Further Studies on the Relationship of Vitamin B₁₂ to Methionine Synthesis in Extracts of Escherichia coli*

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(Received for publication, October 21, 1960)

It has been reported (2) that extracts of Escherichia coli PA15 (a mutant requiring serine or glycine for growth) prepared from cells grown on a medium containing vitamin B₁₂, contain a factor which is required for methionine synthesis from homocysteine. The need for this factor is apparent only when tetrahydrofolate is employed as a carrier of precursors of the methyl group. Either carbon 3 of serine or formaldehyde can serve as precursor. The present paper extends the earlier report and includes data on the purification of the factor which is a vitamin B₁₂-containing protein.

EXPERIMENTAL PROCEDURE

Growth of E. coli PA15—This organism was grown without addition of ammonium sulfate (0.21 g per ml) was added with stirring over a 30-minute period, and the stirring was continued for 30 minutes at 3°. The precipitate was collected by centrifugation (0 to 30% saturation) and the supernatant was added 0.07 g of ammonium sulfate per ml and the resultant precipitate again collected (30 to 40% saturation). This latter step was repeated 2 more times (to provide fractions precipitating at 40 to 50 and 50 to 60% saturation). Each precipitate was dissolved in 0.01 M potassium phosphate, pH 7.8.

(b) Ca₅(PO₄)₃ Gel—To 1 g of freshly prepared Ca₅(PO₄)₃ gel suspended in 17 ml of H₂O, were added 16 ml of a 0 to 35% ammonium sulfate fraction containing 13 mg of protein per ml. The mixture was stirred for 30 minutes at 3°, centrifuged, and the supernatant (gel supernatant) retained. The precipitate was stirred with 20 ml of 0.01 M potassium phosphate for 30 minutes and centrifuged. This procedure was repeated with 0.05, 0.1, and 1.0 M potassium phosphate.

(c) DEAE Chromatography—A DEAE-cellulose (Eastman Organic Chemicals) column, 7.5 x 12 cm, was washed first with 4 liters of 1 M potassium phosphate, pH 7.8, then with 4 liters of water, and chilled to 5° in the cold room where the chromatography was carried out. Sonic extract, 60 ml (21.5 mg of protein per ml), was pipetted onto the column and eluted batchwise with increasing concentrations of potassium phosphate, pH 7.8, as follows: Fraction 1, 200 ml, 0.01 M; Fraction 2, 200 ml, 0.05 M; Fraction 3, 200 ml, 0.1 M; Fraction 4, 200 ml, 0.2 M; Fraction 5, 200 ml, 0.3 M; Fraction 6, 100 ml, 0.4 M; Fractions 7 to 13, 50 ml, 0.4 M; Fraction 14, 400 ml, 1.0 M.

In Experiment IV (Table I), 100 ml of the 0.05 M potassium phosphate eluate from the Ca₅(PO₄)₃ gel was further fractionated on DEAE-cellulose as described in the preceding paragraph. The most active fractions (200 to 330 ml of 0.4 M potassium phosphate in this particular run) were pooled and dialyzed for 3 hours against distilled water (4 liters changed every hour) at 3°. This solution (volume now 100 ml) was lyophilized, the solid was suspended in 5 ml of H₂O, and the insoluble material removed by centrifugation.

Materials—d,L-Tetrahydrofolic acid was prepared as described (3). It was 95% pure as measured by its ability to bind formaldehyde (3). Oxidized tetrahydrofolic acid was prepared by allowing 1 ml of a 0.01 M solution in 1 M potassium phosphate, pH 7.8, to stand exposed to air overnight at room temperature, followed by shaking on a mechanical shaker for 3 hours. The solution changed from a light yellow to a deep orange color during this treatment.

