THE ENZYMATIC SYNTHESIS OF COBAMIDE COENZYMES*

Roscoe O. Brady+ and H. A. Barker

Department of Biochemistry, University of California, Berkeley, California

Received April 4, 1961

The participation of B_{12} coenzymes has been established in the isomerization of glutamate to β-methylaspartate (Barker et al., 1958), the conversion of methylmalonyl-CoA to succinyl-CoA (Smith and Monty, 1959; Gurnani et al., 1960; Stadtman et al., 1960; Stern and Friedman, 1960), and the dismutation of glycols to the corresponding deoxyaldehydes (Abeles and Lee, 1961). Evidence for the biosynthesis of B_{12} coenzymes in vitro has been briefly mentioned by Pawelkiewicz et al. (1960) and Bernhauer et al. (1960). These investigators have stated that extracts of Propionibacterium shermanii catalyze the conversion of aquocobamides to coenzymes in the presence of ATP and Mg^{++}. No experimental details were given in these reports. We have undertaken a detailed investigation of the enzymatic formation of cobamide coenzymes. The present communication describes the preparation and partial purification of the requisite enzymes from extracts of P. shermanii and establishes the nature of the substrates and cofactors required for coenzyme synthesis.

P. shermanii (ATCC 9614) was grown anaerobically in a medium containing yeast extract, glucose, $CaCO_3$, and salts essentially as described by Perlman and Barrett (1958) for 48 hours at 30° with stirring. At this time, additional glucose and $CoCl_2$ were added and the incubation was continued for 8 hours with aeration. The pH was maintained near neutrality with NH_4OH.

---

*This investigation was supported in part by research grants from the National Institutes of Health (E-563), United States Public Health Service, and the National Science Foundation (G-7500) and by a research contract with the Atomic Energy Commission.

+Permanent address: National Institute of Neurological Diseases and Blindness, Bethesda, Maryland.

The washed cells and 16% of their weight of grade FFF Corundum were suspended in 1.5 volumes of 0.01 M potassium phosphate buffer (pH 7.0) and oscillated in a Raytheon 10 KC sonic oscillator for 20 minutes at 0°. The mixture was centrifuged at 18,000 x g for 15 minutes and the supernatant solution was then centrifuged at 80,000 x g for 1 hour. The enzymes which catalyze the synthesis of B_{12} coenzymes were found in the supernatant solution. They were precipitated between 35 and 40% saturation with $(NH_4)_2SO_4$ at pH 6.5 and redissolved in 0.05 M potassium phosphate buffer (pH 7.0). When this fraction was supplemented with the requisite cofactors, its specific activity was 9 times greater than that of the crude extract.

The amount of B_{12} coenzyme synthesized was determined by the spectrophotometric glutamate isomerase coenzyme assay (Barker et al., 1960a). Because of the favorable K_m of benzimidazolylcobamide coenzyme (BC coenzyme) in this assay (Barker et al., 1960b), benzimidazolylcobamide hydrate or cyanide was used as a substrate in most of the experiments. The optimum pH for BC coenzyme synthesis is 7.0. Phosphate buffer is superior to Tris, triethanolamine, or collidine buffer.

Experiments with the protein fraction precipitated by 35-40% saturation with $(NH_4)_2SO_4$ revealed that synthesis of coenzyme proceeded at maximum velocity only when supplemented with benzimidazolylaquocobamide, adenosine triphosphate (ATP), flavin adenine dinucleotide (FAD), reduced glutathione (GSH), and reduced diphosphopyridine nucleotide (DPNH) (Table I).

The observation that benzimidazolylcyanocobamide is only two-thirds as effective as the aquo form suggests that the cyano group must be displaced from the vitamin before conversion to the coenzyme can occur. Adenosine diphosphate was utilized as well as ATP, while adenosine-5'-monophosphate, adenosine, adenine, uridine triphosphate, guanosine triphosphate and inosine triphosphate were inactive. These results suggest the participation of a phosphorylated intermediate in the reaction. The addition of the adenine nucleoside produced by photolysis of BC coenzyme

TABLE I

ENZYMATIC SYNTHESIS OF BENZIMIDAZOLYLCOBAMIDE COENZYME

Except as indicated, the reaction mixtures contained 5 μmoles of potassium phosphate buffer (pH 7.0), 150 mμmoles of ATP, 150 mμmoles of $MnCl_2$, 25 mμmoles of DPNH, 1 mμmole of benzimidazolylaquocobamide, 2.5 mμmoles of FAD, 0.5 μmole of GSH, and a protein fraction precipitating between 35 and 40% saturation with ammonium sulfate (40 μg of protein) in a final volume of 0.05 ml. After incubating for 30 minutes at 37° in the dark, the reaction was stopped by the addition of 0.01 ml of 10% $HClO_4$. After centrifugation, the supernatant solutions were neutralized by the addition of 0.008 ml of 2.5 M KOH. After an additional centrifugation to remove $KClO_4$, the amount of BC coenzyme in aliquots of the supernatant solution was determined by the spectrophotometric glutamate isomerase coenzyme assay (Barker et al., 1960a).

