METHANE FERMENTATION OF FORMATE BY *METHANOBACILLUS OMELIANSKII*[1]

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The pathway of the methane fermentation of formate was investigated in several organisms by Stephenson and Stickland (1933), Barker (1941) and Stadtman and Barker (1951). Since in several instances H_2 and CO_2 were generated during fermentation, the decomposition of formate would probably involve some prior oxidation to CO_2 and subsequent reduction to CH_4 by the CO_2-reduction scheme of Van Niel and Barker (Barker, 1956). However, the alternate possibility of a direct reduction of formate was never investigated satisfactorily. Stadtman and Barker (1951) found C^{14} impractical as a tracer for a possible reductive pathway with *Methanococcus vannielii* because of the vigorous isotopic exchange between formate and CO_2.

In this study, with deuterium as a tracer and hypophosphite as a selective inhibitor of formic dehydrogenase, both oxidative and reductive incorporations of formate into methane are clearly demonstrable with *Methanobacillus omelianskii* (Barker, 1956) previously refered to as *Methanobacterium omelianskii*. Normally the organism couples the reduction of CO_2 to CH_4 with the oxidation of alcohols to acids or ketones. Unlike typical formate fermenters, it does not metabolize formate rapidly enough to use it as a CO_2 substitute for growth (Barker, 1941).

MATERIALS AND METHODS

Incubation of cultures and suspensions was carried out at 37 C. *M. omelianskii* was grown in the medium of Barker (1941), substituting 0.05 per cent Na thioglycolate and 0.001 per cent ferrous versenate for the original sulfide and ferrous salts. The organisms were centrifuged after 2 days incubation, suspended in freshly boiled 0.05 M potassium phosphate buffer, pH 7.0 with 0.01 per cent $Na_2S \cdot 9H_2O$ and recentrifuged at low and at high speeds to remove sediment from the cells. Suspensions were made in 0.6 final volume of buffer, preincubated with $NaPH_2O_2$, and then supplemented with buffer and remaining substrate and incubated *in vacuo*. Concentrations and volumes refer to the final values. Unless specified, inhibitors were preincubated 1 hr before addition of formate.

The composition of the evolved gases and the CDH_3 enrichment at the mass/charge (m/e) ratio of 17 were determined with the Consolidated model 21-401 mass spectrometer by direct inspection of the CO_2-free gas stored over P_2O_5. The methane yield was determined from the volume of the total gas harvest measured manometrically during its transfer over Hg. Corrections were made for the mass spectrometric contributions of contaminating gases (m/e = 2 to 44). The total D enrichment was alternatively determined by inspection of the water of combustion (Washburn *et al.*, 1953). Formate was similarly analyzed after oxidation with Br_2 and dilution with carrier H_2O. Na deuteroformate was prepared by a modification of the procedure of du Vigneaud *et al.* (1946) and contained at least 90 atom per cent D.

In experiments involving serine recovery, suspensions were boiled and the solutions desalted in a modification of the apparatus of Wood (1956). After evaporation to near dryness, the serine was recrystallized from 90 per cent alcohol and filtered while boiling. It was chromatographed in the *sec.* butanol-formic acid-H_2O system of Roberts *et al.* (1955), and identified by its fluorescence after warming. The β carbon of serine was converted to formaldimethone according to the procedure of Aronoff (1956).

Further details of methods are described by Pine and Vishniac (1957).

RESULTS AND DISCUSSION

Requirements for the reductive incorporation of formate. Hypophosphite inhibits the several systems producing CO_2 from formate in the coli-aerogenes bacteria (Peck and Gest, 1957). High

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concentrations approaching unphysiological limits are necessary for the greatest suppression of the dehydrogenase and hydrogenlyase systems (Pinsky, 1952), and should minimize the labilization competing with the intact utilization of formate. Such competition in *M. omelianskii* is evident from experiment 1, table 1, in the hypophosphite-induced yield of CDH_3 in the methane fermentation of deuteroformate. The D enrichment in the product increases correspondingly with increase in hypophosphite concentration to practicable limits, and in experiment 2 is barely detectable without the inhibitor. As indicated in experiment 3 and in the first value in experiment 1 compared to the first values in the remaining experiments, the highest formate concentration effects the greatest labeling. The potential incorporation is probably greater than the maximum CDH_3 enrichments indicate, since there is no indication of their leveling off with increasing hypophosphite concentration. In experiment 3, the incorporation is insensitive to the folic antagonists aminopterin and amethopterin. They also do not affect the methane fermentation of H_2 and CO_2.

