Enzymatic Synthesis of the Methyl Group of Methionine

I. IDENTIFICATION OF THE ENZYMES AND COFACTORS INVOLVED IN THE SYSTEM ISOLATED FROM ESCHERICHIA COLI*

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The mechanism of formation of the methyl group of methionine is of particular interest at the present time since the reaction may be studied in cell-free systems and is dependent on the direct participation of vitamin B12. The reported systems for study of methionine biosynthesis in vitro have been derived from mammalian, avian, and bacterial sources (4-10). Serine and formaldehyde have been the most commonly studied precursors of the 1-carbon fragment that appears in the methyl group of methionine. Homocysteine has been the required acceptor in all the systems.

The studies in both animal and bacterial systems have established cofactor requirements for pyridoxal-P (when serine is the 1-carbon unit donor), tetrahydrofolic acid, adenosine triphosphate, Mg++, and a pyridine dinucleotide. Vitamin B12 has been shown to be a required cofactor only in the system derived from a mutant strain of Escherichia coli which requires vitamin B12 or methionine for growth (11, 12). These studies have not identified the necessary enzymes or the reaction steps in the pathway for methionine synthesis.

This paper reports a system of three partially purified enzyme fractions from E. coli which are essential for methionine biosynthesis from serine. One of the required enzymes is serine hydroxymethylase (13-15), and another has been found to have a genetic block in the synthesis of vitamin B12 which results in a secondary phenotypic block at a stage between homocysteine and methionine.

Experimental Procedures

Coenzymes, nucleoside phosphates, homocysteine thiolactone, and S-adenosylhomocysteine were obtained from the California Corporation for Biochemical Research, Mann Research Laboratories, Sigma Chemical Company, and Pabst Laboratories, Inc. L-Serine-3-Cl4 was obtained from Nuclear-Chicago Corporation. Folic acid derivatives and antagonists were gifts of Dr. T. Jukes and Dr. R. Angier of Lederle Laboratories, Inc., Division of American Cyanamid Corporation. S-Adenosylhomocysteine was kindly provided by Dr. W. Sakami.

Glucose 6-phosphate dehydrogenase was prepared from yeast in collaboration with Dr. W. T. Jenkins by a modification of the method of Kornberg and Horecker (16). Ethanol dehydrogenase was obtained from Sigma Chemical Company, DNase from Worthington Biochemical Corporation, and RNase from Armour and Company, Research Division.

Tetrahydrofolic acid was prepared by the method of Blakley (17) and stored in 0.02 M 2-mercaptoethanol at -15° in a Thunberg tube under nitrogen. N5,N10-Methylene tetrahydrofolate acid was prepared by the method of Osborn, Talbert, and Huenekens (18). Homocysteine (19) was prepared freshly each day from L-homocysteine thiolactone. Two equivalents of KOH were added to a 0.2 M solution of the thiolactone which was then heated at 45° for 6 minutes while nitrogen was bubbled through the mixture. The solution was neutralized with one equivalent of HCl and cooled to 0°. Calcium phosphate gel was prepared by the method of Keilin and Hartree (20). DEAE-cellulose was purchased from the Brown Company, Berlin, New Hampshire, and was treated with ethylenediaminetetraacetic acid and alkali according to the procedure of Camiener and Brown (21).

Mutant Strains of E. coli—The mutant strains of E. coli utilized in the experiments were kindly provided by Dr. B. D. Davis. Strain 113-3 is a mutant strain which requires either vitamin B12 or methionine for growth (22). The nutritional requirement is not satisfied by homocysteine. This strain is considered to have a genetic block in the synthesis of vitamin B12 which results in a secondary phenotypic block at a stage between homocysteine and methionine.

Strain 205-2 is a double mutant which requires both p-aminobenzoic acid and methionine. The two mutations in this strain were selected in separate experiments and are not interrelated. Therefore, as long as sufficient p-aminobenzoic acid is supplied in the culture medium, strain 205-2 can be considered purely as a methionine-requiring mutant. The nutritional requirement of this strain is not satisfied by either homocysteine or vitamin B12.

