ENZYMATIC SYNTHESIS OF A B_{12} COENZYME*

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The cobamide coenzymes, discovered by Barker and his colleagues (Barker et al., 1958; Weissbach et al., 1959), contain a deoxyadenosine moiety linked covalently via the 5'-carbon to the central cobalt atom. The enzymatic conversion of the various forms of vitamin B_{12} to the corresponding cobamide coenzymes has been investigated previously with preparations from <u>Propionibacterium shermanii</u> (Brady et al., 1962) and <u>Clostridium tetanomorphum</u> (Weissbach et al., 1962). The above workers observed that the overall reaction was stimulated by DPNH, FAD or FMN, glutathione, ATP and Mg^{++}. These cofactor requirements suggested a multi-step pathway involving the reduction of B_{12} followed by adenosylation of a reduced form of the vitamin**. This hypothesis is supported by the fact that the chemical synthesis of cobamide coenzymes has been achieved by reducing vitamin B_{12} to the Co^{I} stage prior to its reaction with adenosine-5'-tosylate (Müller and Müller, 1962; Johnson et al., 1963). Alternatively, Weissbach et al. (1962) have proposed

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** In the case of B_{12a} (5,6-dimethylbenzimidazolylhydroxocobamide), in which the oxidation state of the cobalt atom is +3 (Co^{III}), the corresponding reduced forms have been designated B_{12r} and B_{12s}. The oxidation state of the cobalt atom is +2 (Co^{II}) and +1 (Co^{I}), respectively, in these reduced forms.

that the vitamin ⟶ coenzyme conversion occurs as a concerted reaction in which the bound cyanide anion is replaced by the adenosyl group of ATP.

The present communication defines further the cobamide coenzyme-synthesizing system in C. tetanomorphum, and presents evidence on the validity of the stepwise mechanism involving the +1 oxidation state of the cobalt as the reactant with ATP.

Table I shows the cofactor requirements for the conversion of B_{12a} (5,6-dimethylbenzimidazolylhydroxocobamide) to the cobamide coenzyme in crude extracts of C. tetanomorphum. Confirming the results of previous investigators, DPNH and FAD can serve as reductants for the overall reaction (Expt. 1). Reduced lipoic acid, however, is a much better reductant,

Table I

Cofactors for B_{12} Coenzyme Synthesis

Experiment	Reductant added	B_{12} coenzyme synthesized
		mμmoles
1	DPNH + FAD	9.0
2	Lipoic acid	50.0
3	KBH_4	64.0

Each system contained in a Thunberg tube: 200 μmoles of potassium phosphate buffer, pH 7.5, 60 mμmoles of vitamin B_{12a}, 10 μmoles of 2-mercaptoethanol, 4 μmoles of $MgCl_2$, 0.2 μmoles of ATP and 0.5 mg. of a protamine-treated sonicate of C. tetanomorphum in a total volume of 1.0 ml. Where indicated, the following reductants were added: 0.4 μmoles of DPNH, 0.2 μmoles of FAD, 2 μmoles of reduced lipoic acid, 3 mg. of KBH_4. Blanks were identical except for the omission of B_{12a} or the omission of reductant. The tubes were evacuated, flushed several times with N_2, and incubated in the dark at 37° C. for 30 min. The amount of B_{12} coenzyme synthesized was measured in aliquots of the above mixtures, using the glutamic-β-methyl aspartic isomerase system described by Barker et al. (1960).

as shown by Expt. 2. In either instance, synthesis of the B_{12}-coenzyme is completely abolished if ATP and Mg^{++} are omitted. The requirement for enzymatic reduction can be obviated if the B_{12a} is reduced chemically to B_{12s} with borohydride prior to interaction of the latter compound with ATP (Expt. 3). These experiments suggest that the overall reaction proceeds in a step-wise fashion:

$$B_{12a} \longrightarrow B_{12r} \longrightarrow B_{12s} \qquad (1)$$

$$B_{12s} + \text{ATP} \longrightarrow B_{12} \text{ coenzyme} \qquad (2)$$

Further evidence on the existence of separate enzymes for reactions (1) and (2) was provided by the observation that, upon aging, preparations lose their ability to synthesize B_{12} coenzyme from B_{12a}, but not from B_{12s}. Accordingly, our initial efforts were directed toward purification of the adenosylating enzyme responsible for reaction (2). Sonicates of C. tetanomorphum were treated with protamine to remove nucleic acids, fractionated with solid ammonium sulfate (45-75% precipitate), and chromatographed on Sephadex and DEAE cellulose. The enzyme was stabilized by the presence of 2 x 10^{-3} M mercaptoethanol at all stages of purification. By this procedure, the adenosylating enzyme has been purified about 130-fold with a 30% overall recovery. The most purified preparation had a specific activity of 10.8 mμmoles/min./mg. protein at 37° C.