In experiment 4, the requirement for an abundant H supply is evidenced by the almost complete suppression of CDH_3 in the absence of ethanol. Endogenous H donors and the residual formic dehydrogenase activity presumably mediate the reduction necessary for the methane recovered. The requirement of the formate-incorporating system for a reductant above this supply may indicate prior metabolism of formate to a more reduced level before incorporation.

In the absence of hypophosphite the D label is probably rapidly lost, since the C^{14} label of 0.01 M radioformate is substantially equilibrated with CO_2 within 3 hr under conditions similar to those of the D experiments. In contrast, in 0.02 M hypophosphite at least half the label of 0.01 M deuteroformate can still be recovered from the substrate after incubation overnight. It therefore appears that the increased recovery of CDH_3 with concentrations of deuteroformate or hypophosphite above these values is not due entirely to longer preservation of the substrate label. Rather the formate appears to have a low affinity for the incorporating system, and the competition for labilization by exchange or destruction can occur at the site of utilization.

The omission of deuteroformate in experiment 4, or its substitution by normal formate in experiment 2 reduces the *m/e* 17 peak to the value found for normal methane. This attests to the authenticity of the peak as an indication of CDH_3. A further substantiation was made by comparing the combustion and direct scanning procedures for a CDH_3 sample. Three milliliters of gas were collected overnight from a fermentation in 0.125 M hypophosphite, 0.2 M deuteroformate, and 2 per cent ethanol. The H-containing constituents were estimated as 38.8 per cent normal H_2 and 61.2 per cent methane containing 6.06 mole per cent CDH_3 excess. The over-all D enrichment would be 1.16 atom per cent D excess. The remainder of the sample was divided into two aliquots combusted and bracketed between DOH standards. The enrichment was 1.20 atom per cent D excess, in experimental agreement with the calculated value.

The incorporation of the formate label cannot be attributed to transhydrogenation by formic dehydrogenase. No bacterial formic dehydrogenase is known to be phosphopyridine nucleotide-linked, and in *M. omelianskii* the enzyme exchanges the label, as is evident from the hypophosphite experiments.

TABLE 1

Incorporation of deuteroformate into methane by Methanobacillus omelianskii

Expt No.	Supplements			CDH_3 Enrichment
	$NaPH_2O_2$	DCO_2Na	Ethanol	
	M	M	%	*mole % excess*
1	0.02	0.01	1.0	0.11
	0.075	0.01	1.0	0.18
	0.3	0.01	1.0	0.42
2	0.02	0.1	1.0	5.3
	None	0.1	1.0	0.2
	0.02	0.1, unlabeled	1.0	<0.1
3	0.02	0.1	1.0	2.3
	0.02	0.01	1.0	0.2
	0.02, + antifolics	0.1	1.0	2.5
4	0.02	0.1	1.0	1.9
	0.02	None	1.0	<0.1
	0.02	0.1	None	0.2

Experimental conditions: Suspensions containing 0.05 to 0.2 ml packed cell volume in 10 ml of buffer were preincubated 30 to 60 min with $NaPH_2O_2$ and incubated 16 hr with the remaining substrates. Antifolic agents were M $\times 10^{-3}$ aminopterin and amethopterin. No CO_2 was provided.

TABLE 2

Simultaneous incorporation of deuteroformate and CO_2 into methane by Methanobacillus omelianskii

Tube No.	Additions			Methane Yield	CDH_3 Enrichment
	DCO_2Na	$NaPH_2O_2$	$NaHCO_3$		
				μL	*mole % excess*
1	+	+	+	270	4.6
2	+	+		17	4.0
3	+			220	0.1
4	+		+	300	0.1
5				10	<0.1

Experimental conditions: Cells were derived from a culture grown with a 0.1 per cent Na formate supplement. Suspensions of 0.1 ml packed cell volume in a final volume of 1 ml were incubated 135 min in 0.5 M deuteroformate and 2 per cent ethanol. $NaPH_2O_2$, 0.1 M was preincubated 90 min. Addition of 0.1 M $NaHCO_3$ was followed by bubbling with CO_2 to neutrality.