Culture Methods for E. coli Mutant Strains

Stock Cultures—Stock cultures of strain 205-2 of E. coli were maintained on a modification of the basal medium described by Dr. B. D. Davis, personal communication.
Supplements to basal medium for culture of mutant strains of E. coli

<table>
<thead>
<tr>
<th>Strain</th>
<th>Purpose of culture</th>
<th>Supplements</th>
<th>Amount of supplements/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>113-3</td>
<td>Enzyme preparation and cofactor determin</td>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>1 µg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methionine</td>
<td>250 µmoles</td>
</tr>
<tr>
<td>205-2</td>
<td>Cells deficient in vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Methionine</td>
<td>250 µmoles</td>
</tr>
<tr>
<td></td>
<td>Assay of enzyme lacking in strain 205-2</td>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>1 µg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methionine</td>
<td>250 µmoles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-Aminobenzoic acid</td>
<td>100 µg</td>
</tr>
</tbody>
</table>

Strain 113-3 was employed as the source of the methionine-synthesizing enzyme system in order that studies of the general aspects of methionine synthesis and of the special function of vitamin B<sub>12</sub> might be carried out with the same organism. The washed cells were frozen in liquid nitrogen and broken in the Hughes press (27). This method provided a means for the reproducible breakage of cells and yielded a concentrated solution of protein with high activity of the several desired enzymes. The viscosity of the pressed material was reduced by incubation of the solutions with DNase at 37<sup>°</sup> for 10 minutes in the presence of 0.005 M Mg<sup>++</sup>. The enzyme was added at the level of 1 mg per 100 ml of extract. The treated extract was then diluted with an equal volume of cold 0.05 or 0.1 M phosphate or Tris buffer and centrifuged at 78,000 to 105,000 X g (average) in the model L Spinco ultracentrifuge to sediment cell debris and large ribonucleoprotein particles. All subsequent preparative procedures were carried out at 3 to 5° unless otherwise noted.

Designation of Enzymes

Aside from serine hydroxymethylase, two additional enzymes have been recognized as participating in the biosynthesis of the methyl group of methionine from serine. Since the catalytic function is not yet known for either of the new enzymes, temporary rather than properly descriptive names have been assigned to them.

B<sub>12</sub> Enzyme—The first of the enzymes has been designated the "B<sub>12</sub> enzyme" because vitamin B<sub>12</sub> or a derivative is associated with the protein as a tightly bound prosthetic group (28). The activity of this enzyme in extracts of strain 113-3 is dependent upon the presence of vitamin B<sub>12</sub>

205-2 Enzyme—The second of the new enzymes has been designated the "205-2 enzyme." This enzyme is lacking or inactive in extracts of mutant strain 205-2 and can be purified by fractionation from extracts of strain 113-3. Addition of fractions containing 205-2 enzyme to extracts of strain 205-2 completes the enzyme complement required for the synthesis of methionine.

Enzyme Incubation Procedure

The incubation mixture containing all the required components is described in Table II. The least stable component, folate-H<sub>4</sub>, was added last. Anaerobic conditions were obtained.
by performing the incubations in Thunberg tubes or in a desicator that had been evacuated and filled with prepurified nitrogen or with helium. At the end of a 2 or 3 hour incubation period at 37° the incubation mixtures were autoclaved simultaneously with the microbiological assay medium. The tubes were then centrifuged to remove coagulated protein. Four graded aliquots of the supernatant solution of the incubation mixture were transferred with a sterile long-tip pipette to the sterilized microbiological assay tubes.

The methionine content of the incubation vessels was assayed with *Leuconostoc mesenteroides* P60 (American Type Culture Collection No. 8042) in synthetic methionine assay medium (Difco Laboratories) in a final volume of 1 ml. After the tubes had been incubated for 18 hours at 37° the absorbancy at 650 mµ was determined in a Beckman model DU spectrophotometer. Absorbancy readings were converted to equivalent values in terms of µmoles of methionine by means of a standard curve covering the range from 0.5 to 25 µmoles per ml.

### RESULTS

#### Substrates for Methionine Biosynthesis

Serine was utilized as the donor of the 1-carbon unit in most of the experiments to be described. The required concentration for optimal methionine formation was about 0.005 M. Formaldehyde also serves as a source of the 1-carbon unit for methyl group formation. In the first step of the metabolism of these compounds, both are converted to N5, N10-methylene tetrahydrofolic acid. Optimal synthesis of methionine was obtained when homocysteine, the acceptor of the 1-carbon unit, was present in a concentration between 0.002 M and 0.02 M.

