

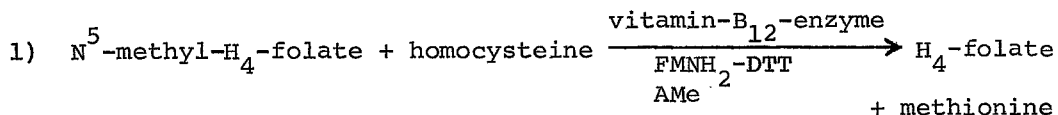
ISOLATION OF METHYL-B₁₂¹ FROM ESCHERICHIA COLI B N⁵-METHYL-H₄-
FOLATE-HOMOCYSTEINE VITAMIN-B₁₂ TRANSMETHYLASE

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Recently, we reported a procedure (1) to obtain μmole quantities of vitamin-B₁₂ transmethylase from extracts of E. coli B and a procedure (2) to propylate chemically its vitamin B₁₂ prosthetic group. E. coli B vitamin-B₁₂ transmethylase catalyzed Reaction 1 at an optimal rate in the reducing system employed for chemical alkylation (1).



Using this reducing system and μmole amounts of enzyme in the presence of Ame, evidence was then obtained that N⁵-methyl-¹⁴C-H₄-folate reacts with the vitamin-B₁₂ protein in the absence of homocysteine to form a radioactive complex containing methyl-¹⁴C groups (3). Approximately 80-90% of the bound methyl-¹⁴C could be transferred to homocysteine yielding ¹⁴CH₃-methionine (3) and these bound methyl-¹⁴C groups were not labilized by exposure to light (3). This communication presents evidence that a methyl-¹⁴C-B₁₂ prosthetic group

¹Abbreviations: methyl-B₁₂, 5,6-dimethylbenzimidazolyl-methyl cobamide; DTT, dithiothreitol; Ame, S-adenosyl-L-methionine iodide; TCA, trichloroacetic acid; OH-B₁₂, 5,6-dimethylbenzimidazolyl-aquo (hydroxo) cobamide.

is formed upon incubation with either N^5 -methyl- ^{14}C -H₄-folate or methyl- ^{14}C -AMe.

MATERIALS AND METHODS

The vitamin-B₁₂ enzyme, N^5 -methyl- ^{14}C -H₄-folate, and methyl- ^{14}C -B₁₂ were prepared as described in references 1, 4, and 5, respectively. The enzyme used here had a specific activity of 2,500 (1) and contained 1 μ mole of bound vitamin-B₁₂ derivative per mg of protein (1). Methyl- ^{14}C -AMe (50 μ c/ μ mole) was purchased from the New England Nuclear Corp. Radioactive dimedone reactive compounds were estimated as in reference 6. All radioactivity measurements were made with a liquid scintillation spectrometer employing the counting fluid of Bray (7).

Reaction mixtures to obtain a ^{14}C -enzyme with N^5 -methyl- ^{14}C -H₄-folate contained potassium phosphate buffer, pH 7.4, 20 μ moles; N^5 -methyl- ^{14}C -H₄-folate (16,500 cpm/ μ mole), 10 μ moles; AMe, 10 μ moles; DTT, 5 μ moles; FMNH₂, 50 μ moles; platinum oxide, 0.1 mg; and vitamin-B₁₂-enzyme, 0.55 mg. Incubations were for 15 minutes at 37° under H₂ as described previously (1,3).

A similar reaction mixture was used to obtain a ^{14}C -enzyme from methyl- ^{14}C -AMe except that N^5 -methyl- ^{14}C -H₄-folate was omitted and 10 μ moles of methyl- ^{14}C -AMe were employed (18,500 cpm/ μ mole).

The radioactive protein was precipitated with 10% TCA and collected on millipore filters in the dark or else separated by Sephadex G-25 chromatography as described recently (3).

