The Role of Vitamin B₁₂ in Methionine Biosynthesis in Avian Liver

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A consideration of numerous studies in vivo indicates that the metabolism of methionine, folic acid, and cyano-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 cyano-B₁₂ requirement in chickens and rats (1–4). A more direct demonstration of an impairment in methyl group biosynthesis de novo was the inability of cyano-B₁₂-deficient rats and pigs to convert 3-¹⁴C-serine and 2-¹⁴C-glycine to the trimethylamine moiety of choline (5, 6). This effect was not upon transmethylation (7). The formimino group of formiminoglutamic acid, a catabolic product of histidine, is normally transferred to folate-H₄ so that little, if any, excretion of FGA occurs in response to a histidine load. As a consequence of cyano-B₁₂ 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 cyano-B₁₂ interrelationship may occur. The methyl transfer from methylfolate-H₄ to homocysteine (12), the terminal step in this biosynthesis, 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 cyano-B₁₂ requirement in the enzyme system obtained from methionine-cyano-B₁₂ 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 methylfolate-H₄ transferase 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 cyano-B₁₂ 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 cyano-B₁₂ 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 cyano-B₁₂-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 enzymatic activity, catalysing the methyl transfer from methylfolate-H₄ to homocysteine to form methionine, is designated methyl-B₁₂ transferase.
Purification of Methylfolate-\( \text{H}_4 \) and Methyl-\( \text{B}_12 \) 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 \( \text{M} \) 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 \( \text{M} \) Tris buffer, pH 7.4. This fraction was dialyzed against 4 liters of 0.005 \( \text{M} \) 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 \( \text{M} \) potassium phosphate buffer, pH 7.4, enzyme activity was eluted from the gel with 60 ml of 0.2 \( \text{M} \) potassium phosphate, pH 7.4. The gel eluate was fractionated with neutralized saturated ammonium sulfate, and the preparement precipitated between 35 and 50\% saturation was dissolved in 0.01 \( \text{M} \) Tris buffer, pH 7.4. This fraction (50 mg of protein per ml) was diluted 10-fold with 0.005 \( \text{M} \) 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 \( \text{M} \) Tris buffer, pH 7.4. Following preliminary washings of the column with 10 ml of 0.01 \( \text{M} \) potassium phosphate buffer, pH 7.4, and 20 ml of 0.01 \( \text{M} \) potassium phosphate buffer, pH 7.4, containing 0.1 \( \text{M} \) NaCl, the enzyme was eluted with 30 ml of 0.01 \( \text{M} \) potassium phosphate buffer, pH 7.4, containing 0.3 \( \text{M} \) 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 \( \text{M} \) Tris buffer, pH 7.4 (11 mg of protein per ml).

Methods and Materials

Methyl-\( \text{B}_12 \) was synthesized from methyl iodide by the general procedure of Smith et al. (28). In the same manner \( \text{H}^4 \text{C-methyl iodide} \) was prepared from \( \text{H}^4 \text{C-methyl iodide} \). \( \Delta \text{L-Methylfolate-} \text{H}_4 \) was prepared according to the procedure of Keresztesy and Donaldson (29). Solutions of methylfolate-\( \text{H}_4 \) were stored in 0.1 \( \text{M} \) mercaptoethanol.

The methylfolate-\( \text{H}_4 \) and methyl \( \text{B}_12 \) 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, \( \text{H}^4 \text{C-methyl-labeled methylfolate-} \text{H}_4 \) and permits the product, \( \text{H}^4 \text{C-methyl-labeled methionine} \), to pass through. The methylfolate-\( \text{H}_4 \)-transferase assay is based on the retention of \( \text{H}^4 \text{C-methionine} \) on a Dowex 50-H\(^+\) resin and its subsequent elution after photolytic decomposition of the substrate, \( \text{H}^4 \text{C-methyl-} \text{B}_12 \).

