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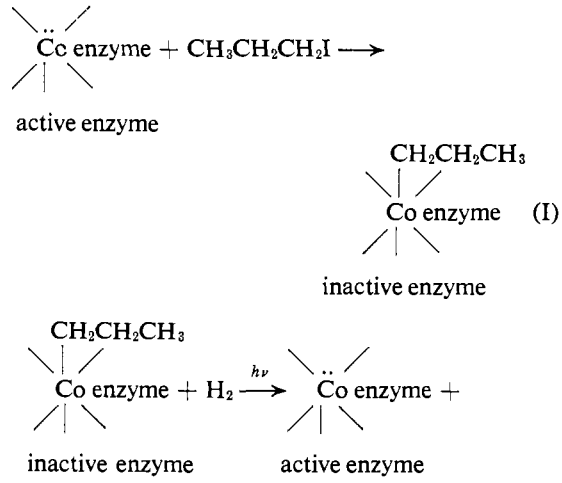
Propylation and Purification of a B_{12} Enzyme Involved in Methane Formation*

J. M. Wood and R. S. Wolfe

ABSTRACT: A new technique for the isolation of a B_{12} enzyme has been developed by use of a radioactive propyl donor which labels and stabilizes the B_{12} enzyme during purification. [1-^{14}C]Propyl iodide was used to purify a [1-^{14}C]propyl-B_{12} enzyme complex; removal of the [1-^{14}C]propyl group as [^{14}C]propane, by photolysis under hydrogen, yielded a B_{12} enzyme which was active in the final methyl-transfer reaction leading to the formation of CH_4 in *Methanobacillus omelianskii*.

Brot and Weissbach (1965) recently have confirmed that a B_{12} enzyme complex participates in the methyl-transfer reaction leading to the biosynthesis of methionine in *Escherichia coli*. This cobamide enzyme was shown to be strongly inhibited by alkylation with low concentrations of propyl iodide, and this inhibition could be reversed by cleavage of the carbon–cobalt bond with light. In a preliminary report we have presented evidence which supports the existence of a similar enzyme which is involved in the final methyl-transfer reaction leading to the formation of CH_4 in *Methanobacillus omelianskii*. Propylation of this enzyme and its reactivation by light led us to the detection of propane as the anaerobic photolysis product (Wood and Wolfe, 1966). The proposed mechanism of inhibition of this enzyme by propyl iodide, followed by its reactivation by light, is summarized as follows.

Extraction of the [1-^{14}C]propyl-B_{12} compound from the [1-^{14}C]propyl-B_{12} enzyme was accomplished, and this alkyl corrinoid was shown to have similar spectral properties to propyl-Factor III (propyl-Co-5-hydroxybenzimidazolylcobamide). Removal of this [1-^{14}C]propyl group as [1-^{14}C]propane, by photolysis under H_2, followed by oxidation of the resulting reduced B_{12} compound in air, gave a product with identical properties to aquo-Factor III (aquo-Co-5-hydroxybenzimidazolylcobamide).



To obtain the evidence presented below the sensitive propane assay was used to locate B_{12} enzymes during ammonium sulfate fractionation procedures, and [1-

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^{14}C]propyl iodide was used to label the B_{12} enzymes in order to follow their chromatographic separation on columns.

Experimental Section

Culture Methods and Preparation of Extracts. M. omelianskii (kindly provided by H. A. Barker) was cultured, harvested, and washed by the method outlined by Wolin *et al.* (1963). Cell-free extracts were prepared by exposing 100-ml batches, containing 1 g of cells (wet weight)/ml of 0.5 M potassium phosphate buffer, pH 7.0, to the maximum frequency output of a Branson sonic probe for 2 min at 0°. Cell debris was removed by centrifugation at 23,000*g* for 20 min at 0°. Crude extracts prepared in this manner contained from 50 to 60 mg of protein/ml. Protein was determined by the method of Lowry *et al.* (1951). The latter method was used since protein-bound corrinoid compounds were found to contribute to the extinction of these proteins at 280 mμ.

