ENZYMIC FORMATION OF METHYLCOBALAMIN IN METHANOSARCINA BARKERII EXTRACTS

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In the methane-producing organism, Methanosarcina barkerii, the methyl group of methanol is converted to methane by a mechanism which does not involve exchange of hydrogen (Pine and Vishniac, 1957). The discovery that methylcobalamin is reduced to methane by extracts of M. barkerii (Blaylock and Stadtman, 1963) suggested that the methanol fermentation may involve an enzymic transfer of the methyl group to a cobamide compound, forming a methyl-B_{12} type derivative, followed by a reductive cleavage to yield methane. The requirements for the conversion of both methanol and methylcobalamin to methane in crude extracts of M. barkerii include a reducing agent, either hydrogen or pyruvate; an energy source, either ATP or ADP; and coenzyme A. Other evidence for a role of cobamide compounds in the methanol fermentation includes the inhibition of the reduction of methanol to methane by intrinsic factor and the decrease in specific activity of C^{14}-methane formed from C^{14}-methanol on the addition of unlabeled methylcobalamin[2/] (Blaylock and Stadtman, 1964b).

The present note reports the enzymic formation of methylcobalamin from a reduced B_{12} compound and methanol.

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[2] There is some doubt as to the significance of these results since addition of unlabeled formaldehyde decreases the specific activity of methane formed when C^{14}-methylcobalamin is the radioactive substrate, but has no effect on the incorporation of label from C^{14}-methanol into methane. Formaldehyde is also converted to methane by these extracts.

Materials and Methods - M. barkerii cells were grown and harvested as described previously (Blaylock and Stadtman, 1963). The enzyme source in all experiments is the 30,000 x g supernatant from a French press extract prepared from cells suspended in .05 M potassium phosphate buffer, pH 7.0, containing 1 mM $Na_2S.9H_2O$ and 10 mM potassium pyruvate. Methylcobalamin was prepared by the method of Smith et al. (1962) and the purity determined by comparison with the spectral data of Müller and Müller (1962). Hydroxo (aquo)cobalamin was prepared from vitamin B_{12} by the method of Bernhauer and Wagner (1963).

Experimental and Results - All reaction and isolation procedures were carried out in dim light and during the reaction the vessel was continuously flushed with helium. Hydroxocobalamin (1-2 μmoles) was reduced to the grey-green stage of B_{12s} (Beaven and Johnson, 1955) by the addition of a few milligrams of $NaBH_4$ in the side arm of a Warburg vessel; the enzyme was incubated in the main compartment for 10 minutes with 100 μmoles potassium phosphate buffer, pH 7.0, 120 μmoles C^{14}-methanol, 10 μmoles ATP and 0.1 μmole CoA. The reaction was started by tipping the reduced B_{12} compound into the reaction mixture (2 ml final volume); after various periods of incubation the reaction was stopped by the addition of ethanol. The B_{12} compounds were extracted with 80% ethanol for 30 minutes at 70°; after centrifugation, the supernatant solution was evaporated to dryness and the residue taken up in a minimum amount of water-saturated phenol. The salts were removed by extraction into water and the phenol was removed by an ether extraction; the B_{12} compounds were recovered in the aqueous phase. The aqueous phase was chromatographed by descending paper chromatography in the solvent system: 100 parts sec.-butanol/50 parts water/3 parts glacial acetic acid; authentic methylcobalamin was run as a standard on each chromatogram. Most of the red color on the chromatogram was located in two distinct areas, one corresponding to hydroxocobalamin and the other to methylcobalamin. The streak at the R_f of methylcobalamin was eluted with water and the spectrum recorded with a Cary model 14 spectrophotometer. The spectrum was similar to methylcobalamin, but had approximately twice the expected absorbancy in the ultraviolet region;

the change in spectrum on acidification was typical of methylcobalamin and on exposure to light a spectrum of hydroxocobalamin was obtained. Several of these eluates, which had been protected from light, were combined and rechromatographed by descending paper chromatography using water-saturated sec.-butanol as solvent. The red band at the R_f of methylcobalamin was eluted with water and the spectrum of this eluate is shown in figure 1a. The only noticeable difference between the spectrum of the enzymically prepared compound and that of the chemically prepared methylcobalamin (figure 1b) is the slightly higher absorbancy below 330 mμ exhibited by the enzymically prepared compound. This higher absorbancy is probably due to contamination with ultraviolet absorbing compounds eluted from the chromatography paper which had not been exhaustively acid-washed. As shown in figure 1a, the spectrum after exposure to light is typical of hydroxocobalamin. The