Simultaneous utilization of formate and CO_2. Since hypophosphite inhibits the production of CO_2 from formate, its unmasking of the intact route of formate incorporation observed in table 1 could be due to a decreased supply of CO_2 as an alternate unlabeled source of methane. The experiments summarized in table 2 do not support such an explanation. In the presence of hypophosphite, the CO_2 supplement in tube 1 does not grossly decrease the CDH_3 enrichment from deuteroformate alone as obtained in tube 2, even though the rate of methane production is increased approximately 16-fold. Without the inhibitor, the cells in formate in tube 3 can evolve methane almost at the rate effected with the CO_2 supplement in tube 4. Again the requirement of hypophosphite for appreciable CDH_3 enrichments is evident. Although hypophosphite strongly suppresses the formate fermentation to a value not appreciably above the endogenous in tube 5, it has relatively little effect on the fermentation rate in tube 1 as compared to tube 4, when a CO_2 supplement is present. In agreement in manometric experiments, 0.1 M hypophosphite does not in the least alter the methane fermentation of a 1:1 H_2-CO_2 atmosphere.

The experiments do not permit evaluation of a possible inhibition of intact formate incorporation by hypophosphite; however, it must be considerably less than the inhibition of formic dehydrogenase because of the increased CDH_3 recovery. Therefore, the action of hypophosphite is confined selectively to formic dehydrogenase, and it may be concluded that formate is normally metabolized for the most part through prior conversion to CO_2. Although the CDH_3 enrichments produced by the alternate reductive pathway represent only minimal limits, the reaction does not contribute extensively to methane production. Rather the persistence of similar CDH_3 enrichments in spite of wide fluctuation in the alternate CO_2 supply suggests that the incorporation is mostly an exchange reaction.

Incorporation of CO_2 and formate labels into serine. The limited effectiveness of formate as a methane precursor is not necessarily incompatible with the existence of a one-carbon transfer system directly operative in the methane fermentation. Free formate is not a direct intermediate in one-carbon transfers in the mammal and its active conversion can be relatively limited (Mitoma and Greenberg, 1952). In replacing the serine requirement of *Leuconostoc mesenteroides* strain P60 with glycine, Lascelles and Woods (1950) found formate an ineffective substitute for CO_2 as a one-carbon supplement.

Formate and CO_2 were compared for their ability to incorporate into serine in *M. omelianskii*. Duplicate tubes containing 0.1 ml of wet cell volume in a total of 1.5 ml of buffer with 0.1 M hypophosphite were exposed 1.5 hr to 10 mg each of glycine and carrier serine, 6 mg of Na formate and 10 mg of $NaHCO_3$, the latter two substrates alternately labeled with 20 to 40 μc of C^{14}. From several experiments the relative specific activities in the β carbon of serine averaged 8.3×10^{-5} in comparison to the C^{14} formate supplied. The corresponding activities derived from labeled CO_2 were consistently lower, averaging only 23 per cent as much.

Although the pathway of serine biosynthesis in *M. omelianskii* was not investigated further, the preferential involvement of formate as a C^{14} donor argues in favor of its function in a folic acid-catalyzed one-carbon transfer system, as for example the one described by Kisliuk and Sakami (1955). If a folic acid-dependent system were involved in the reduction of CO_2 to methane, CO_2 should be equivalent if not superior to formate as a one-carbon donor in the more limited incorporating system demonstrated for serine. Assuming that the formate incorporations into methane and serine are biochemically related, the one-carbon incorporating system demonstrated with formate presumably does not

function directly in the CO_2 reduction scheme for methane formation, but can enter it by a side reaction.

