The Role of Vitamin B₁₂ in Methionine Biosynthesis in Avian Liver

HERBERT DICKERMAN,* BETTY G. REDFIELD, J. G. BIERI, AND HERBERT WEISSBACH

From the Laboratory of Clinical Biochemistry, National Heart Institute, and the Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, 14 Maryland

(Received for publication, January 24, 1964)

A consideration of numerous studies in vivo indicates that the metabolism of methionine, folic acid, and cyanocobalamin (B₁₂) is interrelated. The vitamin has a sparing effect on the methyl group requirement of young chickens, and, conversely, methionine has a sparing effect on the cyanocobalamin requirement in chickens and rats (1-4). A more direct demonstration of an impairment in methyl group biosynthesis de novo was the inability of cyanocobalamin deficient rats and pigs to convert [³¹⁴C]-serine and [²¹⁴C]-glycine to the trimethylamin moiety of choline (5, 6). This effect was not upon transmethylation (7). The formimino group of folic acid, a catabolic product of histidine, is normally transferred to folate-H₄ so that little, if any, excretion of formimino glutamic acid occurs in response to a histidine load. As a consequence of the cyanocobalamin deficiency in rats and chickens, an increased FGA excretion occurs which is diminished by an increased dietary intake of methionine (8, 9). Elevated FGA excretions in response to histidine loads have also been reported in patients with pernicious anemia and megaloblastic anemia of pregnancy (10, 11).

The elucidation of methionine biosynthesis in Escherichia coli mutants has indicated a site at which the methionine, folic acid, and cyanocobalamin interrelationship may occur. The methyl transfer from methylfolate-H₄ to homocysteine (12), the terminal step in the biosynthetic pathway, is one of the known metabolic reactions which requires a cobamide prosthetic group (13-16). Other cofactors required for this reaction are reduced flavin (15) and S-adenosylmethionine (17, 18). Guest et al. (19) demonstrated that methyl-B₁₂ could replace the cyanocobalamin requirement in the enzyme system obtained from methionine-cyanocobalamin auxotrophs of E. coli W, and, of more significance, the methyl group of methyl-B₁₂ was transferred enzymatically to homocysteine to form methionine. The active form of the vitamin in the methyltransferase reaction is as yet unknown, but recent studies suggest that it is a reduced cobamide derivative on the enzyme (16, 20).

Various investigators have demonstrated the formation of methionine from homocysteine by extracts of pigeon, chicken, sheep, and hog liver with serine or formaldehyde as donor of the 1 carbon unit (21-24). Sakami and Ustins (25) have shown that, in hog liver, as in E. coli (12), the transfer of the methyl group from methylfolate-H₄ to homocysteine is the terminal step in this biosynthesis. A cobamide requirement in animal tissues in vivo has not been reported, but AMe (17) and a reducing system (26) are known requirements. Until recently, none of the reported enzyme systems of animal tissues has been extensively purified. During the progress of this study, Buchanan et al. (26) reported the purification of methylfolate-H₄ homocysteine transferase from hog liver, which was identical to the bacterial system in substrate specificity and cofactor requirements. The cyanocobalamin content of the enzyme fractions was determined with Lactobacillus leichmannii as the test organism. The cobamide content was found to be proportional to the methylfolate-H₄ transferase activity throughout the purification.

The present studies were undertaken to elucidate the role of cyanocobalamin in methionine biosynthesis in animal tissues. They include the partial purification and characterization of methylfolate-H₄ transferase and methyl-B₁₂ transferase activities from chicken liver and nutritional factors affecting these enzyme levels.

EXPERIMENTAL PROCEDURE

Nutritional Studies

Female Arbor Acres chickens were obtained from a commercial hatchery 1 day after hatching and kept eight in a cage. Food and water were supplied ad libitum, and weights were determined at weekly intervals. The diet employed was described by Spivey Fox et al. (27). The cyanocobalamin-supplemented diet contained 0.1 mg of the vitamin per kg of diet.