The following additional compounds were tested with crude cell extracts for activity as donor substrates: DL-3-phosphoserine, cystathionine, leucovorin (N5 formyl folate H4), and anhydroleucovorin (N4, N10-methenyl-folate-H4). Phosphoserine was moderately effective as a substrate, but it may have been broken down to serine before being utilized. The other named compounds were inactive. N10-Methyl tetrahydrofolic acid did not serve as a donor of the methyl group in the presence of the B12 enzyme and 205-2 enzyme.

#### Cofactors for Methionine Biosynthesis

The study of the cofactor requirements for methionine biosynthesis has been made with enzyme preparations treated by dialysis or with activated charcoal (Norit) and an anion exchange resin (Dowex 1-Cl). Treatment of an extract of strain 113-3 that had been cultured on vitamin B12 plus methionine with a combination of the methods described above revealed an requirement for ATP, pyridoxal-P, folate-H4, and DPNH. Although the addition of a flavin coenzyme stimulated the reaction considerably, there was a moderate synthesis of methionine with a combination of the methods described above revealed a requirement for ATP, pyridoxal-P, folate-H4, and DPNH. Although the addition of a flavin coenzyme stimulated the reaction considerably, there was a moderate synthesis of methionine with this type of enzyme preparation in the absence of added FAD. When purified enzyme fractions were used without charcoal or resin treatment, however, an absolute requirement was observed for all the cofactors indicated in Table III.

Reducing Coenzymes for Methionine Biosynthesis—The reducing agents for formation of the methyl group of methionine appear to be a coupled system of pyridine and flavin nucleotides (3). When the enzyme preparation had been treated so as to remove the pyridine nucleotides but not endogenous flavin, there was an absolute and specific requirement for DPNH. However, when both types of nucleotides had been removed and external flavin coenzyme was added, as in the system of purified enzyme fractions, either DPNH or TPNH was an effective reducing agent. The former compound was significantly more active, however.

The addition of flavin coenzyme could be made in the form of flavin mononucleotide or FAD, the former being somewhat more effective than the latter. A report has been made previously on the use of catalytically reduced flavin mononucleotide and FAD (3).

Further evidence for participation of a flavin coenzyme in methionine formation was provided by the finding that quinacrine (atebrin) inhibited the system markedly. The inhibition was reversed in part by reincubation of the enzymes with FAD. Chloroquine (Aralen) caused negligible inhibition.

#### Other Cofactors—Pyridoxal-P is a required cofactor for the serine hydroxymethylase reaction. In this reaction, which is an initial step in methionine biosynthesis, the 3-hydroxymethyl group of serine is transferred to tetrahydrofolic acid. The product of the transfer reaction is considered to be N5, N10-methylene tetrahydrofolic acid (18, 29, 30). As will be shown in a later section of this paper, this compound serves as the substrate for the next step in formation of the methyl group.

### Table III

<table>
<thead>
<tr>
<th>Component omitted</th>
<th>Methionine synthesized (µmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>71</td>
</tr>
<tr>
<td>L-Serine</td>
<td>10</td>
</tr>
<tr>
<td>L-Homocysteine</td>
<td>9</td>
</tr>
<tr>
<td>Pyridoxal-P</td>
<td>12</td>
</tr>
<tr>
<td>Folate-H4</td>
<td>9</td>
</tr>
<tr>
<td>DPNH</td>
<td>7</td>
</tr>
<tr>
<td>FAD</td>
<td>11</td>
</tr>
<tr>
<td>ATP</td>
<td>13</td>
</tr>
</tbody>
</table>

The abbreviation used is: folate-H4, tetrahydrofolic acid.
Pteroylglutamic acid was reduced to the tetrahydro level and tested as a cofactor. The optimal concentration of tetrahydropteroylglutamic acid appeared to be somewhat lower than that for folate-H4 as a cofactor for methionine biosynthesis. The existence of experiments on the involvement of vitamin B12 in methionine biosynthesis appears in the second paper of this series (28).

The optimal concentration of Mg++ for methionine synthesis was found to be approximately 0.005 M, and marked inhibition occurred at 0.1 M. No inhibition of methionine biosynthesis occurred in the presence of 0.01 M fluoride ion.

