**CHEMICAL STUDIES OF VITAMIN B₁₂
AND ITS RELATED COMPOUNDS**
**X. NATURAL OCCURRENCE OF COBAMIDE COENZYME
AND OTHER VITAMIN B₁₂-ACTIVE SUBSTANCES
IN THERMOPHILIC METHANE BACTERIA**

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The formation of a large quantity of vitamin B₁₂ group compounds during methane fermentation has been studied by several investigators in recent years. Lately, interesting observations concerning a functional role of the vitamin in methane formation were reported as follows. The formation of methane was inhibited when vitamin B₁₂ was inactivated by the addition of intrinsic factor to broken cell preparations of *M. barkeri* (1). Methylcobalamin, an artificial analogue of Barker's cobamide coenzyme, acted as a methyl donor in fermentation (1, 2). However, neither the mechanism of the reaction involving vitamin B₁₂ nor the natural occurrence and the functional role of cobamide coenzyme in methane fermentation has been clearly elucidated. This paper deals with the natural occurrence of DBCC² and other vitamin B₁₂-active substances in thermophilic methane fermentation.

EXPERIMENTAL

1. Thermophilic Methane Fermentation Process of Acetone-Butanol Distillate

The thermophilic methane bacterium used in the experiment was kindly supplied by National Institute of Fermentation. The bacterium was acclimated to the distillate of acetone-butanol fermentation broth by gradual increase of the amount of the distillate fed to a continuous fermenter. After the acclimation, steady continuous fermentation was carried out at 52° by adding 100 ml of the distillate to 1 liter of the fermentation mixture every day. The amount of gas evolved was 8 times as great in volume as the distillate added and the composition determined by gas chromatography was methane 47 %, CO₂ 53 %.

2. Harvesting of the Cells

Five liters of the fermentation broth was centrifuged at 140 × *g* for 3 minutes.

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² Following abbreviation is used: DBCC, 5,6-dimethylbenzimidazolylcobamide coenzyme.

The precipitate was discarded. The supernatant was further centrifuged at $1,000 \times g$ for 20 minutes. The precipitate mainly consisting of the bacterial cells was washed twice with distilled water, yielding 265 g of the wet cells.

3. Extraction of Vitamin B₁₂-active Substances from the Cells

It should be considered probable that transformation of all the native forms of the vitamin B₁₂ group compounds to the cyano type compounds occurs by the extraction procedure using KCN previously employed. In the present study the extraction method with hot ethanol instead of KCN was used.

A mixture of the wet cells and the same quantity of hot ethanol was refluxed for 20 minutes with occasional stirring. A brown supernatant was obtained by centrifugation of the rapidly cooled mixture at $1,000 \times g$ for 15 minutes. The residue was extracted again with hot ethanol in the same way. The combined extract was evaporated to one-twentieth volume *in vacuo*. The concentrate was used as a sample solution for bioautography. The extract by the KCN procedure was used for the comparison with our sample.

4. Paper Electrophoresis and Bioautography of Vitamin B₁₂-active Substances

About 0.01 ml of the sample solution was streaked along the center of a filter paper sheet (Toyo Filter Paper, No. 50, 2×40 cm). Paper electrophoresis was carried out for 3 hours at 12 volts/cm in 0.5 N acetic acid. After the migration was finished, the paper was dried and cut into 40 pieces, 5 mm wide, *i.e.*, 25 pieces on the cathode side and 15 pieces on the anode side. Vitamin B₁₂-active substances were extracted from each piece in the test tube for microbioassay by autoclaving with 5 ml of the medium for *E. coli* No. 215 or *Ochromonas malhamensis* at 120° for 5 minutes.

In these procedure KCN was added to the medium in order to prevent the inactivation of heat-labile non-cyano type vitamin B₁₂ group compounds by converting them to a heat-stable cyano-type. The presence of vitamin B₁₂-active substance was detected by the growth of *E. coli* No. 215 or *Ochromonas malhamensis* measured with photoelectric colorimeter using S 53 filter.

5. Fractionation of Vitamin B₁₂-active Substances by Ion Exchange Chromatography

The sample solution used for bioautography was further purified by the usual phenol treatment and submitted to column chromatography using DEAE-cellulose. Elution was carried out successively with water, 0.1 N acetate buffer and 0.1 M NaCl-acetone mixture.

6. Measurement of Coenzyme Activity

The cobamide coenzyme activity of the fraction of the column chromatography was measured using a propanediol-propionaldehyde isomerase system according to Abeles and Lee (3).

RESULTS AND DISCUSSION

1. Identification of Native Forms of Vitamin B₁₂-active Substances in the Cells of Methane Bacterium

Comparative bioautograms of the alcohol extract and the cyanide extract using

E. coli No. 215 were given in Fig. 1. The alcohol extract exhibited five growth zones. Three of them, tentatively named P1—3, moved to the cathode, P4 stayed near the start, and P5 migrated to the anode. P1, P2 and P4 were the main growth zones. On the other hand, the disappearance of almost all of the

FIG. 1 Bioautogram of Vitamin B_{12} -active Substances in the Cells of *Methane Bacterium* Using *E. coli* No. 215.

