# ENZYMIC SYNTHESIS OF VITAMIN $B_{12}$

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Received March 15, 1968

It is a rather generally accepted view that $P^1$-cobinamide-$P^2$-guanosine pyrophosphate (GDP-cobinamide)* is a direct precursor of vitamin $B_{12}$ formation. According to Barchielli et al. (1960) this compound and vitamin $B_{12}$ might be formed from cobinamide in the following reaction sequence:

Cobinamide + ATP = Cobinamide-P + ADP

Cobinamide-P + GTP = GDP-cobinamide + PP

GDP-cobinamide + ribazole = vitamin $B_{12}$ + GMP

From data obtained in this laboratory the direct conversion of cobyric acid into GDP-cobinamide was proposed (Bartosinski et al.,1967). Nevertheless Ronzio and Barker (1967) have shown quite recently that GDP-cobinamide does form enzymatically from cobinamide-P coenzyme and GTP in the cell-free system isolated from Propionibacterium shermanii. They could not, however, demonstrate the reaction between GDP-cobinamide coenzyme and ribazole.

In the present communication we show that GDP-cobinamide coenzyme is converted into vitamin $B_{12}$ in the cell-free system isolated from E.coli 113-3 in the presence of dimethylbenzimidazole but not its riboside (ribazole). It has also been found that both supernatant as well as ribosomal fractions are indispensable for the active enzymic system. Moreover, the reaction requires NAD in the partly purified system.

## Materials and methods

The commercially available preparations were as follows: ATP/2Na, California Co.Biochem.Research; DMB, Reanal, Hungary; NAD, Serva, Heilderberg, Germany. Cobinamide, cobinamide-P, GDP-cobinamide cyanides and their coenzymic derivatives

*Abbreviations used: GDP-cobinamide, $P^1$-cobinamide-$P^2$-guanosine pyrophosphate; GDP-cobinamide coenzyme, coenzymic form of GDP-cobinamide containing 5 -deoxyadenosyl moiety attached to the cobalt atom; Cobinamide-P, cobinamide phosphate; DMB, 5,6-dimethylbenzimidazole; ribazole, DMB-riboside, 1-α-D-ribofuranoside-5,6-dimethylbenzimidazole; DMB-ribotide, 1-α-D-ribofuranoside-5,6-dimethylbenzimidazole-$3^1$-phosphate.

were isolated by the method of Pawelkiewicz et al.(1961). DMB-riboside was prepared from vitamin $B_{12}$ according to Friedrich and Bernhauer (1957). DMB-ribotide was isolated and purified according to Friedmann and Harris (1965). $^{14}$C-2-DMB was prepared from 1,2-dimethyl-4,5-diaminobenzene and sodium $^{14}$C-formate by the method of Phillips (1928).

Cell-free extracts of E.coli 113-3 were prepared by the sonication of suspended cells in 0.02 M potassium phosphate buffer, pH 8.6, for 5 min. at 0-4°. The suspension was then centrifuged at 32 000 x g for 20 min. The bacteria were grown at 36° for 12 hrs on the aerated medium containing enzymic casein hydrolysate, potassium phosphate, magnesium sulphate and glucose. They were harvested in a Sharples centrifuge.

Vitamin $B_{12}$ was determined enzymatically by the method of Pawelkiewicz and Schneider (1967) modified by the present authors. The details of it will be described elsewhere. Protein was determined by the tannin method of Mejbaum-Katzenellenbogen (1955).

## Results and discussion

The results of vitamin $B_{12}$ synthesis in a cell-free system of E.coli 113-3 are shown in table 1. Under the conditions used the best precursors of vitamin $B_{12}$ formation appeared to be GDP-cobinamide coenzyme and free DMB. DMB could be substituted by its ribotide but the yield dropped three-fold in that case. DMB-riboside which was postulated as an intermediate of vitamin $B_{12}$ biosynthesis (Barbieri et al.1962) was inactive in our experiments. GDP-cobinamide cyanide as well as cobinamide-P and cobinamide in their cyanide and coenzymic forms were inactive. However, when ATP, glucose and ammonium lactate were added to the incubation mixture all the above mentioned corrinoids were transformed into vitamin $B_{12}$ although with a lower yield. This observation may explain the first data given by Sanders et al.(1959) in a short communication on the synthesis of vit. $B_{12}$ from cobinamide and DMB in E.coli 113-3 cell-free extracts.