Attempts to implicate other one-carbon donors and metabolites in the methane fermentation were unsuccessful. In manometric experiments with H_2 as a H donor, glycine, serine, histidine, methionine, β,β dimethyl propiothetin, S-methyl methionine, choline, betaine, methyl phosphate, acetone, N^{10}-formyl folic acid, sarcosine, and S-adenosyl methionine do not substitute for CO_2. Formaldehyde in low concentrations strongly but temporarily inhibits the methane fermentation. As a single substrate it slowly evolves CO_2 and H_2 but no methane initially. The products are fermented as the substrate disappears. The toxicity of formaldehyde and its side reactions precluded further studies on its incorporation into methane.

Of a number of intermediates in the tricarboxylic acid cycle and the Meyerhof-Embden system tested, pyruvate effects a limited methane fermentation with H_2. However, the ultimate source of the methane is probably CO_2, which is the major constituent of the gas produced from pyruvate alone. Furthermore pyruvate behaves equally well as a H donor, actively reducing methylene blue and CO_2. Miscellaneous compounds such as hydroxypyruvate, carbamyl phosphate, and the condensation product formed from formaldehyde and homocysteine are likewise ineffective as CO_2 substitutes.

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SUMMARY

The methane fermentation of formate by *Methanobacillus omelianskii* has been studied with hypophosphite as a selective inhibitor of formic dehydrogenase. Formate is primarily converted to CO_2 before reduction to methane. In addition, a limited intact incorporation of deuteroformate into methane occurs. Although formate is inferior to CO_2 as a direct source of methane, it is incorporated more effectively than CO_2 into the β carbon of serine. A one-carbon transfer system may be related to the methane fermentation, but a direct involvement could not be demonstrated.

REFERENCES

ARONOFF, S. 1956 Serine degradation. In *Technics of radio-biochemistry*. Iowa State College Press, Ames, Iowa.

BARKER, H. A. 1941 Studies on the methane fermentation. V. Biochemical activities of *Methanobacterium omelianskii*. J. Biol. Chem., **137**, 153–167.

BARKER, H. A. 1956 Biological formation of methane. In *Bacterial fermentations*. John Wiley and Sons, New York.

DU VIGNEAUD, V., CHANDLER, J. P., SIMMONDS, S., MOYER, A. W., AND COHN, M. 1946 The role of dimethyl- and monomethyl-aminoethanol in transmethylation reactions *in vivo*. J. Biol. Chem., **164**, 603–613.

KISLIUK, R. I. AND SAKAMI, W. 1955 A study of the mechanism of serine biosynthesis. J. Biol. Chem., **214**, 47–57.

LASCELLES, J. AND WOODS, D. D. 1950 Synthesis of serine by microorganisms. Nature, **166**, 649–650.

MITOMA, C. AND GREENBERG, D. M. 1952 Studies on the mechanism of the biosynthesis of serine. J. Biol. Chem., **196**, 599–614.

PECK, H. D. AND GEST, H. 1957 Formic dehydrogenase and the hydrogenlyase enzyme complex in coli-aerogenes bacteria. J. Bacteriol., **73**, 706–721.

PINE, M. J. AND VISHNIAC, W. 1957 The methane fermentations of acetate and methanol. J. Bacteriol., **73**, 736–742.

PINSKY, M. J. 1952 The formation of hydrogenlyase and other enzymes in bacteria. Thesis, Indiana University.

ROBERTS, R. B., COWIE, D. L., ABELSON, P. I., BOLTON, E. T., AND BRITTEN, R. J. 1955 Chromatography. In *Studies of biosynthesis in Escherichia coli*. Carnegie Institute of Washington, Washington, D. C.

STADTMAN, T. C. AND BARKER, H. A. 1951 Studies on the methane fermentation. X. A new formate-decomposing bacterium, *Methanococcus vannielii*. J. Bacteriol., **62**, 269–280.

STEPHENSON, M. AND STICKLAND, L. H. 1933 Hydrogenase. III. The bacterial formation of methane by the reduction of one-carbon compounds by molecular hydrogen. Biochem. J. (London), **27**, 1517–1527.

WASHBURN, H. W., BERRY, C. E., AND HALL, L. G. 1953 Measurement of deuterium oxide concentration in water samples by the mass spectrometer. Anal. Chem., **25**, 130–134.

WOOD, T. 1956 A laboratory electrodialyser and desalter. Biochem. J. (London), **62**, 611–613.