The standard incubation mixture for the methylfolate-\( \text{H}_4 \)-transferase reaction included, in a total volume of 0.2 ml, enzyme, 0.01 to 0.1 ml; \( \text{H}^4 \text{C-methyl-labeled methylfolate-} \text{H}_4 \) (specific activity, 600 to 1800 c.p.m. per mpmole), 30 mpmoles; cyano-\( \text{B}_12 \), 10 mpmoles; FMN or FAD, 1 mpmole; AMe, 5 mpmoles; \( \Delta \text{l-homocysteine} \) (prepared from the thiolactone derivative), 50 mpmoles; \( \beta \)-mercaptoethanol, 40 mmoles; and potassium phosphate buffer, pH 7.4, 10 mmoles. Anaerobic experiments were performed in Thunberg tubes in a nitrogen atmosphere.

The standard incubation mixture for the methyl \( \text{B}_12 \)-transferase reaction included, in a total volume of 0.2 ml, enzyme, 0.04 to 0.14 ml; \( \text{H}^4 \text{C-methyl-labeled methyl-} \text{B}_12 \) (specific activity, 1800 c.p.m. per mpmole), 15 mpmoles; \( \Delta \text{l-homocysteine} \), 50 mpmoles; and potassium phosphate buffer, 10 mmoles, pH 7.4.

A unit of methylfolate-\( \text{H}_4 \) transferase or methyl-\( \text{B}_12 \) transferase catalyzes the formation of 1 mpmole of methionine per hour at 37\(^\circ\). Protein concentrations were determined by the method of Warburg and Christian (31).

Cyano-\( \text{B}_12 \), FMN, FAD, \( \Delta \text{l-homocysteine thiolactone} \), DPNH, and TPNH were purchased from the California Corporation for Biochemical Research. Deoxyadenosyl-\( \text{B}_12 \) and Factor B were gifts 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 \( \text{V}_1 \) was the gift of Dr. J. L. Peal, Agricultural Microbiological Unit, Oxford University.

All radioactive determinations were done in a Packard Tri-Carb liquid scintillation counter with a naphthalene-dioxane scintillation fluid (32).

RESULTS

Nutritional Studies

Methylfolate-\( \text{H}_4 \) and Methyl-\( \text{B}_12 \) Transferase Levels Following Cyano-\( \text{B}_12 \) 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-\( \text{B}_12 \) (4, 27). In the present experiments also, a marked difference in weights was observed between animals on basal and cyano-\( \text{B}_12 \)-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-\( \text{B}_12 \)-deficient diet on the 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-\( \text{B}_12 \)-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-\( \text{B}_12 \)-deficient animals.

Restoration in vivo of either the methylfolate-\( \text{H}_4 \) or methyl-\( \text{B}_12 \) transferases from the livers of deficient chickens was not observed when cyano-\( \text{B}_12 \), deoxyadenosyl-\( \text{B}_12 \), methyl-\( \text{B}_12 \), or \( \text{B}_12 \) were added to incubation mixtures.

The failure to restore enzyme activities in vivo 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-\( \text{H}_4 \)-transferase was increased 6-fold over that seen in cyano-\( \text{B}_12 \)-deficient animals if cyano-\( \text{B}_12 \) was administered at 3 and 24 hours before death. Animals which received only a single dose of cyano-\( \text{B}_12 \) at 3 hours prior to sacrifice show no increase in enzyme level as compared to animals maintained on the deficient diet. Cyano-\( \text{B}_12 \) 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:

$$S = \sqrt{\frac{n\bar{x}^2 - (\bar{x})^2}{n(n-1)}}$$

Cyano-Bl2-deficient + injec-
tion of cyano-Br2*   3.36 ± 0.6 0.23 ± 0.06

* At 3 and 24 hours prior to death, 7.75 \(\mu\)moles of cyano-Bl2 were injected.

cyano-Bl2 at 3 and 24 hours. There was no significant weight difference between the repleted animals (those receiving the vitamin for 24 hours) and those maintained on a deficient diet. A 2- to 3-fold increase was observed in the methyl-Bl2 transferase activity following administration of cyano-Bl2 in vivo.

