# ISOLATION OF METHYL-$B_{12}$[1] FROM *ESCHERICHIA COLI* B $N^5$-METHYL-$H_4$-FOLATE-HOMOCYSTEINE VITAMIN-$B_{12}$ TRANSMETHYLASE

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Recently, we reported a procedure (1) to obtain mμmole quantities of vitamin-$B_{12}$ transmethylase from extracts of *E. coli* B and a procedure (2) to propylate chemically its vitamin $B_{12}$ prosthetic group. *E. coli* B vitamin-$B_{12}$ transmethylase catalyzed Reaction 1 at an optimal rate in the reducing system employed for chemical alkylation (1).

1) $N^5$-methyl-$H_4$-folate + homocysteine $\xrightarrow[\text{FMNH}_2\text{-DTT}\ \text{AMe}]{\text{vitamin-}B_{12}\text{-enzyme}}$ $H_4$-folate + methionine

Using this reducing system and mμmole amounts of enzyme in the presence of AMe, evidence was then obtained that $N^5$-methyl-$^{14}$C-$H_4$-folate reacts with the vitamin-$B_{12}$ protein in the absence of homocysteine to form a radioactive complex containing methyl-$^{14}$C groups (3). Approximately 80-90% of the bound methyl-$^{14}$C could be transferred to homocysteine yielding $^{14}CH_3$-methionine (3) and these bound methyl-$^{14}$C groups were not labilized by exposure to light (3). This communication presents evidence that a methyl-$^{14}$C-$B_{12}$ prosthetic group

[1] Abbreviations: methyl-$B_{12}$, 5,6-dimethylbenzimidazolyl-methyl cobamide; DTT, dithiothreitol; AMe, S-adenosyl-L-methionine iodide; TCA, trichloroacetic acid; OH-$B_{12}$, 5,6-dimethylbenzimidazolyl-aquo (hydroxo) cobamide.

is formed upon incubation with either $N^5$-methyl-$^{14}C$-$H_4$-folate or methyl-$^{14}C$-AMe.

## MATERIALS AND METHODS

The vitamin-$B_{12}$ enzyme, $N^5$-methyl-$^{14}C$-$H_4$-folate, and methyl-$^{14}C$-$B_{12}$ 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 mμmole of bound vitamin-$B_{12}$ derivative per mg of protein (1). Methyl-$^{14}C$-AMe (50 μc/μmole) 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-$^{14}C$-$H_4$-folate contained potassium phosphate buffer, pH 7.4, 20 μmoles; $N^5$-methyl-$^{14}C$-$H_4$-folate (16,500 cpm/mμmole), 10 mμmoles; AMe, 10 mμmoles; DTT, 5 μmoles; $FMNH_2$, 50 mμmoles; platinum oxide, 0.1 mg; and vitamin-$B_{12}$-enzyme, 0.55 mg. Incubations were for 15 minutes at 37° under $H_2$ 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-$^{14}C$-$H_4$-folate was omitted and 10 mμmoles of methyl-$^{14}C$-AMe were employed (18,500 cpm/mμmole).

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 $N^5$-methyl-$^{14}C$-$H_4$-folate and methyl-$^{14}C$-AMe

| Enzyme incubated with | Reaction mixture during incubation | After incubation - prior to TCA | During TCA precipitation | TCA ppt. cpm | $^{14}C$-Bound mμmoles |
|---|---|---|---|---|---|
| $N^5$-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 mμmoles of bound vitamin-$B_{12}$ derivative.

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

$N^5$-methyl-$^{14}C$-$H_4$-folate or methyl-$^{14}C$-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. $^{14}C$-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 $^{14}C$-enzyme which was first isolated by Sephadex G-25 filtration, i.e. the protein-bound $^{14}C$ 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 $^{14}C$ per equivalent of vitamin-$B_{12}$ 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 mμmoles 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 mμmoles, 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 mμmoles) incubated with $N^5$-methyl-$^{14}$C-$H_4$-folate plus unlabeled AMe yielded 103,000 cpm (6.4 mμmoles) of $^{14}$C in the final concentrated

alcohol extract and the vitamin-$B_{12}$ enzyme (6.6 mμmoles) incubated wit methyl-$^{14}$C-AMe alone yielded 79,000 cpm (4.3 mμmoles) of $^{14}$C in the fi concentrated extract.

In Table II the ability of the $^{14}$C-material present in the two ethanol extracts to form a $^{14}$C-dimedone derivative before and after photolysis agrees with the results obtained with a chemically synthesized sample of methyl-$^{14}$C-$B_{12}$. Correcting for the dimedone dependent recovery of $^{14}$C with a sample of H$^{14}$CHO, the methyl-$^{14}$C-AMe extract ar the $N^5$-methyl-$^{14}$C-$H_4$-folate extract yielded 58% and 61%, respectively, of their $^{14}$C as a dimedone derivative upon photolysis; the photolytic yield for the methyl-$^{14}$C-$B_{12}$ standard was 64%.

Table II. Dimedone reactivity of $^{14}$C-compound in the ethanol extract and of methyl-$^{14}$C-$B_{12}$ 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-$B_{12}$ protein as methyl-$B_{12}$ was obtained by paper chromatography ($H_2O$, n-butanol, isopropanol, acetic acid, 100:100:70: water, 2-butanol, 28% $NH_4OH$, 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-$B_{12}$; this was true of the extracts obtained after incubating the vitamin-$B_{12}$-enzyme with either methyl-$^{14}$C-AMe or $N^5$-methyl-$^{14}$C-$H_4$-folate. If the respective ethanol extract samples and the methyl-$^{14}$C-$B_{12}$ standard were photolyzed prior to paper chromatography or electrophoresis, essentially all the radioactivity was lost and well separated spots of OH-$B_{12}$ were seen. More recently, the prosthetic group on the $N^5$-methyl-$H_4$-folate treated enzyme has been isolated in the same overall yield without a prior TCA precipitation and without the use of carrier methyl-$B_{12}$ (9). The spectrum of this material closely resembles that of methyl-$B_{12}$ and OH-$B_{12}$ is formed upon photolysis (9).

## DISCUSSION

The present studies show for the first time that both $N^5$-methyl-$H_4$-folate and AMe can react with the E. coli vitamin-$B_{12}$-enzyme to yield a methyl-$B_{12}$ enzyme. AMe is believed to function in methyl transfer from $N^5$-methyl-$H_4$-folate by methylating a specific site on the enzyme (10). Nevertheless, the ability of AMe to form a methyl-$B_{12}$ 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 $N^5$-methyl-$H_4$-folate, AMe was only 1/10 as effective in forming a methyl-$B_{12}$-enzyme. In addition the methylation of the vitamin-$B_{12}$-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.

2) $N^5$-Methyl-$H_4$-folate + vitamin-$B_{12}$-enzyme $\xrightarrow[\text{Reducing system}]{\text{AMe}}$ methyl-$B_{12}$-enzyme + $H_4$-folate

3) Methyl-$B_{12}$-enzyme + homocysteine → methionine + vitamin-$B_{12}$-enzyme

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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