When the animals were killed, the livers were excised and kept at approximately 0° for subsequent procedures. The tissues were homogenized in a Waring Blender for 1 minute, and the resultant homogenates were centrifuged for 60 minutes at 105,000 × g. The supernatant fraction was assayed for methylfolate-H₄ and methyl-B₁₂ transferase activities. The methyl-B₁₂ enzyme activity, catalyzing the methyl transfer from methylfolate-H₄ to homocysteine to form methionine, is designated methyl-B₁₂ transferase.
transferase reaction incubations were carried out for 2 hours while the methylfolate-\(H_4\) transferase reaction incubations were done for periods up to 1 hour.

**Purification of Methylfolate-\(H_4\) and Methyl-\(B_12\) Transferases**

Because both enzyme activities were found to fractionate in the same manner, a common purification procedure was developed. Chicken livers from a commercial source (40 g) were homogenized in 2.5 volumes of 0.1 M Tris, pH 7.4, and the homogenate was centrifuged at 105,000 \(\times g\) for 60 minutes. The supernatant fraction was treated with a neutralized saturated solution of ammonium sulfate, and the protein that precipitated between 30 and 50% saturation was dissolved in 0.01 M Tris buffer, pH 7.4. This fraction was dialyzed against 4 liters of 0.005 M Tris buffer, pH 7.4, for 3 hours, and adjusted to a protein concentration of 20 mg per ml. The dialyzed fraction (60 ml) was then adsorbed onto calcium phosphate gel, 1 mg of gel per mg of protein. Following washing of the gel with 0.01 M potassium phosphate buffer, pH 7.4, enzyme activity was eluted from the gel with 60 ml of 0.2 M potassium phosphate, pH 7.4. The gel eluate was fractionated with neutralized saturated ammonium sulfate, and the precipitate formed between 35 and 50% saturation was dissolved in 0.01 M Tris buffer, pH 7.4. This fraction (50 mg of protein per ml) was diluted 10-fold with 0.005 M Tris buffer, pH 7.4. The diluted enzyme solution was applied slowly onto a DEAE-cellulose column (1 \(\times 9\) cm) that had been equilibrated with 0.005 M Tris buffer, pH 7.4. Following preliminary washings of the column with 10 ml of 0.01 M potassium phosphate buffer, pH 7.4, and 20 ml of 0.01 M potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl, the enzyme was eluted with 30 ml of 0.01 M potassium phosphate buffer, pH 7.4, containing 0.3 M NaCl. The eluate (1.4 mg of protein per ml) was treated with neutralized saturated ammonium sulfate, and the protein that precipitated between 0 and 66% saturation was dissolved in 2 ml of 0.01 M Tris buffer, pH 7.4 (11 mg of protein per ml).

**Methods and Materials**

Methyl-\(B_12\) was synthesized from methyl iodide by the general procedure of Smith et al. (28). In the same manner \(\text{\(^{14}\)}\text{C}\)-methyl-\(B_12\) was prepared from \(\text{\(^{14}\)}\text{C}\)-methyl iodide. \(L\)-Methylfolate-\(H_4\) was prepared according to the procedure of Keresztesy and Donaldson (29). Solutions of methylfolate-\(H_4\) were stored in 0.1 M mercaptoethanol.

The methylfolate-\(H_4\) and methyl \(B_12\) transferase assay procedures have been described in previous publications (16, 30). The former involves passage of the reaction mixture through a Dowex 1-chloride resin bed which retains the substrate, \(\text{\(^{14}\)}\text{C}\)-methyl labeled methylfolate-\(H_4\), and permits the product, \(\text{\(^{14}\)}\text{C}\)-methyl labeled methylmethionine, to pass through. The methyl-\(B_12\)-transferase assay is based on the retention of \(\text{\(^{14}\)}\text{C}\)-methionine on a Dowex 50-H\(^+\) resin and its subsequent elution after photolytic decomposition of the substrate, \(\text{\(^{14}\)}\text{C}\)-methyl-\(B_12\).