**Fractionation of Enzyme System**

The use of serine as donor of the 1-carbon unit for methyl group formation implicated serine hydroxymethylase as an essential component of the enzymatic system. The existence of the two different mutant strains of E. coli, 113-3 and 205-2, indicated a requirement for at least two additional enzymes for methionine synthesis. Fractionation and partial purification of these three enzymes were carried out as described in the following sections. For ammonium sulfate precipitations, additions of solid ammonium sulfate were calculated from a table of fractional saturations at 25°C. The actual level of saturation at the working temperature of 3 to 4°C was slightly higher than the figures reported. The phosphate buffers referred to in subsequent sections were made by dilution of 1 M solutions. The buffer of pH 7.2 was made by diluting a solution with the ratio of K2HPO4 to KH2PO4 of 18:7 to the stated concentrations; that of pH 6.8 by diluting a solution with the ratio 1:1.

**Purification of Serine Hydroxymethylase**—A radioactive tracer assay for serine hydroxymethylase was based upon the observation that N5,N10-methylene folate-H4 formed by the action of serine hydroxymethylase readily yielded its 1-carbon unit to form a precipitate with 5,5-dimethyl-1,3-cyclohexanedione (Dimedon) (31). For the preparation of partially purified serine hydroxymethylase, the extract of strain 113-3 was treated with DNase as described and fractionated with ammonium sulfate; the highest specific activity was found in the fraction precipitating at 50 to 70% saturation. This ammonium sulfate fraction was dialyzed against 0.01 M phosphate buffer, pH 7.2, and the dialyzed solution (30 ml) was chromatographed on a 2.2 × 10-cm column of hydroxylapatite (32) which had been equilibrated with 0.02 M potassium phosphate buffer, pH 7.2. Elution was performed with a convex gradient apparatus. The reservoir contained 125 ml of 0.1 M phosphate buffer, pH 7.2, and the mixer contained 100 ml of 0.02 M buffer, pH 7.2. Serine hydroxymethylase began to be eluted from the column in Fraction 26. A total of 75 0.5-ml fractions was collected.

The product had a specific activity 44 times that of the original extract and was obtained in 33% yield. The partially purified serine hydroxymethylase was found to be substantially contaminated with dihydrofolate reductase, but free from two enzyme fractions discussed below.

**Preliminary Fractionation of B12 Enzyme and 205-2 Enzyme**—The extract of strain 113-3 which had been cultured on B12 plus methionine was prepared with the Hughes press and treated with DNase as described above. The pressed material was diluted with an equal volume of buffer (Tris, 0.05 M; succinic acid, 0.02 M; Mg++, 0.01 M; final pH 7.5). The pH of the diluted extract was adjusted to 7.5 by addition of 1 N KOH. This solution was centrifuged for 15 minutes at 15,000 × g to remove cell debris. The sediment was washed with a small volume of the buffer and the supernatant solution of the wash was combined with the original supernatant solution. The combined extract was then centrifuged in the model L Spinco preparative ultracentrifuge for 90 minutes at 78,000 to 105,000 × g (average) to remove the larger ribonucleoprotein particles that appeared in the tubes as a translucent, brown, gelatinous sediment. Ethylenediaminetetraacetic acid at a final concentration of 0.02 M was added to the supernatant solution from this centrifugation to chelate the Mg++ ions that interfered with the action of RNase. Phosphate buffer (pH 7.2) was added to a final concentration of 0.1 M.

The extract was then incubated with crystalline RNase (0.2 mg per ml) for 10 minutes at 37°C followed by 30 minutes at room temperature. A copious gray precipitate of degraded ribonucleoprotein appeared. This digestion sharpened the fractionation of the proteins with ammonium sulfate in the succeeding step and permitted partial separation of the 205-2 and B12 enzymes.

After digestion with RNase, the solution was cooled in ice and centrifuged. The supernatant fluid was brought to 25% of saturation with ammonium sulfate by addition of 1/2 volume of a neutralized saturated solution of the salt. Solid ammonium sulfate was immediately added to bring the mixture to 35% of saturation. After the solution was stirred for 15 minutes, the precipitate was removed by centrifugation and discarded. A total of 3 ml of 1 N NH4OH were added per 100 ml of centrifuged solution, and solid ammonium sulfate was then added to 45% of saturation. Again 3 ml of 1 N NH4OH per 100 ml were added. The precipitated protein contained practically all the B12 enzyme and nearly one-fifth of the 205-2 enzyme. The supernatant solution was adjusted to 35% of saturation with solid ammonium sulfate. The precipitated protein of the 45 to 55% fraction contained the remainder of the 205-2 enzyme and was practically free from B12 enzyme. Some serine hydroxymethylase was present in this fraction, but the exact amount has not been measured. The ammonium sulfate fractions precipitating at 35 to 45% and at 45 to 55% of saturation represented the starting materials for further purification of the B12 and 205-2 enzymes, respectively.

