BIOSYNTHESIS OF METHANE FROM THE METHYL MOIETY OF METHYLCOBALAMIN

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Methanosarcina barkeri, originally isolated and studied by Schnellen (1947), can grow at the expense of either of the following two reactions:

$$4\ CH_3OH \longrightarrow 3\ CH_4 + CO_2 + 2\ H_2O \qquad (1).$$

$$CH_3COOH \longrightarrow CH_4 + CO_2 \qquad (2).$$

Tracer studies with C^{14} showed that in the fermentation of acetate methane is derived almost exclusively from the methyl carbon and the carboxyl carbon is converted to carbon dioxide (Buswell and Sollo, 1948; Stadtman and Barker, 1949). Similarly, methanol is reduced to methane by a mechanism which does not involve equilibration with carbon dioxide in the system (Stadtman and Barker, 1951). Experiments with deuterium-labeled substrates revealed that the methyl moiety of methanol (reaction 1) or of acetate (reaction 2) is transferred to methane without loss of hydrogen (Pine and Barker, 1956; Pine and Vishniac, 1957).

The methane-producing bacteria as a group are known to be especially rich in cobamide derivatives and M.

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barkeri in particular contains levels sufficient to render the cells pink in color[2]. A functional role of the cobamides in methane formation is indicated by the observation that the over-all process in inhibited by the addition of intrinsic factor to broken cell preparations of M. barkeri[2].

The discovery that methylcobalamin (methyl-B_{12}) can serve as methyl donor to homocysteine in the biosynthesis of methionine (Guest et al., 1962) suggested that the above observations could be explained by an enzymic transfer of the methyl moiety of the fermented substrates to a cobamide compound forming a derivative of the methyl-B_{12} type (Smith et al., 1962; Müller und Müller, 1962) followed by a cleavage yielding methane.

As a first approach to this problem methylcobalamin[3,4] was added in substrate level amounts to disrupted cell preparations of M. barkeri to determine if it could serve as a source of methane. On the assumption that methane formation from methyl-B_{12} might require an external reducing agent, pyruvate[5] was added as electron donor.

2/ B.A. Blaylock, unpublished experiments.

3/ The original material employed in these experiments was a generous gift from H. Weissbach.

4/ Vitamin B_{12} after reduction with $NaBH_4$ was methylated with CH_3I or $C^{14}H_3I$ according to the procedure of Smith et al. (1962). Both the radioactive and non-labeled isolated derivatives exhibited spectral properties characteristic of methyl-B_{12} (Müller und Müller, 1962), were cleaved by cyanide and were rapidly destroyed by light. At least 90% of the radioactivity of the C^{14}-methyl derivative was lost as a volatile product (presumably HCHO) when dried in the light at pH 4.5.

5/ This keto acid is actively decomposed by extracts of various methane-producing bacteria including M. barkeri, Methanobacterium omelianskii and Methanococcus vannielii; furthermore it can serve as sole carbon substrate for growth of M. barkeri.

The data in Table I show that methane is produced under these conditions and its formation is dependent on both methyl-B_{12} and pyruvate. The stoichiometric relationship between the added methyl-B_{12} and the methane

TABLE I

Pyruvate dependent conversion of methyl-B_{12} to methane

Experiment No.	CH_3-B_{12} added	Pyruvate added	Methane produced
	μmoles	μmoles	μmoles
1	3.0	30	2.31
	0*	30	0
2	0.6	30	0.54
	1.5	30	1.25
	3.0	30	1.80
3	0.8	0	0
	0.8	10	0.43
	0.8	30	0.64

* In this sample 3 μmoles of free vitamin B_{12} replaced the methyl derivative.

M. barkeri was cultured anaerobically in a mineral medium (Stadtman and Barker, 1951a) containing 1% methanol. Sarcinae were harvested from 200 l cultures by centrifugation in a Sharples supercentrifuge; the cell paste was frozen in liquid N_2 and stored either at -80° or in liquid N_2. Cells were disrupted in a French pressure cell and the resulting preparation employed as enzyme source. Both the soluble and particulate fractions are required; intact cells do not form methane from methyl-B_{12}.

Reactions were carried out in double side arm Warburg vessels; one arm was fitted with a serum cap to allow withdrawal of gas samples. The main compartment contained the enzyme preparation (0.7 ml) and K phosphate buffer, pH 6.8 (100 μmoles). K pyruvate was in one side arm and methyl-B_{12} in the other. Total fluid volume, 2.0 ml; 37°; gas phase, helium; protected from light. Reactions were terminated when methane production ceased (30 to 60 min. incubations). Aliquots of the gas phase were withdrawn and assayed for methane in a flame ionization apparatus.

produced suggests that the methylated vitamin serves as the actual source of the methane. When the free vitamin replaced the methyl derivative (Exp't. 1, Table I) or when only pyruvate was added (experiments not shown here) no methane could be detected.

Employing C^{14}-labeled methylcobalamin as substrate we could demonstrate transformation of the methyl moiety to methane in good yield (Table II). In contrast very little label appeared in carbon dioxide.

TABLE II

$C^{14}H_4$ formation from $C^{14}H_3$-cobalamin

Experiment No.	$C^{14}H_3$-cobalamin* added	Total $C^{14}H_4$ formed	Total $C^{14}O_2$ formed
	counts/min.	counts/min.	counts/min.
1**	600,000	228,000	3870
2**	595,000	183,500	3520
3	312,000	92,700	1050

* Specific activity, 600,000 counts/min./μmole.

** These values represent the average of two flasks.

Experimental conditions are described in Table I. M. barkeri cells disrupted in a Branson Instruments, Inc. Sonifier were employed for these experiments. The preparations, in contrast to those described in Table I, evolved methane (6 to 10 μmoles) from endogenous materials for 1 1/2 to 2 1/2 hours. Therefore, the preparations were first incubated in buffer only, the methane formed was flushed from the vessels with helium and then the substrates were introduced from the side arms; pyruvate, 30 μmoles and methyl-B_{12} as indicated. The incubations with substrates were terminated when methane production ceased (60 to 90 min.). Aliquots of the gas phase were assayed as before in a flame ionization apparatus; in addition 0.4 or 0.8 ml aliquots were diluted with carrier methane (45 μmoles), freed of CO_2 by equilibration with alkali and combusted over hot copper oxide. The resulting carbon dioxide was trapped in alkali, converted to $BaCO_3$ and examined for radioactivity. The C^{14}-methane isolated in these experiments had been diluted 4 to 10-fold by unlabeled methane formed when pyruvate was added to the preparations.

The data of the above experiments cannot be compared directly because they were obtained from three different experiments conducted on different days with enzyme preparations of varying age. It is therefore, fortuitous that about 30% of the labeled substrate was converted to methane in each case.

The amount of pyruvate required for maximum methane formation (ca. 1.5 x 10^{-2} M) is many times greater than the amount of product formed (Table I). A similar relationship has been observed in nitrogen-fixation studies (Carnahan et al., 1960).

It remains to be demonstrated that the decomposition of methylcobalamin reported here is direct; rather it may involve an intermediate transfer (enzymic or non-enzymic) of the methyl moiety to the normal enzyme-bound carrier.

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