Chemical and Analytical Methods. Methylcobalamin[1] was prepared by the method of Müller and Müller (1962). 5-CH_3-H_4-folate[1] was prepared and standardized by the methods described by Wood *et al.* (1965). Aquo-Factor III was synthesized by the method of Wood *et al.* (1966). Methane and propane were assayed by the gas chromatographic technique previously described by Wood *et al.* (1965, 1966). [1-^{14}C]Propyl iodide was obtained from Nuclear Research Chemicals Co., Inc. Cellex T (TEAE-cellulose) was treated as described by Guest *et al.* (1964), and was obtained from Bio-Rad. Hydroxylapatite, exchange capacity 65 mg/g, was obtained from the Clarkson Chemical Co., Inc. Phosphocellulose was treated as described by Lezius and Barker (1965) and was obtained from Bio-Rad. In certain experiments elution of protein from the gel was obtained by passing the enzyme fraction up the gel column by use of an LKB Re-Cy-Chrom peristaltic pump.

Isotope Assay. [1-^{14}C]Propyl-B_{12} enzymes were detected by applying a 0.1-ml sample of each fraction to a separate Bac-T-Flex membrane filter, diameter 27 mμ, Schleicher and Schuell Co., Keene, N. H. Each membrane filter was dried by the hot air blast from a hair dryer, in the dark, before being counted in a Packard Tri-Carb scintillation spectrometer with a scintillation fluid composed of 0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene and 4.0 g of 2,5-diphenyloxazole/l. of toluene.

Photolysis. Photolysis of the propylcobamide–protein was carried out conveniently in a scintillation vial fitted with a serum stopper. After the addition of 1.0 ml of propylated protein fraction to each scintillation vial, the vial was flushed for 15 min with a continuous stream of O_2-free H_2. The vial was placed on its side, and each fraction was subjected to 20-min illumination from a 200-w tungsten filament lamp at a distance of 15 cm. The cold air blast from a hair dryer was used to prevent heating of protein fractions during the photolysis process.

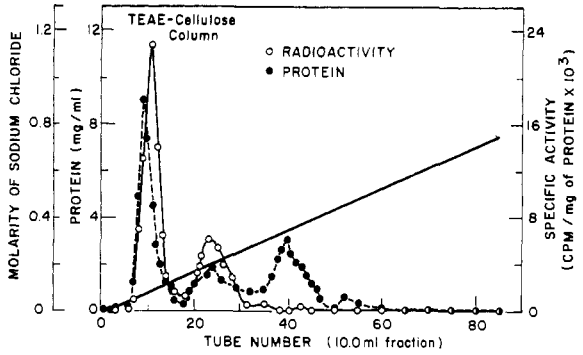

FIGURE 1: Chromatographic separation of [1-^{14}C]-propyl-B_{12} enzymes on TEAE-cellulose.

Assay of Enzymatic Activity. The standard assay system for methane formation consisted of: crude extract, 32.0 mg of protein; ATP, 10.0 μmoles; methylcobalamin, 5.0 μmoles (or *dl*-5-CH_3-H_4-folate, 10.0 μmoles); photolyzed fraction as desired; and 760 μmoles of potassium phosphate buffer, pH 7.0; total liquid volume, 2.2 ml; gas phase, H_2; incubation temperature, 40°. Crude extract for the standard assay system was prepared from a batch of washed cells which was stored at 4° under H_2 to preserve consistent CH_4-forming ability. In the above assay system the amount of crude extract used was chosen so as to catalyze only a low rate of methane formation; stimulation of methane formation by addition of the B_{12} enzyme could then be followed.

The specific activity of the B_{12} enzyme (micromoles of CH_4 per milligram of protein per hour) was determined by measuring the rate of CH_4 formation in reaction mixtures which contained photolyzed B_{12} enzyme, and subtracting the rate of CH_4 formation by the standard assay mixture which contained no added B_{12} enzyme. This assay was found to be consistent, and the rate of CH_4 formation was repeatedly shown to be directly proportional to B_{12} enzyme concentration. Reactions catalyzed by the standard assay mixture alone never exceeded a rate of 0.10 μmole of CH_4/mg of protein per hr, and was never less than 0.08 μmole of CH_4/mg of protein per hr.