Table II shows the effect of several cobamides on the restoration of cyano-Br2 following administration of cyano-Br2 in vivo.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Methylfolate-(H_4) transferase specific activity</th>
<th>Methyl-Bl2 transferase specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>4.40 ± 1.1</td>
<td>0.48 ± 0.05</td>
</tr>
<tr>
<td>Cyano-Bl2-deficient</td>
<td>0.53 ± 0.4</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Cyano-Bl2-deficient + injection of cyano-Br2*</td>
<td>3.36 ± 0.6</td>
<td>0.23 ± 0.06</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Restoration of specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methylfolate-(H_4) transferase</td>
</tr>
<tr>
<td>Cyano-Bl2</td>
<td>40</td>
</tr>
<tr>
<td>Methyl-Bl2</td>
<td>65</td>
</tr>
<tr>
<td>Deoxyadenosyl-Bl2</td>
<td>49</td>
</tr>
<tr>
<td>Factor B</td>
<td>9</td>
</tr>
</tbody>
</table>

This table presents a summary of results obtained in three experiments. For each compound a total of 24 animals were evaluated for the methylfolate-\(H_4\) transferase percentage of restoration and 20 animals for the methyl-Bl2 transferase restoration. The animals were kept on a cyano-Bl2-deficient diet for 4 weeks. At 24 and 48 hours prior to death, 7.75 \(\mu\)moles of the indicated compounds were injected. The activity observed in extracts of control chicks (maintained on a diet with cyano-Bl2 present) was used as the 100% value.

Table III shows the effect of several cobamides on the restoration of activities of the enzyme or enzymes in vivo in deficient animals. It is apparent that those cobamides which have a nucleotide moiety, i.e. cyano-Bl2, deoxyadenosyl-Bl2, and methyl-Bl2, were more effective than Factor B, which is devoid of a nucleotide moiety. This observed specificity agrees with studies in vivo in a variety of organisms (24) which have shown that the nucleotide-free Bl2 analogues are generally ineffective.

**Influence of Dietary Methionine on Methylfolate-\(H_4\) and Methyl-Bl2 Transferase**—The sparing effect of methionine on the growth rates of cyano-Bl2-deficient newborn chickens has been demonstrated in previous studies (1, 4). In the present studies, methionine supplements comparable to those reported (4) were fed to young chickens in order to study the effect of the amino acid on the specific activities of the two transferases. Of particular interest is the fact that methionine is the product of both transferase reactions. Four groups were studied: those on complete diets with and without a methionine supplement, and those on a cyano-Bl2-deficient diet with and without a methionine supplement. The results are summarized in Table IV.

The addition of methionine to the cyano-Bl2-deficient diet did not lead to a restoration of either methylfolate-\(H_4\) or methyl-Bl2 transferase activities, although the animals on a diet supple-
TABLE V

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 Methy1folate-H4 transferase</th>
<th>Specific activity Methyl-B12 transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>4</td>
<td>6.2 ± 0.50</td>
<td>0.39 ± 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>

TABLE VI

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>30 to 60% (NH4)2SO4</td>
<td>1.28</td>
<td>52</td>
<td>0.61</td>
<td>60</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>