The standard incubation mixture for the methylfolate-\(H_4\) transferase reation included, in a total volume of 0.2 ml, enzyme, 0.01 to 0.1 ml; \(\text{\(^{14}\)}\text{C}\)-methyl labeled methylfolate-\(H_4\) (specific activity, 600 to 1800 c.p.m. per \(\mu\)mole), 30 \(\mu\)moles; cyano-\(B_12\), 10 \(\mu\)moles; FMN or FAD, 1 \(\mu\)mole; AMe, 5 \(\mu\)moles; L-homocysteine (prepared from the thiolactone derivative), 50 \(\mu\)moles; \(\beta\)-mercaptoethanol, 40 \(\mu\)moles; and potassium phosphate buffer, pH 7.4, 10 \(\mu\)moles. Anaerobic experiments were performed in Thunberg tubes in a nitrogen atmosphere.

The standard incubation mixture for the methyl \(B_12\) transferase reaction included, in a total volume of 0.2 ml, enzyme, 0.04 to 0.14 ml; \(\text{\(^{14}\)}\text{C}\)-methyl labeled methyl-\(B_12\) (specific activity, 1800 c.p.m. per \(\mu\)mole), 15 \(\mu\)mole; L-homocysteine, 50 \(\mu\)moles; and potassium phosphate buffer, 10 \(\mu\)moles, pH 7.4.

A unit of methylfolate-\(H_4\) transferase or methyl-\(B_12\) transferase catalyzes the formation of 1 \(\mu\)mole of methionine per hour at 37\(^\circ\). Protein concentrations were determined by the method of Warburg and Christian (31).

Cyano-\(B_{12}\), FMN, FAD, L-homocysteine thiolactone, DPNH, and TPNH were purchased from the California Corporation for Biochemical Research. Deoxynadensyl-\(B_{12}\) and Factor B were giftms from Dr. David Perlman, Squibb Institute of Medical Research. Highly purified intrinsic factor was the gift of Dr. Leon Ellenbogen of the Lederle Division of the American Cyanamid Corporation. Factor \(V_{hi}\) was the gift of Dr. J. L. Peet, Agricultural Microbiological Unit, Oxford University.

All radioactive determinations were done in a Packard Tri-Carb liquid scintillation counter with a naphthalene-dioxane scintillation fluid (33).

**RESULTS**

**Nutritional Studies**

Methylfolate-\(H_4\) and Methyl-\(B_12\) Transferase Levels Following Cyano-\(B_{12}\) Omission and Replenishment—Prior nutritional studies indicated that growth was impaired when newborn chickens were fed a diet suboptimal in methionine content, high in fat content, and devoid of cyano-\(B_{12}\) (4, 27). In the present experiments also, a marked difference in weights was observed between animals on basal and cyano-\(B_{12}\)-deficient diets; the 4-week weights of the control animals averaged about 450 g, and those on the deficient diet averaged 100 to 200 g less. Previous studies in which this diet was used indicated that, under similar experimental conditions, FGA excretion was increased in response to a histidine load (9), but there was no decrease in hemoglobin content, red blood cell number, or hematocrit (33).

The effect of a cyano-\(B_{12}\)-deficient diet on the two transferase activities is shown in Table I. There was a distinct reduction in the level of both transferase specific activities in liver extracts from cyano-\(B_{12}\)-deficient animals. Similar results were obtained when the incubations were done under anaerobic conditions. In addition, there was no evidence of the presence of inhibitory factors in the liver extracts of cyano-\(B_{12}\)-deficient animals.

Restoration in vitro of either the methylfolate-\(H_4\) or methyl-\(B_12\) transferases from the livers of deficient chickens was not observed when cyano-\(B_{12}\), deoxynadensyl-\(B_{12}\), methyl-\(B_12\), or \(B_{12}\) were added to incubation mixtures.