RESULTS

Table I demonstrates that the protein-bound ^{14}C from either

Table I. Effect of light on TCA precipitable cpm from N⁵-methyl-¹⁴C-H₄-folate and methyl-¹⁴C-AMe

Enzyme incubated with	Reaction mixture during incubation	After incubation - prior to TCA	During TCA precipitation	TCA ppt. cpm	¹⁴ C-Bound μmoles
N ⁵ -methyl- ¹⁴ C-H ₄ -folate	Dark	Dark	Dark	5,133	0.31
	Light*	Dark	Dark	5,100	0.31
	Dark	Light*	Dark	5,064	0.31
	Dark	Dark	Light*	938	0.06
Methyl- ¹⁴ C-AMe	Dark	Dark	Dark	11,424	0.62
	Light*	Dark	Dark	11,150	0.61
	Dark	Light*	Dark	11,448	0.62
	Dark	Dark	Light*	2,454	0.13

The enzyme (0.55 mg) contained 0.55 μmoles of bound vitamin-B₁₂ derivative.

* Light - A 10-minute exposure to a 100 W tungsten lamp at 10-15 cm either during incubation or subsequently at 0°.

N⁵-methyl-¹⁴C-H₄-folate or methyl-¹⁴C-AMe is stable to both precipitation with cold 10% TCA and light unless the labeled enzyme is exposed to light subsequent to denaturation with TCA. ¹⁴C-Enzyme, illuminated after the addition of TCA lost about 80% of its radioactivity. The data in Table I was obtained by adding cold 10% TCA to the reaction mixtures and collecting the protein precipitates on a millipore filter (3). However, the same pattern has been observed with ¹⁴C-enzyme which was first isolated by Sephadex G-25 filtration, i.e. the protein-bound ¹⁴C was stable to light before TCA precipitation but was readily lost upon exposure to light after TCA precipitation. It should be noted in Table I that nearly one equivalent of TCA-photolabile ¹⁴C per equivalent of vitamin-B₁₂ enzyme was obtained

with methyl- ^{14}C AMe and one-half this amount per equivalent of vitamin- B_{12} enzyme was obtained with N^5 -methyl- H_4 -folate. In addition only one-tenth the amount of a ^{14}C -protein from methyl- ^{14}C -AMe was obtained when 30 μmoles of unlabeled N^5 -methyl- H_4 -folate was present in the incubation mixture. However, AMe was required for optimum labeling of the protein with N^5 -methyl- ^{14}C - H_4 -folate (3). A reducing system was essential to label the enzyme with either methyl donor.

The above data strongly suggested that the methyl group of both AMe and N^5 -methyl- H_4 -folate had been transferred to a reduced vitamin- B_{12} derivative on the enzyme to form a methyl- B_{12} enzyme. This was confirmed as described below. The folate and AMe incubation systems in Table I were scaled up 36 and 12-fold, respectively, and the corresponding ^{14}C enzyme was isolated in the dark by Sephadex gel filtration as recently described (3). All subsequent steps were also performed in the dark. Enzyme, 19.8 mg, labeled with N^5 -methyl- ^{14}C - H_4 -folate and enzyme, 6.6 mg, labeled with methyl- ^{14}C -AMe were then lyophilized separately and precipitated twice with cold 10% TCA. Two successive extractions with hot 80% ethanol were then carried out according to the method of Takeyama and Buchanan (8). The resulting colorless precipitates contained only 5% of the ^{14}C in the respective ethanol extracts. Unlabeled carrier methyl- B_{12} , 200 μmoles , was added to each ethanol extract which was then taken to dryness at 40° under a stream of N_2 . Each residue was dissolved in 0.25 ml of 90% ethanol and the following recoveries were noted: Vitamin- B_{12} enzyme (19.8 μmoles) incubated with N^5 -methyl- ^{14}C - H_4 -folate plus unlabeled AMe yielded 103,000 cpm (6.4 μmoles) of ^{14}C in the final concentrated

alcohol extract and the vitamin-B₁₂ enzyme (6.6 μ moles) incubated with methyl-¹⁴C-AMe alone yielded 79,000 cpm (4.3 μ moles) of ¹⁴C in the concentrated extract.

In Table II the ability of the ¹⁴C-material present in the two ethanol extracts to form a ¹⁴C-dimедone derivative before and after photolysis agrees with the results obtained with a chemically synthesized sample of methyl-¹⁴C-B₁₂. Correcting for the dimedone dependent recovery of ¹⁴C with a sample of H¹⁴CHO, the methyl-¹⁴C-AMe extract and the N⁵-methyl-¹⁴C-H₄-folate extract yielded 58% and 61%, respectively, of their ¹⁴C as a dimedone derivative upon photolysis; the photolytic yield for the methyl-¹⁴C-B₁₂ standard was 64%.