The natural stereoisomer of tetrahydrofolic acid (L,L-tetrahydro-
### Table I

**Correlation of vitamin B₁₂ content and ability to promote methionine synthesis**

<table>
<thead>
<tr>
<th>Protein Activity units*/mg protein</th>
<th>B₁₂ Activity units*/mg B₁₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>I. (NH₄)₂SO₄ fractionation</td>
<td></td>
</tr>
<tr>
<td>Sonic extract</td>
<td>340</td>
</tr>
<tr>
<td>Saturation, 0-30%</td>
<td>40</td>
</tr>
<tr>
<td>Saturation, 30-40%</td>
<td>95</td>
</tr>
<tr>
<td>Saturation, 40-50%</td>
<td>25</td>
</tr>
<tr>
<td>Saturation, 50-60%</td>
<td>50</td>
</tr>
<tr>
<td>Recovery</td>
<td>56%</td>
</tr>
<tr>
<td>II. Ca₃(PO₄)₂ gel (NH₄)₂SO₄, 0-35% saturation</td>
<td></td>
</tr>
<tr>
<td>Gel supernatant</td>
<td>728</td>
</tr>
<tr>
<td>Buffer supernatant, 0.01 M</td>
<td>68</td>
</tr>
<tr>
<td>Buffer supernatant, 0.05 M</td>
<td>45</td>
</tr>
<tr>
<td>Buffer supernatant, 0.1 M</td>
<td>29</td>
</tr>
<tr>
<td>Recovery</td>
<td>91%</td>
</tr>
<tr>
<td>III. DEAE chromatography</td>
<td></td>
</tr>
<tr>
<td>Sonic extract</td>
<td>1,290</td>
</tr>
<tr>
<td>Fractions 1 to 5 combined</td>
<td>362</td>
</tr>
<tr>
<td>Fraction 6</td>
<td>260</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>42</td>
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<tr>
<td>Fraction 8</td>
<td>37</td>
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<td>Fraction 9</td>
<td>55</td>
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<td>Fraction 10</td>
<td>45</td>
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<td>Fraction 11</td>
<td>30</td>
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<tr>
<td>Fraction 12</td>
<td>22</td>
</tr>
<tr>
<td>Fraction 13</td>
<td>22</td>
</tr>
<tr>
<td>Fraction 14</td>
<td>56</td>
</tr>
<tr>
<td>Recovery</td>
<td>72%</td>
</tr>
<tr>
<td>IV. Combination of I, II, and III</td>
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</tr>
<tr>
<td>Sonic extract</td>
<td>13,080</td>
</tr>
<tr>
<td>(NH₄)₂SO₄, 0-35% saturation</td>
<td>4,340</td>
</tr>
<tr>
<td>Ca₃(PO₄)₂ gel, 0.05 M supernatant</td>
<td>444</td>
</tr>
<tr>
<td>Combined DEAE fractions</td>
<td>50</td>
</tr>
<tr>
<td>Recovery</td>
<td>0.4%</td>
</tr>
</tbody>
</table>

*An activity unit is the ability to synthesize 1 µg of methionine in 3 hours under the assay conditions described in Fig. 1.

Folate) was generated from 9 µmoles of horse liver 5-formyl-L,L-tetrahydrofolate (4) by incubating it with 72 µmoles of L-glutamate, 200 µmoles of potassium phosphate, pH 7.8, and 80 units of transformylase (5) for 3 hours at 37° under hydrogen in a Thunberg tube, in a total volume of 1 ml. Microbiological assay with *Pediococcus cerevisiae* (ATCC 8081) showed that 6 µmoles of 5-formyl-L,L-tetrafolate remained, 3 µmoles having been converted to L,L-tetrahydrofolate, which is destroyed under the assay conditions used. (The 5-formyltetrahydrofolate and the transformylase enzyme were kindly supplied by Drs. M. Silverman and J. C. Keresztesy.) The incubation mixture was used as such as a source of natural tetrahydrofolate. It had been observed¹ that 5-formyl-L,L-tetrahydrofolate is one-tenth as inhibitory to methionine synthesis as is dl,L-tetrahydrofolate.

¹ R. L. Kisliuk, unpublished experiments.

Its presence would therefore not affect the results to be presented in Table III. Further,¹ 5-formyl-L,L-tetrahydrofolate does not serve as a source of 1-carbon units for methionine synthesis in the presence or absence of the B₁₂ protein. A control mixture prepared in the same manner but with 5-formyl-L,L-tetrahydrofolate omitted did not affect methionine synthesis.