The failure to restore enzyme activities in vitro led to an attempt at restoration of the activities of the enzyme or enzymes by administration of the vitamin in vivo. Table II records the results of one such experiment. The specific activity of the methylfolate-\(H_4\) transferase was increased 6-fold over that seen in cyano-\(B_{12}\)-deficient animals if cyano-\(B_{12}\) was administered at 3 and 24 hours before death. Animals which received only a single dose of cyano-\(B_{12}\) at 3 hours prior to sacrifice show no increase in enzyme level as compared to animals maintained on the deficient diet. Cyano-\(B_{12}\) injected at 3, 24, and 48 hours before death gave the same results as in animals that received...
The incubation mixtures and assay procedures are described in "Experimental Procedure." The numbers in parentheses represent the range of specific activities. The standard deviations presented in this and following tables are derived from the equation

\[ S = \sqrt{\frac{\sum x^2 - (\sum x)^2}{n(n-1)}} \]

Table I

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. of animals</th>
<th>Methylfolate-H₄ transferase specific activity</th>
<th>No. of animals</th>
<th>Methyl-B₁₂ transferase specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>30</td>
<td>3.36 ± 1.18 (3.0-9.3)</td>
<td>16</td>
<td>0.61 ± 0.10 (0.41-0.73)</td>
</tr>
<tr>
<td>Cyano-B₁₂-deficient</td>
<td>30</td>
<td>0.55 ± 0.28 (0.1-1.2)</td>
<td>16</td>
<td>0.10 ± 0.04 (0.03-0.16)</td>
</tr>
</tbody>
</table>

Table II

Effect of parenteral administration of cyano-B₁₂ on methylfolate-H₄ and methyl-B₁₂ transferase activities

The incubation mixtures and assay procedures are described in "Experimental Procedure." The numbers in parentheses represent the range of specific activities. The standard deviations presented in this and following tables are derived from the equation

\[ S = \sqrt{\frac{\sum x^2 - (\sum x)^2}{n(n-1)}} \]

Table III

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Restoration of specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylfolate-H₄ transferase</td>
<td>%</td>
</tr>
<tr>
<td>Methyl-B₁₂ transferase</td>
<td>%</td>
</tr>
<tr>
<td>Cyano-B₁₂</td>
<td>40</td>
</tr>
<tr>
<td>Methyl-B₁₂</td>
<td>46</td>
</tr>
<tr>
<td>Deoxyadenosyl-B₁₂</td>
<td>49</td>
</tr>
<tr>
<td>Factor B</td>
<td>9</td>
</tr>
</tbody>
</table>

Table IV

Effect of dietary methionine on methylfolate-H₄ and methyl-B₁₂ transferase activities

A 3-week interval from birth was used as the experimental period in this study. Control intake was the basal diet (27), which included 3 g DL-methionine per kg of diet; in the methionine-supplemented group, an additional 6 g of methionine per kg of diet were fed. The body weight represents the mean weight of each group of eight animals. The incubation mixtures and assay procedures are described in "Experimental Procedure."
Effect of varied amounts of dietary dl-methionine on methylfolate-H₄ and methyl-B₁₂ transferase of chicken liver

The experimental feeding period was 4 weeks. Basal diet included 3 g of dl-methionine per kg of diet while the appropriate dl-methionine supplements are indicated in the left-hand column. Body weight represents the mean of eight animals in each experimental group. The incubation procedures and assay procedures are described in “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Diet</th>
<th>Mean body weight</th>
<th>Specific activity Methylfolate-H₄ transferase</th>
<th>Specific activity Methyl-B₁₂ transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>418</td>
<td>0.31 ± 0.05</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>Basal + 6 g of methionine per kg of diet</td>
<td>507</td>
<td>2.4 ± 0.09</td>
<td>0.40 ± 0.05</td>
</tr>
<tr>
<td>Basal + 12 g of methionine per kg of diet</td>
<td>528</td>
<td>1.8 ± 0.35</td>
<td>0.32 ± 0.08</td>
</tr>
<tr>
<td>Basal + 18 g of methionine per kg of diet</td>
<td>418</td>
<td>1.4 ± 0.36</td>
<td>0.31 ± 0.05</td>
</tr>
</tbody>
</table>