Results

Isolation of the B_{12} Enzyme. To obtain the data presented in Figure 1, 347 g of fresh cells of *M. omelianskii* was washed and disrupted by sonication to give 400 ml of crude extracts. These extracts were fractionated with a saturated solution (pH 7.0) of enzyme-grade ammonium sulfate; the fraction which precipi-

[1] Abbreviations used: propyl-Factor III, propyl-Co-5-hydroxybenzimidazolylcobamide; methylcobalamin, methyl-Co-5,6-dimethylbenzimidazolylcobamide; 5-CH_3-H_4-folate, N^5-methyltetrahydrofolate; ATP, adenosine 5′-triphosphate.

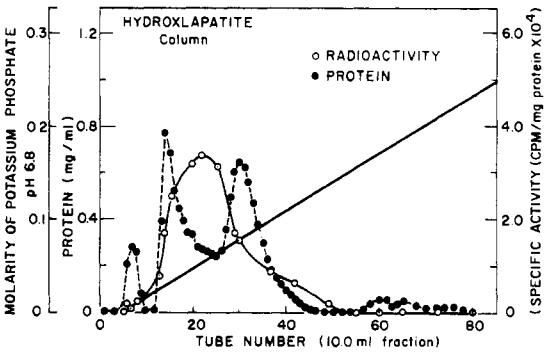

FIGURE 2: Chromatographic separation on hydroxylapatite of the [1-^{14}C]propyl-B_{12} enzyme, which is involved in the methyl-transfer reaction leading to the formation of CH_4.

tated between 80 and 100% saturation was retained, redissolved in 30 ml of 0.05 M potassium phosphate buffer, pH 7.0, and dialyzed against 8 l. of distilled water for 18 hr at 4°. After this treatment, this fraction was stirred vigorously under O_2-free H_2 for 2 hr prior to the addition of 0.5 mc of [1-^{14}C]propyl iodide (sp act. 2.76 mc/mmole). All subsequent procedures were conducted in subdued light. After allowing the [1-^{14}C]-propyl iodide to react with this fraction for 30 min at 25°, the resulting radioactive protein was applied to a TEAE-cellulose column (15 × 3 cm) and eluted with a gradient of 0.01–0.75 M sodium chloride at a flow rate of 60 ml/hr. The elution profile (Figure 1) shows that at least two [1-^{14}C]propyl-labeled B_{12} enzymes are present in the 80–100% ammonium sulfate fraction. However, only the first radioactive peak, which eluted at 0.07 M, was active after photolysis and addition to the CH_4 assay system. No catalytic function has been discovered yet for the second radioactive [1-^{14}C]-propyl-B_{12} enzyme.

The contents of tubes 8–13 from the TEAE-cellulose column were pooled, dialyzed for 18 hr against 8 l. of distilled water at 4°, applied to a hydroxylapatite column (15 × 2 cm), and eluted at a flow rate of 45.0 ml/hr. A gradient of 0.001–0.25 M potassium phosphate buffer, pH 6.8, was used to elute this fraction (Figure 2). Only one radioactive peak was obtained from this column, and the eluate of peak tubes was shown to be active after photolysis and assay for CH_4 formation.

The eluate of tubes 14–26 was pooled, dialyzed for 18 hr against 8 l. of distilled water at 4°, and concentrated to a volume of 12.3 ml by application to a second hydroxylapatite column followed by elution with 0.2 M potassium phosphate buffer, pH 6.8. This protein fraction was subjected to gel filtration by passage at a flow rate of 50 ml/hr up a Sephadex G-200 column (60 × 3 cm) which previously had been washed for 2 days with 0.05 M potassium phosphate buffer, pH 7.0. By use of this technique good resolution of the [1-^{14}C]propyl-B_{12} enzyme was obtained (Figure 3). Only one radioactive peak was found, and this was present in the excluded protein. This result suggests that the [1-^{14}C]propyl-B_{12} enzyme has a molecular weight of at least 200,000. Preliminary studies with a sucrose density gradient, with catalase as standard, suggested that the enzyme has a molecular weight of approximately 800,000. This B_{12} enzyme was found to be active only when added to a standard assay system which contained freshly prepared crude extract and ATP. All attempts to activate the B_{12} enzyme by the addition of numerous cofactors failed.