**Purification of B12 Enzyme**—The principal assay method for the B12 enzyme was based upon the fundamental observation that extracts of strain 113-3 which had been cultured on vitamin B12 or vitamin B12 plus methionine synthesized methionine rapidly with the appropriate incubation mixture (Table II), whereas extracts of 113-3 cultured on methionine formed only a small amount of methionine in vitro. The extracts of strain 113-3 cultured on methionine became fully active for methionine synthesis, however, after the addition in vitro of vitamin B12 or after the addition of a protein fraction designated “B12 enzyme” which was isolated from strain 113-3 cultured with B12 or B12 plus methionine (12, 28). The approximate activity of this protein fraction could be measured by determination of the methionine formed when an aliquot of B12 enzyme fraction was added to a fixed amount (1 mg of protein) of extract of strain 113-3 cultured on methionine. The relationship between the amount of B12 enzyme added to the extract and the amount of methionine synthesized is shown in Fig. 1. Quantitative assays of B12 enzyme fractions were performed at two levels of added enzyme. This procedure is considered to provide fairly accurate comparisons of fractions at different levels of purification. A
The gel suspension was then stirred and centrifuged. The gel was resuspended and eluted twice with ammonium sulfate. Under the conditions of this fractionation, which in-cluded elimination of RNA and pH control, nearly 90% of the radioactive Brz enzyme was added to an extract of strain 113-3 of E. coli. The incubation mixture (1 ml) contained 1 mg of the extract and all necessary substrates and cofactors listed in Table II.

The purification procedure for the Brz enzyme was comprised of four steps. (a) The extract of strain 113-3 cultured on radioactive Brz plus methionine was fractionated with ammonium sulfate. Under the conditions of this fractionation, which include elimination of RNA and pH control, nearly 90% of the radioactivity in the soluble extract appeared in the 35 to 45% ammonium sulfate fraction, about 10% in the 0 to 35% fraction, and negligible quantities above 45% of saturation. (b) The 35 to 45% ammonium sulfate fraction, about 10% in the 0 to 35% fraction, was dissolved in 0.05 M potassium phosphate buffer of pH 6.8 and was dialyzed for 2 hours in a large volume of 0.01 M phosphate buffer. The protein concentration was adjusted to between 5 and 20 mg per ml by dilution, and calcium phosphate gel (20) was added at a gel to protein ratio (by weight) of 1.67. The gel suspension was then stirred and centrifuged. The gel was resuspended and eluted twice with 0.03 M buffer of pH 6.8. (c) The protein solution eluted from the gel was applied to a column (1.5 X 28 cm) of DEAE-cellulose (33) which had been equilibrated with 0.03 M phosphate buffer at pH 7.2. The column was eluted by the linear gradient method in which the reservoir contained 150 ml of 0.5 M potassium phosphate buffer at pH 7.2 flowing into a mixer containing 150 ml of 0.03 M phosphate buffer at pH 7.2. The Brz enzyme emerged somewhat after the bulk of the protein and just after the midpoint of the gradient elution. (d) The most active fractions from the DEAE-cellulose column were pooled and dialyzed for 4 hours against 0.01 M phosphate buffer at pH 7.2. The solution was then loaded on a 0.9- X 26-cm column of hydroxylapatite (32) which had been equilibrated with the same 0.01 M buffer. The protein was eluted with use of a linear gradient apparatus in which the reservoir contained 30 ml of 0.3 M potassium phosphate buffer, pH 7.2, flowing into a mixer containing 30 ml of 0.01 M phosphate buffer, pH 7.2. The active fractions were eluted when about two-thirds of the buffer had been passed through the column. These fractions were pooled and constituted the final product. The solution was subdivided into small aliquots and stored at -15°. The purification procedure is summarized in Table IV. The ratios of absorbancies, 280 m to 260 m, were 0.55 in the crude extract and 1.62 in the final product, an indication that the nucleic acid had been almost completely eliminated in the latter fraction (34).

