% of the molecules with $^{13}C_2$ shows that at least two intact acetate units must have been incorporated into F1 during its biosynthesis. The incorporation of three or more acetates into F1 is excluded by the structure of F1, since there is no way for three intact acetate units to fit into the structure.

The question then arises as to where the two acetate units are incorporated into the F1 molecule. This cannot be unambiguously determined from the mass spectral data reported in Table I; however, the number of possibilities can be reduced by the observation that the m/z 80 ion still contains >10% $^{13}C_2$ units and no detectable excess of single ^{13}C atoms. Since the m/z 80 ion must originate from the loss of the C-5 of the furan, no ^{13}C could have been incorporated at this position. Thus, the two acetate units would have to be at either the C-2 methylene and the C-2 of the furan and the C-4 methylene and the C-4 of the furan, the C-2 methylene and the C-2 of the furan and the C-3 and C-4 of the furan, or the C-4 methylene and the C-4 of the furan and the C-2 and C-3 of the furan.

The correct alternative was determined after considering the [2,2,2-$^2H_3$]acetate incorporation data presented in Table II. As can be seen from the data for the molecular ion at m/z 211, up to three deuteriums are incorporated into the F1 molecule from the labeled acetate. Since the deuterium content of the $M^+ - HOAc$ m/z 151 and $M^+ - HOAc - CH_2CO$ m/z 109 ions is as large as that measured in the M^+ ion at m/z 211, none of the incorporated deuterium must reside on the C-2 methylene group, which I have shown to furnish the proton radical for the concerted loss of acetic acid. Thus, there must be either two deuteriums on the C-4 methylene and one on either the C-3 or C-5 of the furan ring or one deuterium at the C-4 methylene and one at both the C-3 and C-5 of the furan ring. This latter possibility, however, can be eliminated since only two acetate units were incorporated into the F1 and, thus, only two positions in the molecule could be labeled.

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

compound	distribution of ^2H[a]					ion used for measurement[b]
	0	1	2	3	4	
F1 diacetyl derivative	43.2 (100)	27.5 (76.5)	21.9 (60.3)	7.4 (25.7)	ND[c]	m/z 211, M^+
	42.3 (100)	25.3 (84.0)	23.1 (77.6)	9.3 (41.4)	0 (10.6)	m/z 151, $M^+ - CH_3COOH$
	45.4 (100)	25.5 (81.1)	21.9 (67.4)	7.2 (32.0)	0 (6.2)	m/z 109, $M^+ - CH_3COOH - CH_2CO$
alanine	80.8 (100)	9.2 (45.9)	5.5 (12.6)	4.5 (8.1)		m/z 140, $M^+ - COOC_4H_9$
proline	76.7 (100)	22.6 (37.5)	1.5 (4.9)			m/z 166, $M^+ - COOC_4H_9$

[a] The numbers in parentheses are the observed normalized ion abundances. [b] Ion abundances in unlabeled samples are as reported in Table I. [c] Ion abundance was too small for accurate measurement.

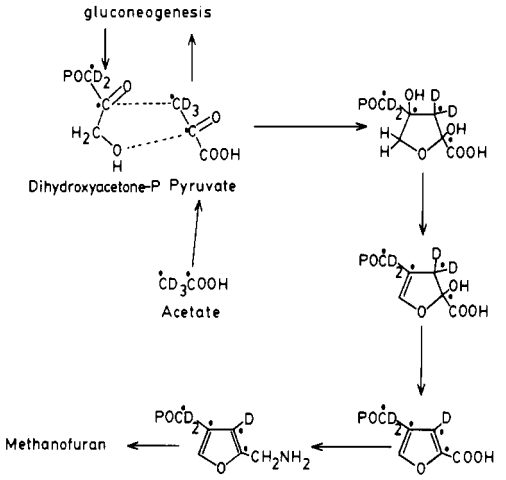

FIGURE 4: Proposed biosynthetic pathway for the generation of the 2-(aminomethyl)-4-(hydroxymethyl)furan component of methanofuran.

Evidence in support of two deuteriums at the C-4 methylene also comes from the recorded deuterium abundance, which shows a disproportionately higher number of F1 molecules having two deuteriums than would be expected by the incorporation of deuterium at three separate sites. Thus, one of the labeled positions must arise from acetate via a pathway in which two of the protons are partially retained at one site. The possibility that one of the deuteriums from the acetate is incorporated at C-5 can also be excluded in that no ^{13}C was incorporated at this position and the m/z 80 ion contained molecules with three deuteriums (data not shown).