Reaction (2) is demonstrated spectrophotometrically in Fig. 1. In an anaerobic cuvette, B_{12a} was reduced with borohydride and the resulting B_{12s} (solid line) was allowed to react with ATP, Mg^{++} and purified adenosylating enzyme. The mixture was incubated at 37° C. for 60 minutes in order to insure completion of the reaction. The spectrum of the product, shown by the dashed line, may be compared to that of an authentic sample of 5,6-dimethylbenzimidazolylcobamide coenzyme, kindly supplied by Dr. D.

Perlman. When the above experiment was repeated with B_{12r}, prepared by the anaerobic photolysis of B_{12} coenzyme (Brady and Barker, 1961), no reaction was observed over a period of several hours.

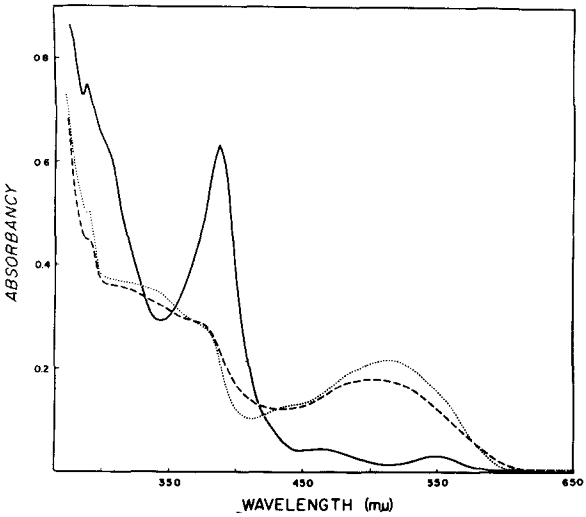
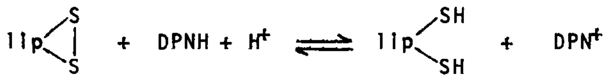


Figure 1. Spectrophotometric Demonstration of Reaction (2).

The experimental cuvette contained 60 mμmoles of B_{12a}, 400 μmoles of potassium phosphate buffer, pH 7.5, 4 mg. of KBH_4, 0.2 μmoles of ATP, 10 μmoles of mercaptoethanol, 4 μmoles of $MgCl_2$ and 0.18 mg. of purified adenosylating enzyme in a total volume of 2.0 ml. The blank was identical except for the omission of the B_{12a}. Spectra were taken with a Cary recording spectrophotometer, Model 14.

It is of interest that a <u>vicinal</u> dithiol, lipoic acid, is used to provide the reducing power for converting B_{12a} to B_{12s}. Dolphin and Johnson (1963) have shown that B_{12a} reacts non-enzymatically with monothiols to produce compounds in which the cobalt is in a lower oxidation state and perhaps complexed with the thiol. Preliminary studies have been undertaken on the isolation of the lipoic acid-dependent B_{12} reductase responsible for reaction 1. It is possible that the requirement for DPNH and FAD, noted in Table 1, may be due to the presence in the crude system of an FAD-dependent lipoyl dehydrogenase (reaction 3). A similar



$$\text{lip}\left\langle {S \atop S} \right| + \text{DPNH} + H^+ \rightleftharpoons \text{lip}\left\langle {SH \atop SH} \right. + \text{DPN}^+ \qquad (3)$$

requirement for DPNH and FAD, which could be replaced by reduced lipoic acid, has been noted previously by Reichard (1962) in the bacterial system responsible for the direct conversion of CDP to dCDP. If the system for reduction of CDP is, in fact, the same as that reported herein, one would expect B_{12s} to be the actual reductant of the nucleotide.

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