The results of the purification procedure are presented in Table I. The increase in radioactive specific activity compared to enzymic specific activity during each purification step was similar when either methylcobalamin or 5-CH_3-H_4-folate was used as methyl donor. The spectrum of the purified [1-^{14}C]propyl-B_{12} enzyme (Figure 4) is very similar to that reported for the most active form of the B_{12} enzyme which catalyzes

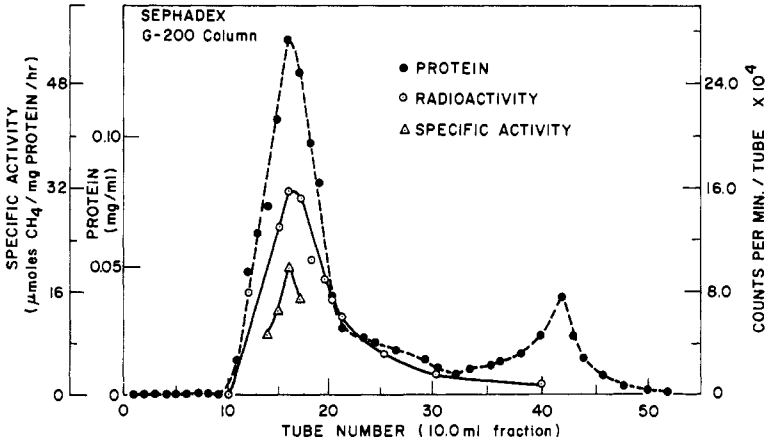

FIGURE 3: Comparison of enzymic and radioactive specific activities of the [1-^{14}C]propyl-B_{12} enzyme during gel filtration on Sephadex G-200.

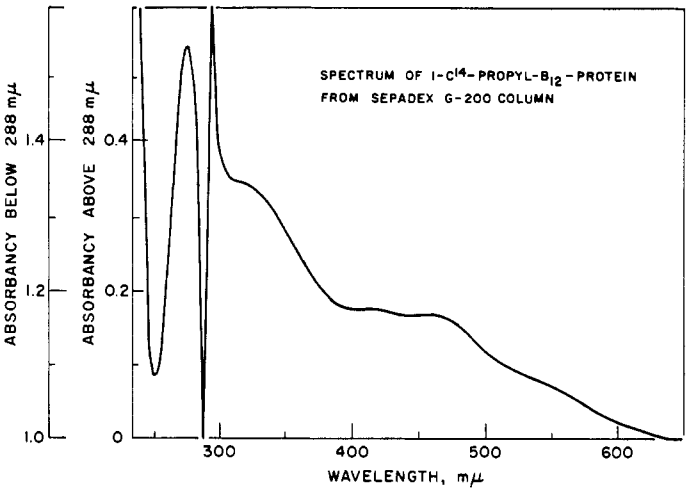

FIGURE 4: Absorption spectrum of [1-^{14}C]propyl-B_{12}-protein at a concentration of 0.86 mg of protein/ml was determined in 0.05 M potassium phosphate buffer, pH 7.0.

TABLE I: Purification of [1-^{14}C]Propyl-B_{12} Enzyme.

	Vol. (ml)	Protein (mg)	Sp Act. (μmoles of CH_4/ mg per hr)	Sp Radio-activity (cpm/mg)	% Yield of Enzyme in Pooled Fractions[a]
Crude extracts	400	8400	0.18	—	100 (−)
$(NH_4)_2SO_4$, 80–100%	30	720	1.14	([1-C^{14}]Iodopropane added, 0.5 mc)	54.3 (−)
TEAE-cellulose, tube 11	10	50	3.48	22.4×10^3	36.3 (8–13)
Hydroxylapatite, tube 22	10	2.6	6.53	33.5×10^3	16.4 (14–26)
G-200 Sephadex, tube 16	10	1.4	18.90[b]	113.9×10^3	4.8 (14–18)