Purification of methylfolate-H₄–homocysteine and methyl-B₁₂–homocysteine transferase

Incubation and assay procedures were used as described in “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>0.41</td>
<td>100</td>
<td>0.12</td>
<td>100</td>
</tr>
<tr>
<td>20 to 60% (NH₄)₂SO₄</td>
<td>1.28</td>
<td>52</td>
<td>0.61</td>
<td>56</td>
</tr>
<tr>
<td>Calcium phosphate eluate</td>
<td>3.5</td>
<td>58</td>
<td>0.73</td>
<td>42</td>
</tr>
<tr>
<td>35 to 55% (NH₄)₂SO₄</td>
<td>4.1</td>
<td>32</td>
<td>1.6</td>
<td>42</td>
</tr>
<tr>
<td>DEAE eluate</td>
<td>13.6</td>
<td>24</td>
<td>5.2</td>
<td>31</td>
</tr>
<tr>
<td>0 to 66% (NH₄)₂SO₄</td>
<td>15.9</td>
<td>19</td>
<td>6.2</td>
<td>26</td>
</tr>
</tbody>
</table>

Requirements for methylfolate-H₄–homocysteine transferase

The incubation mixtures and assay were performed as described in “Experimental Procedure.” As an enzyme source, 190 pg of chicken liver was used. The anaerobic determinations were done in Thunberg tubes in a nitrogen atmosphere.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Methionine formed</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmole/50 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>2.11</td>
<td>3.17</td>
<td></td>
</tr>
<tr>
<td>– β-Mercaptoethanol</td>
<td>0.50</td>
<td>2.16</td>
<td></td>
</tr>
<tr>
<td>– Cyano-B₁₂</td>
<td>0.33</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>– S-Adeoxymethionine</td>
<td>0.37</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>– Homocysteine</td>
<td>0.30</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>– FMN</td>
<td>1.90</td>
<td>2.78</td>
<td></td>
</tr>
</tbody>
</table>

During the course of the present study, β-mercaptoethanol was found to stimulate markedly the methyl transfer from methylfolate-H₄ aerobically with the more purified enzyme preparations (calcium phosphate eluate or beyond). Even under anaerobic conditions, there was a 50% stimulation in the presence of this sulfhydryl reagent. In the presence of β-mercaptoethanol, the stimulations of FMN, FAD, DPNH, or TPNH under aerobic or anaerobic conditions are relatively inconsistent and of a small
magnitude. The reducing system may be needed to keep the enzyme-bound cobamide in a reduced state, but this is as yet unproven in animal systems.

The enzymatic reaction was linear with time up to 60 minutes and was also proportional to enzyme concentration (Fig. 1). The pH optimum of the methylfolate-H₄ transferase activity was between 6.8 and 7.5. Kₘ determinations of the required compounds under aerobic conditions indicated the following values: n1-methylfolate-H₄, 2.25 × 10⁻⁴ M; AMe, 7.5 × 10⁻⁴ M; and L-homocysteine, 9 × 10⁻⁶. The requirements for the enzymatic transfer of the methyl group of methyl-B₁₂ to homocysteine were only the methyl donor, methyl-B₁₂, and the methyl acceptor, homocysteine. The optimal pH range was between pH 6.8 and 8.0. The Kₘ for homocysteine was 2 × 10⁻⁴ M, while that for methyl-B₁₂ was 5.2 × 10⁻⁴ M.

**Effects of Cobamides on Methylfolate-H₄ Transferase**

Previous observations with extracts of chicken, rat, and hog liver indicated that addition of cyanob-B₁₂ and other cobamide compounds in vitro markedly stimulated the methylfolate-H₄ transferase in an air but not in a nitrogen atmosphere (16, 26). MethylhBlz was 5.2 × 10⁻⁴ M. The liver indicated that addition of cyano-B₁z and other cobamide derivatives, including those which lack a nucleotide, indicates the participation of a cobamide in methionine biosynthesis in chickens. Since the methylfolate-H₄ transferase, an alternative approach was undertaken to demonstrate the participation of a cobamide in methionine biosynthesis in chickens. The methylfolate-H₄ transferase activity of chicken liver decreased during cyano-B₁₂ deficiency and was partially restored with cyano-B₁₂ replenishment in vivo,

2 Recently, Buchanan et al. (26) have been able to satisfy partially the requirement for reduced FAD with B-mercaptoethanol in the transferase from hog liver enzyme. Similar results have been obtained in E. coli 113-3W and type B (36, 30).