Condition, 0.5 *N* acetic acid, 13 volts/cm, 3 hr

growth zones corresponding to P1 and P2 and the concomitant increase of the zone near the start were observed in the extract with cyanide solution. From these findings it seems likely that vitamin B_{12} -active substances for *E. coli* No. 215 giving P1 and P2 zones are non-cyano type compounds easily convertible to cyano forms.

Fig. 2 shows the bioautogram using *Ochromonas malhamensis* of the alcohol extract. It is well known that the organism exhibits a more strict response than *E. coli* which responds to a variety of the analogues of vitamin B_{12} in addition to

FIG. 2 Bioautogram of Vitamin B_{12} -active Substances in the Cells of Methane Bacterium Using *Ochromonas malhamensis*
Condition, 0.5 *N* acetic acid, 13 volts/cm, 3 hr

5,6-dimethylbenzimidazolyl cobamide. P2 and P3 of the bioautogram using *E. coli* were not clearly observed in that using *Ochromonas*. The comparative size of the area of the growth zones, P1—P5, in the three bioautograms mentioned above are qualitatively shown in Table I. The form of vitamin B_{12} -active substance

TABLE I
Comparison of the Sizes of the Growth Zones in the
Bioautograms of Vitamin B_{12} -Active Substances in the
Cells of Methane Bacterium

Sample	Test organism	Growth zones				
		P1	P2	P3	P4	P5
Extract with alcohol	<i>E. coli</i> No. 215	+++	++	+	+++	+
Extract with cyanide	<i>E. coli</i> No. 215	±	±	*	+++	+
Extract with alcohol	<i>Ochromonas malhamensis</i>	+++	+	±	+++	+

* In the bioautogram of the cyanide extract, the separation of P3 from P4 is incomplete.

corresponding to P1—P5 might be supposed on the basis of the activity on the microorganisms and the change by the cyanide treatment as follows: (a) P1 shows the presence of a non-cyano type vitamin B_{12} with the activity for both *E. coli* and *Ochromonas*. The compound might be considered to be DBCC from its behavior in paper electrophoresis and its coenzyme activity as mentioned later. (b) P2, a non-cyano type vitamin B_{12} analogue lacking 5,6-dimethylbenzimidazolyl cobamide structure which has no *Ochromonas* activity. (c) The zones, P3 and P4

are probably due to pseudo-cobalamin and cyanocobalamin, respectively, from their behaviors of paper electrophoresis and against the cyanide treatment. (d) The presence of the vitamin B₁₂-active substance migrating to the anode was interesting though the amount was small. Its behavior was analogous to that of vitamin B₁₂ coenzyme M of Takeyama and Buchanan (4) or the protein bound form reported in our previous paper (5). The detailed studies concerning the structure of these vitamin B₁₂ analogues will be dealt with in the following paper.

2. Coenzyme Activity of the Partial Purificate of the Vitamin B₁₂-active Substance in Methane Bacterium

The orange-red colored fraction eluted with water from DEAE-cellulose column chromatography exhibited the coenzyme activity in a propanediol-propionaldehyde intramolecular oxidation-reduction system of Abeles and Lee (Fig. 3). The fraction

FIG. 3 Coenzyme Activity Curve to the Graded Amounts of the Orange-red Fraction

corresponded to P1 of the bioautogram.

From the findings of the bioautography and the coenzyme activity measurement DBCC is assumed to be contained in the cells of methane bacterium.

SUMMARY

1. Little has been known about the native forms of vitamin B₁₂-active substances and the natural occurrence of Barker's cobamide coenzyme in the cells of

methane bacteria, since most of non-cyano type vitamin B₁₂ were transformed to the cyano type by the extraction procedure using cyanide solution previously employed.

The authors employed an extraction method using hot ethanol instead of the cyanide treatment in order to avoid the transformation of cyanide-labile vitamin B₁₂ analogues.

2. A purified extract was obtained by the usual phenol treatment of the alcohol extract. By paper ionophoresis performed in 0.5 N acetic acid, five zones active to *E. coli* No. 215 were observed: three of them (tentatively named P1—P3 in the order of the mobilities) moved toward the cathode, one (P4) stayed near the start and the fifth (P5) migrated toward the anode. On the other hand, disappearance of P1 and P2 and concomitant increase in the area of P4 were observed in the extract with cyanide.

In the bioautogram using *Ochromonas malhamensis* which is known to have more specific response to 5,6-dimethylbenzimidazolyl cobamide than *E. coli*, the growth zones corresponding to P2 and P3 were not detected.

3. The partial purificate was fractionated by DEAE-cellulose column chromatography. A fraction eluted with water contained the vitamin B₁₂-active substance corresponding to the zone P1. The fraction exhibited the coenzyme activity in Abeles-Lee's propanediol-propionaldehyde conversion.

4. From these results it seems appropriate to conclude as follows: P1 corresponds to DBCC; P2, non-cyano type B₁₂ analogue lacking 5,6-dimethylbenzimidazolyl cobamide structure; P3, pseudocobalamin; P4, cyanocobalamin and P5, an unidentified B₁₂ analogue with anionic character, the paper ionophoretic behavior of which is analogous to that of vitamin B₁₂ coenzyme M of Takeyama and Buchanan.

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