TABLE VII

Requirements for methylfolate-H4-homocysteine transferase

The incubation mixtures and assay were performed as described in “Experimental Procedure.” As an enzyme source, 190 µg of the third ammonium sulfate fraction were 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>Complete</td>
<td></td>
<td>2.11</td>
<td>3.17</td>
</tr>
<tr>
<td>- β-Mercaptoethanol</td>
<td></td>
<td>0.50</td>
<td>2.16</td>
</tr>
<tr>
<td>- Cyan-Bl2</td>
<td></td>
<td>0.33</td>
<td>1.65</td>
</tr>
<tr>
<td>- S-Aminoethylmethionine</td>
<td></td>
<td>0.37</td>
<td>0.38</td>
</tr>
<tr>
<td>- Homocysteine</td>
<td></td>
<td>0.30</td>
<td>0.18</td>
</tr>
<tr>
<td>- FMN</td>
<td></td>
<td>1.90</td>
<td>2.78</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 30% stimulation in the presence of this sulphydryl 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 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. $K_m$ determinations of the required compounds under aerobic conditions indicated the following values: naphthyl-methylfolate-H₄, $2.25 \times 10^{-4}$ M; AMe, $7.5 \times 10^{-4}$ M; and l-homocysteine, $9 \times 10^{-5}$ M. 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 $K_m$ for homocysteine was $2 \times 10^{-4}$ M, while that for methyl-Blz was $5.2 \times 10^{-4}$ M.

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

Previous observations with extracts of chicken, rat, and hog liver indicated that addition of cyano-B₁₂ and other cobamide compounds in vitro markedly stimulated the methylfolate-H₄ transferase in an air but not in a nitrogen atmosphere (16, 26, 35). This was similar to the observations of Peel (37) on the transferase in an air but not in a nitrogen atmosphere (16, 26, 35). This was similar to the observations of Peel (37) on the transferase in an air but not in a nitrogen atmosphere (16, 26, 35). This was similar to the observations of Peel (37) on the transferase in an air but not in a nitrogen atmosphere (16, 26, 35). This was similar to the observations of Peel (37) on the transferase in an air but not in a nitrogen atmosphere (16, 26, 35).

With the more purified enzyme preparation, the addition of cyano-B₁₂ in vitro resulted in 50 to 100% stimulation in the reaction rate under anaerobic conditions (Table VII). This effect was only observed if $\beta$-mercaptoethanol or either FMN or FAD were present in the incubation. Deoxyadenosyl-B₁₂, B₁₂, methyl-B₁₂, Factor B, and Factor V₁₈ all satisfied the cobamide requirement under anaerobic conditions. Factor B had a maximal effect at $10^{-8}$ M while cyano-B₁₂ stimulated maximally at $10^{-4}$ M. Deoxyadenosyl-B₁₂, B₁₂, and methyl-B₁₂ were essentially equivalent in effectiveness to cyano-B₁₂ while Factor V₁₈ behaved as Factor B. The effectiveness of a variety of cobamide derivatives, including those which lack a nucleotide, indicates that the stimulation in vitro is a nonspecific effect, such as reported by Peel (37), and is not related to a definite coenzyme function. Highly purified intrinsic factor did not inhibit the methylfolate-H₄ transferase reaction either when it was added directly or when it was preincubated with the partially purified enzyme.

**Purification of ⁶⁰Co-Cyanocobalamin-Labeled Methylfolate-H₄ Transferase**

Because a definite coenzymatic function of cyano-B₁₂ or a B₁₂ derivative had not appeared with purification of the methylfolate-H₄ transferase, an alternative approach was undertaken to demonstrate the participation of a cobamide in methionine biosynthesis in chickens. Since the methylfolate-H₄ transferase activity of chicken liver decreased during cyano-B₁₂ deficiency and was partially restored with cyano-B₁₂ replenishment in vivo,

$^3$ Recently, Buchanan et al. (26) have been able to satisfy partially the requirement for reduced FAD with $\beta$-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.

$^5$ This fraction of intrinsic factor bound 4 $\mu$g of cyanocobalamin per mg of protein.