Fractionation of crude E.coli extracts by ultracentrifugation has shown that 105 000 x g supernatant as well as ribosome fractions are indispensable for vitamin $B_{12}$ synthesis (table 2). The role of the ribosomal fraction is not yet clear and remains to be elucidated. It has been found, however, that it firmly binds DMB, and that ribosome-bound DMB is incorporated into vitamin $B_{12}$.

When 0.9 μmole of 2-$^{14}$C-DMB (716 000 cpm/μmole) was preincubated with 100 mg of a ribosomal fraction in 50 ml of 0.02 M K-phosphate buffer, pH 8.6, at 37° for 6 hrs a most of the radioactivity was not washed from the ribosomes with the above buffer. When ribosome-bound $^{14}$C-DMB, suspended in 20 ml of phosphate buffer, was incubated for a further 6 hrs at 37° with 150 mg of protein of 105 000 x g supernatant and 1 μmole of GDP-cobinamide coenzyme, labeled vitamin $B_{12}$ was formed. From the reaction mixture 105 mμmoles of purified cyanocobalamin was isolated.

Table 1

The enzymic synthesis of vitamin $B_{12}$ in a cell-free system of E. coli 113-3

| Corrinoid | DMB or its derivatives | Vitamin $B_{12}$ formation in picomoles |
|---|---|---|
| GDP-cobinamide-coenzyme | DMB | 375 |
| | DMB-riboside | less than 10 |
| | DMB-ribotide | 125 |
| GDP-cobinamide-cyanide | DMB | less than 10 |
| Cobinamide-P coenzyme | DMB | less than 10 |
| Cobinamide-P cyanide | DMB | less than 10 |
| Cobinamide coenzyme | DMB | less than 10 |
| Cobinamide cyanide | DMB | less than 10 |

The incubation mixture contained in a final volume of 1 ml: 20 μmoles of potassium phosphate buffer, pH 8.6; 0.4 μmole of $MgCl_2$ ; 1 mμmole of corrinoid; 5 mμmoles of DMB or its derivative; and 5 mg protein of the E.coli extract. The incubation was carried out at 37° for 6 hrs.

Table 2

Synthesis of vitamin $B_{12}$ from GDP-cobinamide coenzyme and DMB by E.coli 113-3 enzymes

| Sample | Vitamin $B_{12}$ formation in picomoles |
|---|---|
| Complete system | 490 |
| Ribosomal fraction omitted | 60 |
| Supernatant fraction omitted | 50 |

The complete incubation mixture contained in a final volume of 1 ml: 50 μmoles of potassium phosphate buffer, pH 8.6; 0.4 μmole of $MgCl_2$; 5 mμmoles of DMB; 1 mμmole of GDP-cobinamide coenzyme; 2 mg of 105 000 x g supernatant protein and 2 mg ribosomal protein. The reaction mixture was incubated 6 hrs at 37°.

Its specific activity amounted to 600 000 cpm/μmole.

Experiments were next carried out to fractionate the supernatant proteins by ammonium sulphate. Four fraction were obtained as follows: 1 0-20 % saturation,

II 20-30 % sat., III 30-38 % sat., and IV 38-50 % sat. After dialysis against 0.02 M K-phosphate buffer, pH 8.6, each fraction was investigated in the vitamin $B_{12}$ synthesis system. Results are shown in table 3.