A biosynthetic pathway consistent with the observed incorporation data is outlined in Figure 4. This pathway involves the condensation of two three-carbon units, dihydroxyacetone phosphate (DHAP), and pyruvate to generate a dihydroxy-substituted tetrahydrofuran, which is subsequently transformed into F1. Both the DHAP and pyruvate would be generated in the cells from the acetates, labeled as indicated in the figure, by metabolic pathways known to occur in methanogenic bacteria (Fuchs & Stupperich, 1984; Ekiel et al., 1983, 1984). The first committed step in the biosynthesis would involve the addition of the anionic C-3 methyl of pyruvate or phosphoenolpyruvate (PEP) to the ketone group of the dihydroxyacetone (DHAP), as observed during the biosynthesis of aromatic amino acids (DeLeo et al., 1973), followed by the addition of the C-3 hydroxyl group of the DHAP to the carbonyl of the pyruvate. Elimination of 2 mol of water from this dihydroxytetrahydrofuran would produce the phosphate ester of 2-carboxy-4-(hydroxymethyl)furan. Reduction of the carboxylic acid group to an aldehyde and subsequent transamination would produce the phosphate ester of F1. [The reduction of the carboxylic acid to an aldehyde has many biochemical analogues and could, perhaps, proceed through a phosphate ester intermediate, as is the case in the production of the semialdehyde of glutamic acid and aspartic acid from the amino acids (Wriston & Yellin, 1972), or through a thiol ester intermediate, as in the conversion of phosphoglyceric acid to glyceraldehyde phosphate (Walsh, 1979).] Displacement of the phosphate by the phenolic hydroxyl group of tyramine would generate the ether bond between F1 and tyramine.

If the F1 moiety of methanofuran is produced as described above, then the distribution of label observed in the F1 should reflect the distribution of label in the cellular DHAP and pyruvate. We would expect both the cellular DHAP and pyruvate to be labeled to about the same extent with $^{13}C_2$ units; therefore, if we let a represent the mole percent of the pyruvate or DHAP with no ^{13}C and b the mole percent of the pyruvate or DHAP with a $^{13}C_2$ unit, then the distribution of ^{13}C in the F1 molecule derived from one pyruvate and one DHAP would be represented by $(a + b)^2$, where a^2 represents the mole percent of the F1 molecules with no $^{13}C_2$ units, $2ab$ the mole percent of the F1 molecules with one $^{13}C_2$ unit, and b^2 the mole percent of the F1 molecules with two $^{13}C_2$ units. With values for a^2, $2ab$, and b^2 calculated from the m/z 151 and m/z 109 ion abundances (Table I), it was calculated that the combination of two acetate units, containing 5.6 and 6.3% $^{13}C_2$, respectively, would be required to generate the observed distribution of ^{13}C. The measured $^{13}C_2$ in the cellular alanine and proline was found to be 5.8 and 6.1%, respectively (Table I). Since proline is derived from glutamate via pyruvate and alanine is derived directly from pyruvate, this indicates that the pyruvate present in the cells is labeled to the extent of at least 6% $^{13}C_2$. Since only 10% of the fed acetate contained $^{13}C_2$, then 60% of the proline, alanine, and glutamate was derived from the fed acetate. Since pyruvate is the principal source of DHAP in methanogenic bacteria (Ekiel et al., 1983; White, 1985), we would expect it to contain the same percentage of $^{13}C_2$ as does the pyruvate.

Had deuterium from [2,2,2-$^2H_3$]acetate been incorporated into F1 to the same extent as that observed for the $^{13}C_2$-labeled acetate, then the deuterium distribution of the F1 molecules would have been 16% $^2H_0$, 24% $^2H_1$, 24% $^2H_2$, and 36% $^2H_3$, which is clearly much larger than that observed (Table II). [This distribution was calculated from the expression $(a + b)(c + d)$, where b is the mole percent of acetate incorporated with two deuteriums and d is the mole percent of acetate incorporated with one deuterium.] The observed lower percent incorporation is the result of exchange of the original acetate deuteriums with solvent protons during the metabolism of the acetate. This exchange is likely to arise either from the protons of the methyl group of pyruvate, which is the first metabolite formed from acetate (Ekiel et al., 1983) or from the C-3 protons of PEP. This is supported by the known chemical (White, 1978) and enzymatic (Meloche, 1970; Rose, 1960) exchange of pyruvate methyl protons and the enzymatic exchange of the C-3 protons of PEP (Robinson & Rose, 1972). The exchange is confirmed in these cells by the proline and alanine data, which show much less deuterium incorporated into these molecules than would be expected on the basis of

the $^{13}C_2$ acetate incorporation data. The exchange of the acetate methyl protons during its conversion into the methyl of alanine is also clearly demonstrated by the presence of alanine with one, two, and three deuteriums (Table II). The fact that the label incorporated into the alanine is less than would be expected on the basis of the proline data is likely the result of the specific deuterium exchange of those pyruvates undergoing transamination to alanine (Babu & Johnston, 1974).

As a result of the exchange of the pyruvate methyl hydrogens, the deuterium distribution observed in F1, assuming it is derived from pyruvate and DHAP as shown in Figure 4, should be defined by the product $(a + b + c)(x + y)$, representing the deuterium distribution at the C-4 methylene, derived from DHAP, and the deuterium distribution at C-3, derived from pyruvate. [In these expressions, a, b, and c represent the atom percent of the molecules with zero, one, and two deuteriums, respectively, at the C-4 methylene, and x and y represent the mole percent of the molecules with zero and one deuterium at C-3.] By solving for values that give the best fit to the average of the observed deuterium incorporations in the M^+, m/z 151, and m/z 109 ions (Table II), we find $a = 0.64$, $b = 0.08$, $c = 0.28$, $x = 0.69$, and $y = 0.31$. The values for a, b, and c are very close to those calculated for the C-6 carbon of glucose (White, 1987). Since the C-6 position of the glucose is derived from the C-1 of DHAP, this is strong evidence that DHAP is, in fact, the source of this carbon. The y value of 0.31 is only 0.01 less than that expected from the DHAP data, assuming the random loss of one hydrogen. The y value is somewhat higher than expected, based on both the alanine and proline data, due to proton exchange in going from pyruvate to alanine and/or kinetic isotope effects during abstraction of the pyruvate hydrogens.