$^6$ It was anticipated that ⁶⁰Co cyano-B₁₂ injected into deficient chicks would label the enzyme obtained from such animals, if a cobamide compound was attached to the enzyme. Sixteen chicks were maintained on a cyano-B₁₂-deficient diet, and, after a 3-week period, a total of 2.25 mmoles of ⁶⁰Co-labeled cyano-B₁₂ (specific activity, 1.4 $\mu$C per mpmole) were injected per animal over a 48-hour period. Comparable animals which did not receive a cobamide supplement had a specific activity in the original supernatant fraction of 0.26 for the methylfolate-H₄ transferase. The ⁶⁰Co-cyano-B₁₂-treated animals had a specific activity for methylfolate-H₄ transferase of 1.0, again showing the ability of cyano-B₁₂ administration to restore the enzymatic activity in part (see Table II).

The liver supernatant fraction of ⁶⁰Co-cyano-B₁₂-treated animals was purified according to the method described in "Experimental Procedure." Table VIII records the distribution of radioactivity emitted by ⁶⁰Co-cyano-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 ⁶⁰Co 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 ⁶⁰Co-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 ⁶⁰Co-cyano-B₁₂ radioactivity was apparent in the pass-through and 0.01 $\mu$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 ⁶⁰Co radioactivity emitted, and methylfolate-H₄ and methyl-B₁₂ transferase activities, were retained on the column and eluted identically with 0.01 $\mu$M potassium phosphate, pH 7.4, containing 0.3 $\mu$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 ⁶⁰Co-cyano-B₁₂.

The achievement of a constant ratio of ⁶⁰Co per enzyme unit as well as the retention of ⁶⁰Co-cyano-B₁₂ through
TABLE VIII
Distribution of \(^{60}\text{Co}\) radioactivity and methylfolate-\(H_4\) transferase during purification

The nutritional experimental procedure and details of the \(^{60}\text{Co}\) cyano-B12 administration are discussed in the text. The overall purification was 30-fold.

<table>
<thead>
<tr>
<th>Purification fraction</th>
<th>(\text{c.p.m.})</th>
<th>\text{Ratio}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant*</td>
<td>(1.5 \times 10^4)</td>
<td>354</td>
</tr>
<tr>
<td>First (NH(_4))SO(_4) fractionation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 30%</td>
<td>(0.72 \times 10^4)</td>
<td>34</td>
</tr>
<tr>
<td>30 to 50%</td>
<td>(6.5 \times 10^3)</td>
<td>195</td>
</tr>
<tr>
<td>50 to 70%</td>
<td>(2.0 \times 10^3)</td>
<td>246</td>
</tr>
<tr>
<td>Calcium phosphate adsorption and elution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>(0.2 \times 10^4)</td>
<td></td>
</tr>
<tr>
<td>0.01 M phosphate buffer, pH 7.4, eluate</td>
<td>(0.2 \times 10^4)</td>
<td>140</td>
</tr>
<tr>
<td>0.2 M phosphate buffer, pH 7.4, eluate*</td>
<td>(3.9 \times 10^3)</td>
<td>150</td>
</tr>
<tr>
<td>0.3 M phosphate buffer, pH 7.4, eluate</td>
<td>(0.8 \times 10^3)</td>
<td>76</td>
</tr>
<tr>
<td>Second (NH(_4))SO(_4) fractionation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 35%</td>
<td>(0.2 \times 10^4)</td>
<td>102</td>
</tr>
<tr>
<td>35 to 55%*</td>
<td>(2.5 \times 10^4)</td>
<td>102</td>
</tr>
<tr>
<td>DEAE-cellulose, tube 15 (See Fig. 2)</td>
<td>(4.5 \times 10^4)</td>
<td>101</td>
</tr>
</tbody>
</table>

* These fractions have been used for further purification.

The above experiment was done with chickens on a cyanob-12 deficient diet. It was felt that injection of \(^{60}\text{Co}\)-cyano-B12 into control chickens on a diet adequate in cyano-B12 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 \(^{60}\text{Co}\)-cyano-B12 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-B12 intake, the ratio of \(^{60}\text{Co}\) 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-B12 per kg of diet, the enzyme sites are not completely saturated with the cobamide prosthetic group.

DISCUSSION

Of the predicted functions of cyanob-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-B12 (39-41).