L-Homocysteine was purchased from the Nutritional Biochemicals Corporation and vitamin B₁₂ from Merck and Company. All other coenzymes were purchased from the Sigma Chemical Company.

Dimethylbenzimidazole cobamide coenzyme and adenosyl-L-methionine were gifts of Dr. H. Weissbach and Dr. S. Harvey Mudd, respectively. Factor B was supplied by Dr. M. R. S. Fox from a preparation originating at Merck and Company. Chicks fed a synthetic diet supplemented with vitamin B₁₂ were also supplied by Dr. Fox.
March 1961
R. L. Kisliuk
819

Manner of Incubation—Incubations for methionine synthesis were carried out in Thunberg tubes under hydrogen.

Assay—L-Methionine was determined microbiologically with Leucomonost mesenteroides (ATCC 8042) in the oxidized peptone medium of Lyman et al. (6).

Vitamin B₁₂ was determined with Lactobacillus leichmannii (ATCC 7830) in Difco B₁₂ assay medium.

Davis and Mingoli (7) observed that the B₁₂ taken up by E. coli during growth could be recovered on boiling the cells. Under the conditions of the present study, 95% of the B₁₂ added to the medium was taken up by the cells during growth. Virtually all of this could be recovered by autoclaving the cells for 10 minutes. It was possible, therefore, to estimate the B₁₂ content of a given cell fraction by assay with L. leichmannii provided that the sample was added to the assay medium before the latter was autoclaved. The B₁₂ content of non-B₁₂-grown organisms was too small (20 mpg per g of acetone-dried organisms) to cause any error.

Protein was determined by the method of Lowry et al. (8).

Spectrophotometric measurements were carried out with a Beckman model DU spectrophotometer.

RESULTS

Correlation of Ability to Promote Methionine Synthesis with B₁₂ Content

Although extracts of E. coli prepared from cells grown on a medium containing vitamin B₁₂ are known to be able to utilize tetrahydrofolate as a carrier of single carbon units for methionine synthesis (2), it remained to be determined whether or not the factor responsible for this activity actually contained the vitamin. It was desirable therefore to correlate the ability to promote methionine synthesis (which is proportional to the amount of factor present (Fig. 1)) with B₁₂ content as the factor was purified.

The data in Table I show that enzymatic activity is correlated with B₁₂ content throughout purification. The ratio of activity to B₁₂ content does not vary greatly in a given experiment. This means that a large proportion of the B₁₂ present in the sonic extract is enzymatically active. The variability between experiments is due to variable activity in different batches of assay extract.

The turnover number (number of moles of methionine synthesized per mole of B₁₂ per minute) is between 1500 and 2000 based on the data of Experiments III and IV.

By the procedure outlined, preparations have been obtained containing 0.44 μg (0.33 pmole) of B₁₂ per mg of protein. One such preparation assayed for riboflavin with Lactobacillus casei containing 0.36 to 0.4 pmole per mg of protein, which may be significant in view of the results of Hatch et al. (9) indicating a role for flavin in methionine formation in E. coli.

Properties of Active Material—The following evidence suggests that the active material contains protein; it is nondialyzable, heat-labile (2) and is labile to trypsin treatment. The most highly purified fractions have a ratio of absorbancy at 260 μm to 280 μm of 1.3, which indicates a protein content of about 90% (10).

Sonic extracts containing the B₁₂ protein retained full activity for at least 6 months; the purified fractions for at least 1 month when stored at −20°C. Both crude and purified material lost activity when stored in 0.01 M potassium phosphate, pH 7.8, at low protein concentration. A solution of the sonic extract containing 1 mg of protein per ml lost 20% of its activity on storage at −20°C for one month. A solution of the same material containing 10 mg of protein per ml lost no activity under these conditions.

The B₁₂ in the active fractions was unavailable to L. leichmannii if the sample was added to the previously autoclaved assay medium. This is consistent with the earlier observation (2) that the B₁₂ present in active preparations is nondialyzable.