The above data present little information as to how the intact methanofuran molecule is assembled from its component units of 1,3,4,6-hexanetetracarboxylic acid, glutamate, tyramine, and F1. It does, however, suggest that F1 is condensed with the tyramine by displacement of phosphate from F1-P.

ACKNOWLEDGMENTS

I thank Kim Harich for running the mass spectra, Jia Y. Juan for assistance in growing the bacteria, and Linda D. White for editing the manuscript.

REFERENCES

Babu, U. M., & Johnston, R. B. (1974) *Biochem. Biophys. Res. Commun. 58*, 460–466.

Cariou, M. (1978) *Bull. Soc. Chim. Fr.*, 271–272.

Chwala, A. (1948) *Monatsh. Chem. 78*, 172–173.

DeLeo, A. B., Dayan, J., & Sprinson, D. B. (1973) *J. Biol. Chem. 248*, 2344–2353.

Divald, S., Chun, M. C., & Joullie, M. M. (1976) *J. Org. Chem. 41*, 2835–2846.

Dunlop, A. P., & Peters, F. N. (1953) *The Furans*, American Chemical Society Monograph 119, p 232, American Chemical Society, Washington, DC.

Ekiel, I., Smith, I. C. P., & Sprott, G. D. (1983) *J. Bacteriol. 156*, 316–326.

Ekiel, I., Smith, I. C. P., & Sprott, G. D. (1984) *Biochemistry 23*, 1683–1687.

Floss, H.-G., & Mothes, U. (1966) *Phytochemistry 5*, 161–169.

Fuchs, G., & Stupperich, E. (1984) in *Microbial Growth on C_1 Compounds* (Crawford, R. L., & Hanson, R. S., Eds.) pp 199–202, American Society for Microbiology, Washington, DC.

Games, D. E., & James, D. H. (1972) *Phytochemistry 11*, 868–869.

Heyns, K., Stute, R., & Scharmann, H. (1966) *Tetrahedron 22*, 2223–2235.

Hikino, H., & Konno, C. (1976) *Heterocycles 4*, 817–870.

Leigh, J. A., & Wolfe, R. S. (1983) *J. Biol. Chem. 258*, 7536–7540.

Leigh, J. A., Rinehart, K. L., Jr., & Wolfe, R. S. (1984) *J. Am. Chem. Soc. 106*, 3636–3640.

Leigh, J. A., Rinehart, K. L., Jr., & Wolfe, R. S. (1985) *Biochemistry 24*, 995–999.

Lovley, D. R., Greening, R. C., & Ferry, J. G. (1984) *Appl. Environ. Microbiol. 48*, 81–87.

Meloche, H. P. (1970) *Anal. Biochem. 38*, 389–392.

Morris, L. J., Marchall, M. O., & Kelly, W. (1966) *Tetrahedron Lett. 36*, 4249–4253.

Porter, Q. N. (1985) *Mass Spectrometry of Heterocyclic Compounds*, pp 146–197, Wiley, New York.

Rahn, C. H., Sand, D. M., Wedmid, Y., Schlenk, H., Krick, T. P., & Glass, R. L. (1979) *J. Org. Chem. 44*, 3420–3424.

Rau, O., Kern, W., & Spiteller, G. (1984) *Liebigs Ann. Chem. 1984*, 1504–1512.

Robinson, J. L., & Rose, I. A. (1972) *J. Biol. Chem. 247*, 1096–1105.

Rose, I. A. (1960) *J. Biol. Chem. 235*, 1170–1177.

Salomaa, P., Kankaanpera, A., Nikander, E., Kaipainen, K., & Aaltonen, R. (1973) *Acta Chem. Scand. 27*, 153–163.

Sand, D. M., Schlenk, H., Thoma, H., & Spiteller, G. (1983) *Biochim. Biophys. Acta 751*, 455–461.

Shono, T., Matsumura, Y., Tsubata, K., & Takata, J. (1981) *Chem. Lett.*, 1121–1124.

Sullivan, B., Faulkner, D. J., & Webb, L. (1983) *Science (Washington, D.C.) 221*, 1175–1176.

Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, pp 322–330, Freeman, San Francisco.

White, R. H. (1978) *Biochemistry 17*, 3833–3840.

White, R. H. (1985) *Biochemistry 24*, 6487–6493.

White, R. H. (1987) *Biochemistry 26*, 3163–3167.

Wriston, J. C., Jr., & Yellin, T. O. (1972) *Adv. Enzymol. Relat. Areas Mol. Biol. 39*, 185–248.