The present study was undertaken to clarify in a similar manner the role of cyanob-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 cyanob-12 depletion, repletion of the specific activity with administration of cobamide derivatives 24 hours prior to death, and the intimate association of \(^{60}\text{Co}\)-cyano-B12 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. cyano-B_{12}, deoxyadenosyl-B_{12}, and methyl-B_{12}, 
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 holoenzyme 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_{12} 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 to E. coli did stimulate the methyl transfer from 
methylfolate-H_{4} 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 
\(\beta\)-mercaptoethanol for the cobamide stimulation, and (d) the 
relative efficiency of Factors B and V_{4} at low concentrations, 
suggest that the stimulation is similar to the nonenzymatic 
catalysis of monothiol oxidation by cyano-B_{12} derivatives (42).

The relationship of the methyl-B_{12} transferase activity to the 
methylfolate-H_{4} transferase is still in doubt. One major difference 
between the two transferase activities was the suppression of 
methylfolate-H_{4} transferase levels by increased dietary methionine 
without a comparable effect on the levels of methyl-B_{12} transferase. 
On the other hand, the enzyme activities were not 
separated during purification although the methyl-B_{12} transferase activity was purified to a slightly greater extent. This appeared 
to be due to a greater lability of the methylfolate-H_{4} transferase. In 
addition, there was a comparable response of the two transferase 
activities to cyano-B_{12} depletion and repletion.

The suppression of avian liver methylfolate-H_{4} transferase by 
increased dietary methionine would be expected to result in an 
accumulation of methylfolate-H_{4} in vivo. On the contrary, 
dietary methionine has been reported to lower the levels of 
methylfolate-H_{4} in rat liver with a corresponding increase in the 
formation of formylated folate-H_{4} 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_{4} as well as a reduction in the methylfolate-H_{4} transferase.

**SUMMARY**

1. Nutritional deficiency of 5,6-dimethylbenzimidazolyl-
coamide cyanide (cyano-B_{12}) in young chicks leads to a marked 
reduction of \(N\)-methyltetrahydrofolate and 5,6-dimethylbenzimidazolylcoamide methyl (methyl-B_{12}) transferase 
activities; replenishment with cyano-B_{12}, 5,6-dimethylbenzimidazolylcoamide \(N\)-deoxyadenosyl (deoxyadenosyl-B_{12}), and methyl-B_{12} resulted in partial restoration of both activities, while cobaminax 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 methyltetra-
hydrofolate transferase but not of methyl-B_{12} transferase 
activity.

3. The methyltetrahydrofolate and methyl-B_{12} transferase 
activities of chicken liver have been partially purified and char-
acterized. The activities were not separated at this stage of 
purification. The purified methyltetrahydrofolate transferase 
required homocysteine and S-adenosylmethionine, and \(\beta\)-mercap-
tetoethanol as a reducing compound. Methyl-B_{12} transferase 
required only the methyl group acceptor, homocysteine.

4. Cyano-B_{12}, deoxyadenosyl-B_{12}, methyl-B_{12}, Factor B, 
cobyricinic acid a, b, c, d, e, g, hexamide (Factor V_{4}), and a 
reduced form of cyano-B_{12} (B_{12}) all stimulated the methyltetra-
hydrofolate transferase reaction acrbobically and, to a lesser 
extent, anaerobically. The anaerobic cobamide stimulation was 
effective only in the presence of \(\beta\)-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. \(^{14}\)Co-Cyanocobalamin, when injected into vitamin-B_{12}- 
deficient chicks, was incorporated into a protein fraction which 
was found during purification in the same fractions as methyl-
folate-H_{4} transferase. In the nondepleted animal, there was evidence of 'j@Co-cyano-
coamide, when injected into vitamin-B_{12}- 
deficient chicks, was incorporated into a protein fraction which 
was found during purification in the same fractions as methyl-
folate-H_{4} transferase. In the nondepleted animal, there was evidence of 'j@Co-cyano-

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