4 A paradoxical observation was that although flavin compounds stimulated the reaction in the presence of cobamide compounds (see Table VII), the reaction was inhibited by flavin compounds in the absence of cobamide compounds.

3 This fraction of intrinsic factor bound 4 μg of cyanocobalamin per mg of protein.

The liver supernatant fraction of ²⁶Co-cyanob-D₃-treated animals was purified according to the method described in “Experimental Procedure.” Table VIII records the distribution of radioactivity emitted by ²⁶Co-cyanob-B₁₂ units of methylfolate-H₄ transferase, and the ratio of specific activities of these two components. Except for the 0 to 30% fraction of the first ammonium sulfate step, there was a definite correlation between units of enzyme activity and total radioactivity over the entire purification. The ratio of counts per minute of ²⁶Co to number of enzyme units decreased abruptly with the first ammonium sulfate fractionation but then approach a constant value as would be expected if the ²⁶Co-cobamide was attached to the enzyme. Further evidence in this respect is seen by the examination of the DEAE-cellulose (Table VIII, Step 6) fractionation (Fig. 2). A minor component of eluted ²⁶Co-cyanob-B₁₂ radioactivity was apparent in the pass-through and 0.01 M potassium phosphate wash. These fractions contained the bulk of the applied protein and a small fraction of the methylfolate-H₄ transferase units. The bulk of ²⁶Co radioactivity emitted, and methylfolate-H₄ and methyl-B₁₂ transferase activities, were retained on the column and eluted identically with 0.01 M potassium phosphate, pH 7.4, containing 0.3 M sodium chloride. The peak tube represents a 9% recovery of original methylfolate-H₄ transferase activity (30-fold purified) as well as 5% of the original supernatant ²⁶Co-cyanob-B₁₂. The achievement of a constant ratio of ²⁶Co per enzyme unit as well as the retention of ²⁶Co-cyanob-B₁₂ through
TABLE VIII
Distribution of 60Co radioactivity and methylfolate-\(H_4\) transferase during purification

The nutritional experimental procedure and details of the 60Co cyano-B\(_{12}\) administration are discussed in the text. The over-all purification was 30-fold.

<table>
<thead>
<tr>
<th>Purification fraction</th>
<th>60Co (c.p.m.)</th>
<th>Methylfolate-(H_4) transferase (\text{total units})</th>
<th>(c.p.m./\text{enzyme unit})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant*</td>
<td>(1.5 \times 10^4)</td>
<td>4230</td>
<td>354</td>
</tr>
<tr>
<td>First (NH(_4))(_2)SO(_4) fractionation</td>
<td>(0.72 \times 10^5)</td>
<td>2090</td>
<td>34</td>
</tr>
<tr>
<td>0 to 30%</td>
<td>(6.5 \times 10^5)</td>
<td>3340</td>
<td>195</td>
</tr>
<tr>
<td>30 to 50%</td>
<td>(2.0 \times 10^5)</td>
<td>810</td>
<td>246</td>
</tr>
<tr>
<td>Calcium phosphate adsorption and elution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>(0.2 \times 10^5)</td>
<td>140</td>
<td>143</td>
</tr>
<tr>
<td>0.01 M phosphate buffer, pH 7.4, eluate</td>
<td>(0.2 \times 10^5)</td>
<td>2580</td>
<td>150</td>
</tr>
<tr>
<td>0.2 M phosphate buffer, pH 7.4, eluate*</td>
<td>(3.9 \times 10^5)</td>
<td>1050</td>
<td>76</td>
</tr>
<tr>
<td>0.3 M phosphate buffer, pH 7.4, eluate</td>
<td>(0.8 \times 10^5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second (NH(_4))(_2)SO(_4) fractionation</td>
<td>(0.2 \times 10^5)</td>
<td>195</td>
<td>102</td>
</tr>
<tr>
<td>0 to 35%</td>
<td>(2.5 \times 10^5)</td>
<td>2520</td>
<td>102</td>
</tr>
<tr>
<td>35 to 55%*</td>
<td>(4.5 \times 10^5)</td>
<td>400</td>
<td>101</td>
</tr>
<tr>
<td>DEAE-cellulose, tube 15 (See Fig. 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* These fractions have been used for further purification.