Table 3

Vitamin $B_{12}$ synthesis in the system containing proteins of supernatant fraction fractionated by ammonium sulphate

| Fraction | Vitamin $B_{12}$ formation in picomoles | Mixture of fractions | Vitamin $B_{12}$ formation in picomoles |
|---|---|---|---|
| I (0-20 % sat.) | 0 | I + II | 60 |
| II (20-30 % sat.) | 0 | I + III | 80 |
| III (30-38 % sat.) | 30 | I + IV | 50 |
| IV (38-50 % sat.) | 60 | II + III | 70 |
| | | II + IV | more than 300 |
| | | III + IV | more than 300 |

The incubation mixture contained in a final volume of 1 ml: 16 μmoles of K-phosphate buffer, pH 8.6; 0.4 μmole of $MgCl_2$; 1 mμmole of GDP-cobinamide coenzyme; 2 mg of DMB-ribosome complex; and 1 mg of the protein of investigated fraction. The incubation time 6 hrs at 37°.

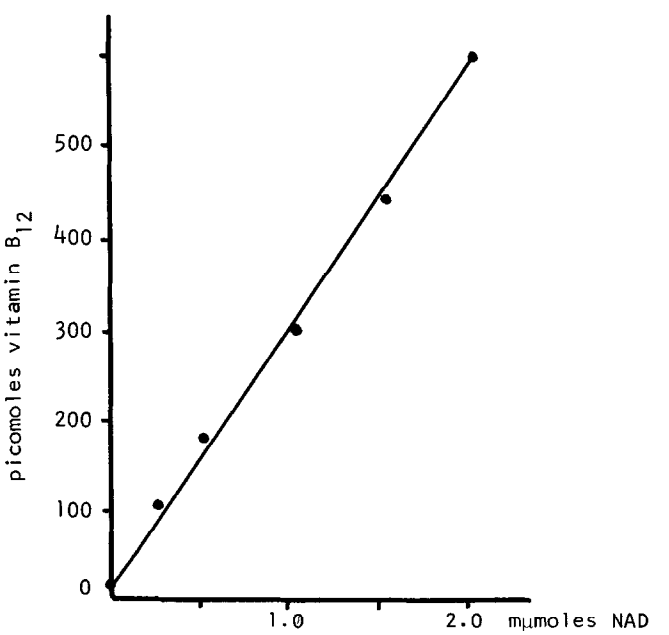


Fig.1. The effect of NAD concetration on the synthesis of vitamin $B_{12}$. Assays contained in a final volume of 1 ml: 16 μmoles of K-phosphate buffer, pH 8.6; 0.4 μmole of $MgCl_2$; 1 mμmole of GDP-cobinamide coenzyme; 2 mg of DMB-ribosome complex; 1.5 mg of fraction III protein and varying concetrations of NAD. Incubation time 6 hrs at 37°.

These data suggested that 105 000 g supernatant might contain two active compounds at least. Fraction IV appeared further to be thermostable and was not inactivated by treatment with isopropanol or dilute acetic acid. The search for the heat stable factor in this fraction has revealed that it could be replaced by NAD in the vitamin $B_{12}$ synthetizing system. This result suggests that fraction IV may contain firmly bound NAD which is released during the synthesis of vitamin $B_{12}$. The effect of NAD concentration on the vitamin $B_{12}$ formation is depicted in Figure 1.

Formation of vitamin $B_{12}$ increases in direct proportion to the amount of added NAD over the range between 0 and 2 mμmoles. Therefore, this compound may be considered as a co-substrate rather than a co-factor in the synthesis. The preliminary experiments with NAD labeled in the ribose moieties seem to indicate that NAD may provide its sugar moiety to the nucleotide part of vitamin $B_{12}$. This fact would also be consistent with the earlier data of Friedmann and Harris (1965) and Friedmann (1965) who have demonstrated that pyridine nucleotides take part in DMB metabolism in Prop.shermanii extracts.

Acknowledgements

One of us (T.K.) is grateful to the Ministry of High Education of Poland for a Research Fellowship.

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