Reactant omitted	Benzimidazolylcobamide coenzyme formed
	mμmole
None	0.53
Benzimidazolylaquocobamide replaced with 1 mμmole of benzimidazolylcyanocobamide	0.34
DPNH	0.16
GSH	0.03
$MnCl_2$	0.03
ATP	0.02
FAD	0.02
Benzimidazolylaquocobamide	0.01
None, reaction product photolysed	0.01
None, glutamate omitted from assay mixture	0.01

(Weissbach et al., 1960) was without effect. It is of interest in this connection that FAD, flavin adenine mononucleotide, and riboflavin were all approximately equally effective at the relatively high concentration (5×10^{-4} M) employed in the present experiments, whereas none of the flavin-containing cofactors were active at 5×10^{-6} M. These results suggest that the ribityl moiety of these components may be serving as a source of the sugar moiety of the newly synthesized coenzyme.

Mn^{++} was the most effective divalent cation. Co^{++}, Mg^{++}, and Zn^{++} exhibited, respectively, 69%, 65%, and 32% of the activity of Mn^{++}.

Oxidized glutathione was inactive in the system, whereas 2-mercaptoethanol appeared to be as effective as GSH. Cysteine was 40% as effective

as GSH. These observations indicate that the requirement for sulfhydryl-containing compounds probably is nonspecific.

Reduced triphosphopyridine nucleotide was only one-fourth as active as DPNH. The oxidized forms of these cofactors were inactive. The requirement for DPNH is consistent with the evidence that conversion of vitamin B_{12} analogues to the respective coenzymes is a reductive process which may involve reduction of the cobalt from the trivalent form in the vitamin to the divalent form in the coenzyme (Pawelkiewicz et al., 1960; Brady and Barker, 1961). Preliminary experiments indicate that DPNH is not required if the reaction is carried out anaerobically in the presence of riboflavin previously reduced with hydrogen and palladium.

Evidence that the product of the enzymatic synthesis is BC coenzyme is provided by the absolute requirement of the system for benzimidazolylaquocobamide or benzimidazolylcyanocobamide. The reaction product is completely inactivated by irradiation with an 100 W tungsten lamp for 10 minutes at 10 cm distance, and there is an absolute dependence upon the presence of glutamate in the coenzyme assay mixture. Additional experiments indicate that when aquocobalamin is substituted for its benzimidazole analogue, the product formed by extracts of P. shermanii is coenzyme B_{12} (5,6-dimethylbenzimidazolylcobamide coenzyme). The formation of coenzyme B_{12} is indicated by the fact that the coenzyme could be detected with the glycol-deoxyaldehyde coenzyme assay of Abeles and Lee (1961) but not with the glutamate isomerase assay, which is relatively insensitive to coenzyme B_{12}. We are indebted to Dr. Joseph R. Stern for the former assays.

REFERENCES

Abeles, R. H., and Lee, H. A., Jr., J. Biol. Chem., 236, PC1 (1961).
Barker, H. A., Smyth, R. D., Weissbach, H., Munch-Petersen, A., Toohey, J. I., Ladd, J. N., Volcani, B. E., and Wilson, R. M., J. Biol. Chem., 235, 181 (1960a).
Barker, H. A., Smyth, R. D., Weissbach, H., Toohey, J. I., Ladd, J. N., and Volcani, B. E., J. Biol. Chem., 235, 480 (1960b).
Barker, H. A., Weissbach, H., and Smyth, R. D., Proc. Nat. Acad. Sci. U.S., 44, 1093 (1958).

Bernhauer, K., Gaiser, P., Muller, O., and Wagner, O., Biochem. Z., 333, 106 (1960).
Brady, R. O., and Barker, H. A., Biochem. Biophys. Res. Comm., in press.
Gurnani, S., Mistry, S. P., and Johnson, B. C., Biochim. Biophys. Acta, 38, 187 (1960).
Pawelkiewicz, J., Bartosinski, B., and Walerych, W., Bull. Acad. Polonaise Sciences, Cl. II, 8, 123 (1960).
Perlman, D., and Barrett, J. M., Canadian J. Microbiol., IV, 9 (1958).
Smith, R. M., and Monty, K. J., Biochem. Biophys. Res. Comm., 1, 105 (1959).
Stadtman, E. R., Overath, P., Eggerer, H., and Lynen, F., Biochem. Biophys. Res. Comm., 2, 1 (1960).
Stern, J. R., and Friedman, D. L., Biochem. Biophys. Res. Comm., 2, 82 (1960).
Weissbach, H., Ladd, J. N., Volcani, B. E., Smyth, R. D., and Barker, H. A., J. Biol. Chem., 235, 1462 (1960).