A spectrophotometric analysis of the extract of the combined DEAE fractions mentioned in Table I, revealed in addition to the protein peak at 280 μm a peak at 405 μm. At present, it is not known whether the 405-μm peak is in any way related to the B₁₂ content (2.6 μg per ml) of the sample.

Comparison of Properties of B₁₂ Protein with Those of Dimethylbenzimidazole Cobamide Coenzyme

The dimethylbenzimidazole cobamide coenzyme, described by Barker et al. (11, 12), does not stimulate methionine synthesis in the system employed even when present at 3700 times the concentration of B₁₂ in the B₁₂ protein (Table II).

The partially purified B₁₂ protein (a DEAE fraction containing 70 μg of B₁₂ per mg of protein) showed no loss in activity.
zyme are inactivated by 0.1 mM cobamide coenzyme (1). No methionine synthesis occurred upon addition of the Brz protein and protection from light. Homocysteine. The results presented in Table IV exclude room temperature. When a sonic extract containing the Brz protein is added, it acts as an inhibitor unless the Blz protein is present.

The serine hydroxymethylase reaction in E. coli extracts proceeds several times faster than when DPN is added. Similar results were obtained with either serine or 5,10-methylene-tetrahydrofolate as substrate. In addition to these reactions, no requirement for Brz protein has been observed for thymidylate synthetase (13) or deoxyxystygidyl hydroxymethylase (14), two other tetrahydrofolate-dependent enzymes from E. coli.

Production of B12 Protein under Various Conditions—Extracts of cells grown on as little as 0.1 µg of B12 per liter of minimal medium supplemented with glycine, contained a detectable amount of the B12 protein. Extracts of cells grown on Factor B (7.3 µg per liter) also contained active material. This was expected, as B12-requiring mutants of E. coli will grow on Factor B (15). When growth took place on minimal medium supplemented with glycine and containing 10⁻⁴ M DL-methionine as well as vitamin B12 (2.3 µg per liter), extracts of the cells contained the usual amount of the B12 protein. The inability of resting cells (16) and cell extracts (17) prepared from cells grown on methionine to synthesize methionine, is therefore not due to lack of the B12 protein.

**Distribution of B12 Protein**—Ultrasonic extracts of E. coli mutants 121/176 (17) and 113-3 (18) are able to synthesize the B12 protein.

Attempts to find a factor active in the E. coli assay system described in Fig. 1 in extracts of acetone-dried, B12-grown L. leichmannii and in extracts prepared from the livers of chicks fed a diet supplemented with vitamin B12 were unsuccessful.

**Inhibition of Methionine Synthesis by Tetrahydrofolate**—It has been proposed that the inhibition of methionine synthesis in the assay extract by tetrahydrofolate (in the absence of the B12 protein) is due to the action of the latter substance as a competitive analogue of the naturally occurring folic acid coenzyme (2). However, the possibility was not excluded that this inhibition was due to an impurity in the dl, l-tetrahydrofolate. The following evidence makes this seem unlikely:

(a) Tetrahydrofolate oxidized in air caused no inhibition. The incubation mixture contained an amount of oxidized material equivalent to 5 × 10⁻⁴ M tetrahydrofolate, 10 times the amount required for 50% inhibition (2).

(b) Folate (5 × 10⁻⁴ M) caused no inhibition and promoted no methionine synthesis on addition of the B12 protein.

(c) l-L-Tetrahydrofolate, generated from 5-formyl-l-L-tetrahydrofolate behaves as does dl-L-tetrahydrofolate (Table III), that is, it acts as an inhibitor unless the B12 protein is present.