[a] Since it was found to be a practical impossibility to assay all peak fractions using the CH_4 assay method, the per cent yield of enzyme recovered was calculated from the relative specific radioactivity recovered in the above pooled fractions. [b] In the above CH_4 assays methylcobalamin was used as methyl-donating substrate. When N^5-CH_3-H_4-folate was used a specific activity of 21.33 μmoles/mg of protein per hr was determined for protein excluded from the G-200 Sephadex column in tube 16.

methylmalonyl isomerization in *Propionibacterium shermanii* (Wood *et al.*, 1964).

Isolation of the Propyl-B_{12} Moiety. Isolation of the [1-^{14}C]propyl-B_{12} compound from the [1-^{14}C]propyl-B_{12} enzyme was accomplished by the following procedure. Freshly harvested cells of *M. omelianskii* (1 kg) were disrupted by sonication to give 1125 ml of crude extract which contained 46.2 mg/ml of protein. The fraction which precipitated between 80 and 100% saturation with ammonium sulfate was resuspended in 153 ml of 0.05 M potassium phosphate buffer, pH 7.0, and dialyzed for 18 hr against 8 l. of distilled water at 4°. After preincubation under O_2-free H_2 for 2 hr this fraction was allowed to react with 0.25 mc of [1-^{14}C]propyl iodide (sp act. 1 mc/mmole) for 30 min. The radioactive protein thus obtained was applied to a TEAE-cellulose column (40 × 5 cm), and the protein which was eluted with 0.07 M sodium chloride was retained. This fraction was previously shown to contain mainly the [1-^{14}C]propyl-B_{12} enzyme which is involved in the methane-forming reaction.

The B_{12} compounds present in this fraction were then extracted by stirring with 2 l. of absolute ethanol at 80° for 30 min. The precipitated protein was removed by centrifugation at 10,000*g* for 1 hr, and the supernatant solution was decreased to a volume of

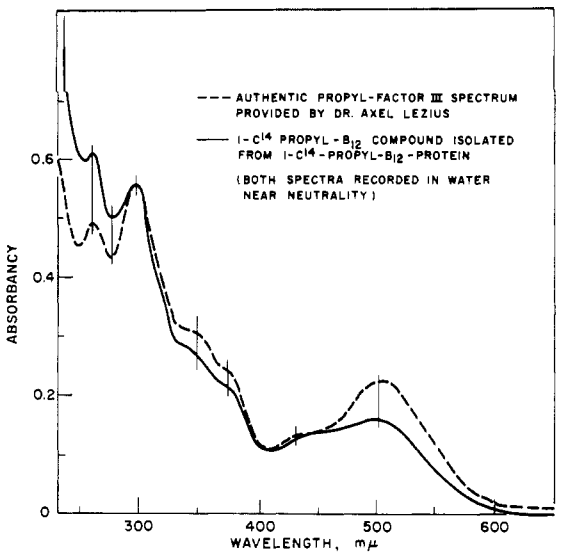

FIGURE 5: Absorption spectra of the isolated [1-^{14}C]-propyl-B_{12} compound and authentic propyl-Factor III.

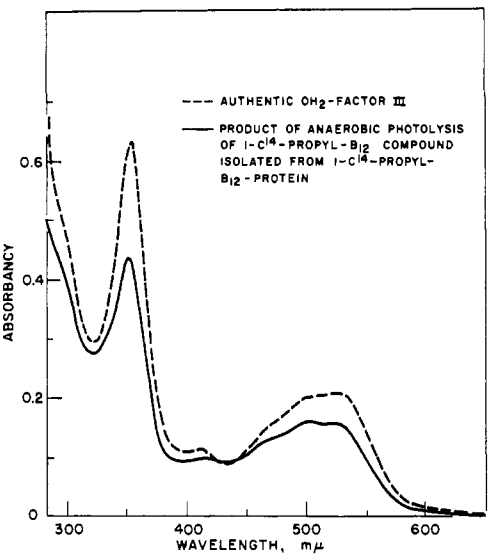

FIGURE 6: Comparison of the spectrum of the oxidized product of photolytic cleavage of the [1-^{14}C]propyl-B_{12} compound with that of authentic aquo-Factor III. Both compounds were dissolved in water.

