**CHEMICAL STUDIES ON VITAMIN B₁₂
AND ITS RELATED COMPOUNDS**
**VI. PAPER-ELECTROPHORETIC EXAMINATION ON COBAMIDES
IN *PROPIONIBACTERIUM SHERMANII* CELLS CULTIVATED
IN THE PRESENCE OF SELECTED PRECURSORS**

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In the previous paper (1) the attempt was made to accelerate cobamide synthesis by *Propionibacterium shermanii* by adding a cobamide-like compound (X factor) and 5,6-dimethylbenzimidazole (DBI) to the medium and the *Escherichia coli* activity was found to be markedly increased, most activity being present in the cells. For measuring the *E. coli* activity, however, all the cobamides present were converted to the cyano form by adding cyanide according to the conventional method, whereby it remained uncertain in what form the cobamide occurred. In the present study, an attempt was made to clarify the kind of the cobamide produced by cultivating *Pr. shermanii* in a medium containing both X factor and DBI and by preparing the extract of the cells, followed by examination using paper electrophoresis and bioautography.

EXPERIMENTAL

1. *Materials*

X factor and DBI were the same as described in the previous paper (1).

2. *Cultivation*

Pr. shermanii IAM 1713 strain was used as the synthesizing organism. As described in the previous paper, 100 ml of the standard medium was placed in a 200-ml conical flask, to which 300 μ g of X factor was added. The whole was sterilized by heating for 10 minutes at 120°, inoculated with 5 ml of the bacterial suspension which had been cultivated for 5 days, and statically cultivated for 5 days at 30°. After ascertaining that the bacteria had proliferated well, 2 mg of DBI was added and the cultivation was made for further 10 days.

3. *Extraction*

The cells in 80 ml of the culture were harvested by centrifugation at 10,000 rpm for 20 minutes, to which 10 ml of absolute alcohol was added. The mixture was homogenized in a glass homogenizer. The homogenate was heated for 5

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minutes at 60°, followed by centrifugation and the supernatant was used as the extract.

4. Electrophoresis

In the center of a 2×40 cm paper strip (Toyo filter paper No. 50) 0.001 ml of the test sample was spotted. Electrophoresis was conducted at constant voltage of 13 V/cm for 3 hours using 0.5 *N* acetic acid as the electrolyte solution. The test sample was prepared by diluting the above extract 125-fold. For testing the effect of cyanide, the same volume of 0.1 % KCN was added to another aliquot of the sample and after adjustment of pH to 6.0, the mixture was heated for 30 minutes at 90°, diluted similarly as given above and subjected to electrophoresis. In this case, cyanide-containing electrolyte solution was employed. In each case authentic samples of cyanocobalamin (CN-B₁₂), hydroxocobalamin (OH-B₁₂) and Factor B (cobinamide) were also used as standards.

5. Bioautography

To the basal medium for vitamin B₁₂ assay using *E. coli* 2.5 % purified agar was added, heat-sterilized, and then poured into sterile Petri dishes, 35 cm in diameter. When it was cooled to 40°, 5 ml of *E. coli* 215 inoculum suspension was added and allowed to solidify, on which the filterpaper after electrophoresis was placed for 15 minutes. After removal of the paper, the agar medium was incubated for 18 hours at 37° and the growth of the bacteria was observed.

6. Measurement of B₁₂ Activity

The supernatant and the cell fraction were separated and the B₁₂ content of each fraction was determined using *E. coli*.

RESULTS AND DISCUSSION

1. Bacterial Yield and Distribution of *E. coli* Activity

Table I shows the distribution of *E. coli* activity in the supernatant and cell fraction of the culture as well as the yield of bacterial cells. The *E. coli* activity was found almost exclusively in the cells, the B₁₂ content of which reached as high as 0.56 µg/mg.

TABLE I
Cell Yield and Distribution of B₁₂ Activity

<i>E. coli</i> -activity in		Yield in dry cells	B ₁₂ in cells
Cells	Supernatant		
	µg/ml	mg/ml	µg/mg
0.80	0.05	1.43	0.56

2. Bioautography

Fig. 1 is an example of the bioautograms of the cyanide-free sample and Fig. 2 is that after addition of cyanide. In the former, 4 spots, I—IV, became apparent, while in the latter only 2 spots, V and VI, were observed. A comparison with the standards showed that V and VI corresponded to cobinamide and CN-B₁₂,

FIG. 1 Bioautogram without Adding Cyanide

FIG. 2 Bioautogram with Addition of Cyanide

respectively, since both spots were observed even after cyanide treatment. The spot II was located at approximately the same position as OH-B₁₂ and cobinamide. But, if it were OH-B₁₂, it should disappear by cyanide treatment and the value determined in the previous assay should be low. It is therefore assumed that the spot II is mostly due to the unreacted cobinamide. The spot III was situated at the position corresponding to CN-B₁₂, but it cannot be definitely said to be CN-B₁₂. Worthy of note are the spots I and IV. Though no standard samples comparable to them were available, the spot I behaved quite similarly to the coenzyme as discovered by Barker *et al.* (2) and the spot IV migrated toward the anode similarly to the bound forms of B₁₂. Both of them, moreover, disappeared and converted to V or VI after cyanide treatment. These findings support the assumption that they are the coenzyme and the bound form, respectively. Thus the presence of at least 4 forms of the *E. coli*-active substance was proven by electrophoresis and bioautography. These findings seem to reveal the natural forms of the cobamides biosynthesized in the cells.

SUMMARY

An attempt was made to elucidate the natural forms of the cobamides synthesized in *Propionibacterium shermanii* grown in the culture medium containing both a cobinamide-like compound (X factor) and 5,6-dimethylbenzimidazole as precursors. The cells were homogenized in alcohol and the extract was examined by paper electrophoresis, whereby at least 4 forms of cobamide, including Barker's coenzyme and a protein-bound form were observed.

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