**Inability of Adenosyl-L-methionine to Serve as Precursor of Methionine**—In view of the ATP requirement for methionine synthesis, it was considered (2) that either S-adenosyl-l-homocysteine (19) or l-homocysteine-AMP (similar to carboxyl-activated methionine (20)) might be the actual acceptor of the methyl group. If the former compound were the acceptor, one would expect that adenosyl-l-methionine would be formed and then either cleaved to methionine or donate its methyl group to homocysteine. The results presented in Table IV exclude such a pathway. Adenosyl-l-methionine was not converted to methionine.
methionine by the extracts, nor did it transfer its methyl group to homocysteine. Incidentally, it was observed that adenosyl-L-methionine did not support the growth of L. mesenteroides even when added to the autoclaved assay medium aseptically. Stevens and Sakami (21) and Nakao and Greenberg (22) have presented evidence that adenosylmethionine is not an intermediate in methionine synthesis in extracts of organisms grown in the absence of Blz. L-Homocysteine-AMP has not been tested as yet.

**DISCUSSION**

It may be postulated that vitamin Blz plays the same role, that of an electron donor and acceptor, in many of the reactions in which it is known to be involved. The reactions in question are listed below:

1. Glutamate $\rightarrow$ $\beta$-methyl aspartate (23) Methyl group formation by isomerization
2. Succinyl-CoA $\rightarrow$ methylmalonyl-CoA (24) Methyl group formation by reduction
3. Formaldehyde + homocysteine $\rightarrow$ methionine (2) Methyl group formation by reduction
4. Formate incorporation into thymine methyl (25) Substituted methyl group formation by reduction
5. Riboside $\rightarrow$ deoxyriboside (26) Substituted methyl group formation by reduction

In the isomerization reactions, a redistribution of electrons in the coenzyme by the metalloporphyrin-like structure of the vitamin. The hypothesis (2) that organisms grown in the presence of vitamin Blz possess a mechanism for methionine synthesis which differs from that in organisms grown in the absence of the vitamin is supported by work cited herein and other recent work. For example, very little Blz can be detected in the assay extracts prepared from cells grown on vitamin Blz and presumably containing vitamin Blz, which promotes methionine methyl group formation in extracts in the presence of tetrahydrofolate that is not replaced by dimethylbenzimidazole cobamide coenzyme and is not light-sensitive.

Recent work by Guest et al. (17) has shown that the dimethylbenzimidazole cobamide coenzyme is more active than vitamin Blz in stimulating methionine synthesis in ultrasonic extracts of E. coli. Possibly, the coenzyme is an intermediate in the formation of the Blz protein. Evidence has been presented by Guest (27) that the cobamide coenzyme itself is not the active form. Inhibition of the activity of the coenzyme by the anilide analogue of vitamin Blz was found to be competitive. However, extracts prepared from cells grown on vitamin Blz and presumably containing the Blz protein are insensitive to the anilide. This evidence is consistent with the results of the present work in which the Blz protein is not replaced by the coenzyme and diffuses from the coenzyme in its sensitivity to light.

The fact that TPN is more active than DPN in the hydroxy methyltetrahydrofolate acid dehydrogenase reaction may explain why TPN inhibits methionine synthesis when added with DPN (2). The TPN-activated dehydrogenase may compete with the methionine-forming system for the available single carbon units.

**SUMMARY**

1. The factor present in Escherichia coli, grown on a medium containing vitamin Blz, which promotes methionine methyl group formation in extracts in the presence of tetrahydrofolate is a vitamin Blz-containing protein. It is not replaced by dimethylbenzimidazole cobamide coenzyme and is not light-sensitive.

2. Further evidence supporting the hypothesis that organisms grown on vitamin Blz are capable of synthesizing methionine by a different mechanism than those grown in the absence of the vitamin is discussed.

3. The natural stereoisomer of tetrahydrofolate (I-l-tetrahydrofolate) affected the system in the same manner as the synthetic compound.

4. Adenosyl-L-methionine is not an intermediate in methionine synthesis in the system studied.

5. The hydroxymethyltetrahydrofolate dehydrogenase of E. coli extracts which showed greater activity with triphosphopyridine nucleotide than with diphosphopyridine nucleotide was not affected by the presence of the Blz protein.

**Acknowledgment**—The author wishes to thank Dr. Milton Silverman and Dr. Herbert Weissbach for helpful discussion and Mrs. M. Romine and Mr. H. A. Bakker for carrying out most of the microbiological assays.

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