approximately 400 ml by evaporation under reduced pressure at 60°. At this point 1 l. of distilled water was added, and the volume was reduced once again to 400 ml. The B_{12} compounds present in this aqueous solution were further extracted with phenol by the method of Johnson *et al.* (1963). After phenol extraction the aqueous fraction which contained the B_{12} compounds was adjusted to pH 3.0 with 6 N acetic acid. This solution was applied to a phosphocellulose column (10 × 1 cm) which had been equilibrated with 0.025 M sodium acetate buffer, pH 3.9.

After washing this column with 100 ml of 0.025 M sodium acetate buffer, pH 3.9, a single red peak which was radioactive was eluted with 0.025 M sodium acetate buffer, pH 4.5. The eluate in peak tubes was pooled, adjusted to pH 7.0 with 6 N ammonium hydroxide, and lyophilized. This residue was dissolved in 0.5 ml of ethanol (95%) and applied to Whatman 3MM paper followed by high-voltage electrophoresis in 4% formic acid at a potential gradient of 30 v/cm and a current of 1.25 ma/cm for 4 hr. After this procedure three yellow spots of mobilities 28.0, 22.5, and 12.5 cm were detected. Autoradiography revealed that only the spot of mobility 12.5 cm was radioactive. Each spot was eluted with water and minor impurities present in the paper were removed from each pure B_{12} compound by passing each eluate down a Sephadex G-10 column (6 × 0.5 cm) equilibrated with distilled water.

Insufficient material was obtained to identify the B_{12} compound of mobility 28.0 cm, but it was shown to have the same mobility as aquo-Factor III. The B_{12} compound with mobility 22.5 cm was identified as Factor III coenzyme after following the spectrophotometric and chromatographic analyses of Lezius and Barker (1965). The radioactive product, mobility 12.5 cm, was shown to have similar spectral properties to propyl-Factor III (Figure 5). Anaerobic photolysis of this radioactive B_{12} compound followed by its oxidation in air yielded a product which was not radioactive, and this compound had a spectrum similar to that of an authentic preparation of aquo-Factor III (Figure 6).

Discussion

Propyl iodide has been used to furnish information on the reaction mechanisms of B_{12}-dependent C_1-transfer reactions (Brot and Weissbach, 1965; Wood and Wolfe, 1966). The process of photolytic cleavage of the carbon–cobalt bond under hydrogen, to give propane as the C_3 product, indicates that a reduced form of B_{12} enzyme functions as C_1 acceptor in these reactions (Hogenkamp, 1965). This view is further supported by the observations that a variety of methyl donors readily alkylate these enzymes (Elford *et al.*, 1965; Wood *et al.*, 1966). The lack of specificity of these enzymes tends to promote the idea of Ingraham (1964) that B_{12} enzymes function as biological Grignard reagents in protein solution. The development of a very sensitive propane assay to locate the presence of B_{12} enzymes during fractionation procedures and the use of [1-^{14}C]propyl iodide to facilitate their purification have far-reaching implications. Although anaerobes lend themselves well to the application of these techniques, owing to the presence of a variety of natural reducing agents, there is no reason why the addition of a reduced flavin–adenine dinucleotide and/or ferredoxin-reducing systems should not prepare aerobic systems for similar treatment (Weissbach *et al.*, 1966). We have successfully applied these techniques to purify a Factor III enzyme. The identification of propyl-Factor III is consistent with the concept that a reduced Factor III enzyme is involved in the final methyl-transfer reaction leading to the formation of CH_4

in *M. omelianskii*. The requirement for the addition of a small amount of crude extract to this Factor III enzyme is not yet understood. It appears that the system requires another component and the possibility that it could be a second carbanion acceptor cannot be ruled out. Studies on the properties of this enzyme are in progress.

Acknowledgments

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