Table II. Dimedone reactivity of ¹⁴C-compound in the ethanol extracts and of methyl-¹⁴C-B₁₂ standard before and after photolysis*

Vitamin-B ₁₂ - enzyme treated with:	CPM in aliquot of alcohol extract	CPM in dimedone derivative	
		Before light	After light
Methyl- ¹⁴ C-AMe	7,000	0	3,495
N ⁵ -Methyl- ¹⁴ C- H ₄ -folate	5,500	0	2,860
-	38,000 (methyl- ¹⁴ C- B ₁₂ standard)	47	20,750
-	7,700 (¹⁴ C-formaldehyde standard)	6,580	-

* Photolysis - 100 W at 10-15 cm for 15 min at 0°.

Further identification of the radioactive material extracted from the vitamin-B₁₂ protein as methyl-B₁₂ was obtained by paper chromatography (H₂O, n-butanol, isopropanol, acetic acid, 100:100:70: water, 2-butanol, 28% NH₄OH, 36:100:24) and paper electrophoretic

separation (0.5 M acetic acid pH 2.5). All of the radioactivity that could be detected after separation in these systems was confined to the single, red, ultraviolet absorbing zone containing the carrier methyl-B₁₂; this was true of the extracts obtained after incubating the vitamin-B₁₂-enzyme with either methyl-¹⁴C-AMe or N⁵-methyl-¹⁴C-H₄-folate. If the respective ethanol extract samples and the methyl-¹⁴C-B₁₂ standard were photolyzed prior to paper chromatography or electrophoresis, essentially all the radioactivity was lost and well separated spots of OH-B₁₂ were seen. More recently, the prosthetic group on the N⁵-methyl-H₄-folate treated enzyme has been isolated in the same overall yield without a prior TCA precipitation and without the use of carrier methyl-B₁₂ (9). The spectrum of this material closely resembles that of methyl-B₁₂ and OH-B₁₂ is formed upon photolysis (9).

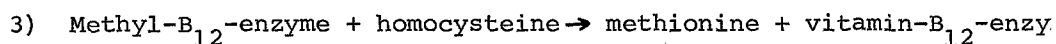
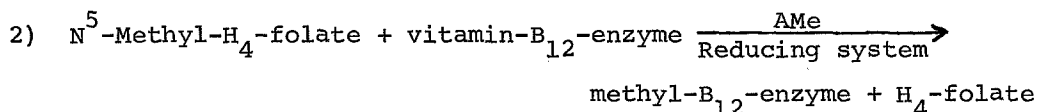
DISCUSSION

The present studies show for the first time that both N⁵-methyl-H₄-folate and AMe can react with the E. coli vitamin-B₁₂-enzyme to yield a methyl-B₁₂ enzyme. AMe is believed to function in methyl transfer from N⁵-methyl-H₄-folate by methylating a specific site on the enzyme (10). Nevertheless, the ability of AMe to form a methyl-B₁₂ enzyme may be due to a chemical reaction of this active methyl donor with the enzyme-bound cobamide which is not related to the catalysis of Reaction 1. As stated above, in the presence of N⁵-methyl-H₄-folate, AMe was only 1/10 as effective in forming a methyl-B₁₂-enzyme. In addition the methylation of the vitamin-B₁₂-enzyme by AMe occurs rapidly even at 0° (9) whereas the reaction with

N^5 -methyl- H_4 -folate requires incubation for 15 minutes at 37° (3). It should be noted that methyl group transfer from neither AMe nor N^5 -methyl- H_4 -folate to $OH-B_{12}$ will occur nonenzymatically in the $FMNH_2$ -DTT reducing system reported here (9).

It is not known why the methyl- ^{14}C -enzyme is not light-sensitive until the protein is denatured with TCA. The ability of other agents to promote photolysis of the bound methyl- ^{14}C group is being examined.

The data presented here support the following partial Reactions 2 and 3 for the overall Reaction 1.



Recently Brodie (11) using catalytic levels of an unpurified pig liver preparation has shown that both N^5 -methyl- H_4 -folate and AMe methyl groups become photolabile as a result of incorporation into carrier methyl- B_{12} . The route of this conversion has not been clarified, however.

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