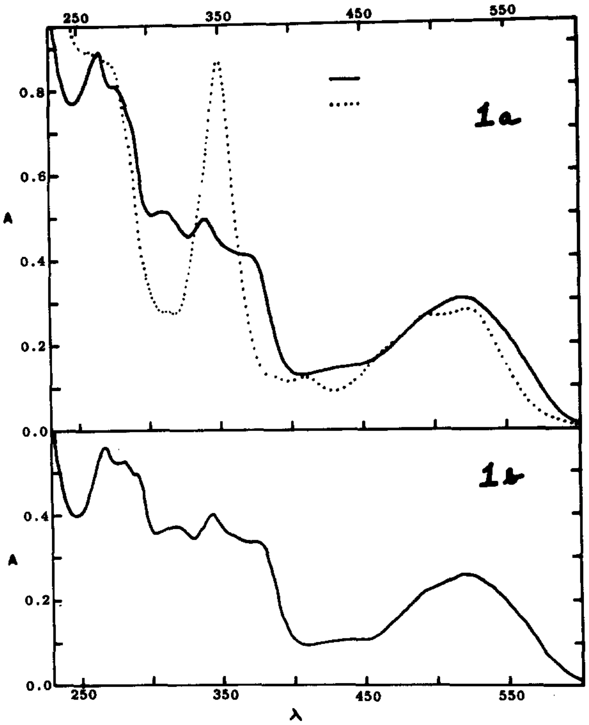

Fig. 1a ___ Enzymically synthesized methylcobalamin; pH 7.

---- Same sample after exposure to visible light.

Fig. 1b ___ Chemically synthesized methylcobalamin; pH 7.

electrophoretic mobility of the red compound in 0.5 N acetic acid was the same as chemically prepared methylcobalamin. The eluate was radioactive and more than 90% of the radioactivity was lost on exposure to light. Based on the loss of radioactivity on exposure to light and the absorbancy at 520 mμ, the specific activity of the methylcobalamin formed was 9 mμcuries per μmole. The specific activity of the C^{14}-methanol added to the reaction mixture was 26 mμcuries per μmole. The dilution by endogenous substrates is consistent with the finding that nearly all extracts active in the production of methane from methanol have high levels of endogenous activity (Blaylock and Stadtman, 1964a). The specific activity of the methane formed from the same extract under optimal conditions for methane formation from C^{14}-methanol (26 mμcuries per μmole) was 11 mμcuries per μmole; however, a comparison of the specific activities of the methylcobalamin and methane may be of little significance, since the reaction conditions were different.

As shown by the data in Table I, not all the reaction mixture components normally required for methanol conversion to methane are necessary for the accumulation of methylcobalamin in these extracts. However since many

TABLE I

Requirements for methylcobalamin formation

Reaction Mixture	methylcobalamin formed#
	mμmoles
Complete*	76
- $NaBH_4$	0
- ATP; -CoA	103
- Hydroxocobalamin + vitamin B_{12}	99
- Enzyme + boiled enzyme	0

* The complete reaction mixture is described in the text; reaction time 10 min; 1.0 ml enzyme containing 45 mg protein.

\# Methylcobalamin was isolated as described in the text; the amount was calculated from the absorbancy at 520 mμ of the eluates from paper chromatograms developed in sec.-butanol/water/acetic acid. The assay method detects 5 mμmoles.

enzyme preparations are capable of catalyzing the over-all reaction at a low rate in the absence of added ATP and CoA, it is possible that these, or other endogenous activating substances, are required for the conversion of methanol to methylcobalamin. No nonenzymic formation of methylcobalamin has been observed in the reaction mixtures employed. Hydroxocobalamin and vitamin B_{12} are equally efficient as starting materials; both are reduced to B_{12s} by $NaBH_4$. Although the reduced B_{12} is added to the reaction mixture in the form of B_{12s}, the actual methyl acceptor may be either B_{12r}, the reaction product of B_{12s} and water under anaerobic conditions (Tackett et al., 1963) or the brown B_{12} compound formed from B_{12r} and sodium hydrogen sulfide[3] (Dolphin and Johnson, 1963).

The yield of methylcobalamin under the above reaction conditions was found to be maximum in short term experiments. For example, in parallel samples, 106 mμmoles were recovered after 15 minutes' incubation and only 43 mμmoles after 30 minutes. This decrease in methylcobalamin may be due to the subsequent reduction of the methylcobalamin to methane by the excess $NaBH_4$ added, or it may reflect the conversion of methylcobalamin to other compounds. When C^{14}-methylcobalamin is added to extracts under conditions for optimal methane formation, several unidentified labeled compounds are formed in addition to methane (unpublished data).

Although the demonstration of the enzymic formation of methylcobalamin from methanol in these extracts is further evidence for a cobamide dependent methanol reduction, it remains to be demonstrated that methylcobalamin, either free or enzyme bound, is an actual intermediate in the reduction of methanol to methane. It is clear, however, that some type of efficient alkylating agent, which can transfer its methyl moiety to reduced B_{12}, is produced enzymically from methanol.

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[3] Evidence that this brown compound is the cobalamin product in the conversion of methylcobalamin to methane in Methanobacillus omelianskii has been reported by Wolin et al. (1964).

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