The above experiment was done with chickens on a cyano-B\(_{12}\) deficient diet. It was felt that injection of 60Co-cyano-B\(_{12}\) into control chickens on a diet adequate in cyano-B\(_{12}\) would yield little, if any, significant activity in the protein since the enzyme sites would be expected to be saturated with the cobamide prosthetic group. Injection of 60Co-cyano-B\(_{12}\) into animals on a control diet did yield radioactivity in a protein fraction that was associated with the enzyme through a 40-fold purification. However, with the chicks on an adequate cyano-B\(_{12}\) intake, the ratio of 60Co radioactivity to number of activity units of the purified enzyme was only 16. This value was one-sixth of that obtained from the vitamin-deficient animals. The data suggest that even at dietary levels of 0.1 mg of cyano-B\(_{12}\) per kg of diet, the enzyme sites are not completely saturated with the cobamide prosthetic group.

**DISCUSSION**

Of the predicted functions of cyano-B\(_{12}\) in animal tissues, only its participation in the methylmalonyl-CoA isomerase reaction has been extensively elucidated by the demonstration of a decrease in enzyme activity following vitamin deficiency, restoration of activity with vitamin replenishment in vivo, purification of a cobamide-containing enzyme, removal of the cobamide prosthetic group in vitro, and restoration of activity in vitro by the addition of a cobamide, i.e. deoxyadenosyl-B\(_{12}\) (39-41).

The present study was undertaken to clarify in a similar manner the role of cyano-B\(_{12}\) in methionine biosynthesis in animal tissues. However, only three of the requisites which would indicate a cobamide participation have been fulfilled. These are the depletion of the methylfolate-\(H_4\) transferase concentrations in avian liver during cyano-B\(_{12}\) depletion, repletion of the specific activity with administration of cobamide derivatives 24 hours prior to death, and the intimate association of 60Co-cyano-B\(_{12}\) radioactivity during purification of the methylfolate-\(H_4\) transferase. Further credence can be assigned to the participation of a cobamide derivative in this reaction because of the structural specificity of the cobamides which were effective in replenishment. Factor B, which lacks the nucleotide moiety,
was essentially ineffective, while those which possessed the nucleotide, i.e. cyanolo-B, deoxyadenosyl-B, and methyl-B, were effective in restoration of liver enzyme specific activity. It should be stressed that the prosthetic group has not as yet been isolated from the homocysteine in these animal studies and that the above data only suggest that a cobamide participates in the reactions. However, other points in favor of this view should be mentioned. The requirements of the reaction are essentially identical with those discussed for the cobamide-dependent reaction in E. coli, in contrast to the non-cobamide-dependent pathway that has been described (13). Also the ability to obtain methyl-B transfer is consistent with the observation seen in E. coli (19, 16).

Despite the failure to resolve the purified enzyme into a cobamide coenzyme and apoenzyme, the addition of a variety of cobamide compounds in vitro did stimulate the methyl transfer from methylfolate-H, to homocysteine under aerobic and, to a lesser extent, anaerobic conditions. The characteristics of this effect, (a) the lack of cobamide specificity, (b) the decrease in the magnitude of the effect during anaerobiosis, (c) the requirement of β-mercaptoethanol for the cobamide stimulation, and (d) the relative efficacy of Factors B and VB, at low concentrations, suggest that the stimulation is similar to the nonenzymatic catalysis of monothiol oxidation by cyanolo-B derivatives (22).

The relationship of the methyl-B transferase activity to the methylfolate-H, transferase is still in doubt. One major difference between the two transferase activities was the suppression of methylfolate-H, transferase levels by increased dietary methionine without a comparable effect on the levels of methyl-B transferase. On the other hand, the enzyme activities were not separated during purification although the methyl-B transferase activity was purified to a slightly greater extent. This appeared to be due to a greater lability of the methylfolate-H, transferase. In addition, there was a comparable response of the two transferase activities to cyanolo-B depletion and repletion.

The suppression of avian liver methylfolate-H transferase by increased dietary methionine would be expected to result in an accumulation of methylfolate-H in vivo. On the contrary, dietary methionine has been reported to lower the levels of methylfolate-H, in rat liver with a corresponding increase in the concentration of formylated folate-H compounds (43). It is difficult to reconcile these two findings. One possibility is that methionine might lead to a reduction in the synthesis of methylfolate-H, as well as a reduction in the methylfolate-H transferase.

SUMMARY

1. Nutritional deficiency of 5,6-dimethylbenzimidazolylcobamide cyanide (cyanolo-B) in young chicks leads to a marked reduction of N4-methyltetrahydrofolate and 5,6-dimethylbenzimidazolylcobamide methyl (methyl-B), transferase activities; replenishment with cyanolo-B, 5,6-dimethylbenzimidazolylcobamide 5'-deoxyadenosyl (deoxyadenosyl-B), and methyl-B resulted in partial restoration of both activities, while cobamid hexa-amide (Factor B) was not effective.

2. Feeding of methionine supplements at levels of 6 g of methionine per kg of diet resulted in reduction of methyltetrahydrofolate transferase but not of methyl-B transferase activity.

3. The methyltetrahydrofolate and methyl-B transferase activities of chicken liver have been partially purified and characterized. The activities were not separated at this stage of purification. The purified methyltetrahydrofolate transferase required homocysteine and S-adenosylmethionine, and β-mercaptoethanol as a reducing compound. Methyl-B transferase required only the methyl group acceptor, homocysteine.

4. Cyanolo-B, deoxyadenosyl-B, methyl-B Factor B, cobalamin acid a, b, c, d, e, g, hexamide (Factor VB), and a reduced form of cyanolo-B (B[V]) all stimulated the methyltetrahydrofolate transferase reaction aerobically and, to a lesser extent, anaerobically. The anaerobic cobamide stimulation was effective only in the presence of β-mercaptoethanol and riboflavin phosphate "flavin mononucleotide" or flavin adenine dinucleotide. This is suggestive of the nonenzymatic catalysis by cobamides of the oxidation of thiols. A cobamide compound has not been isolated from the purified transferases.

5. 160-Cyanocobalamin, when injected into vitamin-B deficient chicks, was incorporated into a protein fraction which was found during purification in the same fractions as methyl-B transferase. In the nondepleted animal, there was evidence of 160-cyano- cobalamin incorporation, but to a much lesser extent than in the replenished animals.

Acknowledgments—The authors are indebted to Mrs. Esther M. Hurley and Mr. Woodrow Duvall for assistance in the nutritional studies. In addition, Mr. Todd Wheeler provided technical assistance during the course of this study. The authors wish to thank Dr. Anthony Mead for his generosity in helping to prepare the tetrahydrofolate.

REFERENCES

18. Buchanan, J. M., Abstracts of the Meeting of the American Chemical Society, Division of Biological Chemistry, Atlantic City, September, 1960, p. 19C.


31. WARBURG, O., AND CHRISTIAN, W., Biochem. Z., 310, 384 (1941–1942).


The Role of Vitamin B$_{12}$ in Methionine Biosynthesis in Avian Liver
Herbert Dickerman, Betty G. Redfield, J. G. Bieri and Herbert Weissbach


Access the most updated version of this article at http://www.jbc.org/content/239/8/2545.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/239/8/2545.citation.full.html#ref-list-1