The specific radioactivity of the final enzyme product indicated that the protein contained about 1.0 pmole of Brz per gram. With the assumptions that the enzyme contains 1 mole of vitamin per mole of protein and has a molecular weight of 1 X 105 the purity would be about 1%. Properties of Brz Enzyme—There was no significant loss of activity upon storage of Brz enzyme fractions for several months in the frozen state as long as the solution was not thawed and refrozen unduly. The enzyme was damaged by exposure to organic solvents in making acetone powder extracts or in the fractionation of extracts with ethanol at low temperature.

Purification of 205-2 Enzyme—Determinations of the approximate activity of fractions containing the 205-2 enzyme could be carried out by addition of these fractions to an extract of strain 205-2 in a procedure analogous to the assay method described for the Brz enzyme. However, the relationship between the amount of added 205-2 enzyme fraction and the amount of methionine formed was not linear over an extended range of concentration of the enzyme.

The preliminary steps in the purification of the 205-2 enzyme

![Fig. 1](http://www.jbc.org/)

**TABLE IV**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Enzymatic activity*</th>
<th>Co60-radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units/mg</td>
<td>Total units X 10^9</td>
<td>Relative improvement</td>
<td>Yield</td>
</tr>
<tr>
<td>Crude extract</td>
<td>260</td>
<td>8840</td>
<td>150</td>
<td>1330</td>
</tr>
<tr>
<td>(NH4)2SO4 (35-45%)</td>
<td>40.5</td>
<td>1700</td>
<td>54.7</td>
<td>93</td>
</tr>
<tr>
<td>Gel eluate</td>
<td>109</td>
<td>310</td>
<td>448</td>
<td>38</td>
</tr>
<tr>
<td>DEAE-cellulose column</td>
<td>54.5</td>
<td>85</td>
<td>6840</td>
<td>164</td>
</tr>
<tr>
<td>Hydroxylapatite column</td>
<td>6.3</td>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One unit of enzyme activity catalyzes the synthesis of 1 mmole of methionine during a 3-hour incubation period under standard conditions described in Table II. The Brz enzyme fraction was added to an incubation mixture containing 1 mg of protein of an extract of strain 113-3 that had been cultured on methionine.
through ammonium sulfate precipitation were identical with those in the preparation of the B12 enzyme. The protein precipitating between 43 and 35% saturation of ammonium sulfate was dissolved in a minimal amount of 0.03 M potassium phosphate buffer, pH 7.2, containing 8 × 10⁻⁴ M FAD and was dialyzed for 3 hours against 8 liters of 0.01 M potassium phosphate buffer, pH 7.2. During the dialysis a copious white precipitate gradually appeared. This was removed by centrifugation and was discarded. The protein concentration of the enzyme solution was adjusted to 10 to 17 mg per ml by the addition of an 8 × 10⁻⁴ M solution of FAD. Calcium phosphate gel (15 mg per ml) was added at a gel to protein ratio of 1:1 (by weight). The suspension was stirred for 30 minutes and the gel collected by centrifugation. The gel was resuspended in a solution of 8 × 10⁻⁴ M FAD, stirred for 15 minutes, and again collected by centrifugation. The supernatant solution was discarded. The gel was suspended in 0.02 M potassium phosphate buffer, pH 7.2, containing 8 × 10⁻⁴ M FAD, stirred for 30 minutes, and collected by centrifugation. This elution was repeated, and the two supernatant solutions were combined. The total volume of the eluting buffer used was roughly equal to the volume of the enzyme solution before the addition of gel. The gel eluant was applied directly to a 1.5-× 32-cm column of DEAE-cellulose that had previously been equilibrated with a solution of 0.02 M potassium phosphate buffer, pH 7.2. The enzyme was eluted by a solution of potassium phosphate buffer, pH 7.2, with a linear gradient ranging in concentration from 0.02 to 0.5 M. The most active fractions were pooled to give a preparation which exhibited a specific activity about 14 times greater than that of the starting material. The over-all yield was less than 10%. Addition of FAD in a concentration of 8 × 10⁻⁴ M to the eluting buffer during chromatography raised the yield significantly.

Properties of 205-2 Enzyme—There was a 30% loss of 205-2 enzyme activity upon storage for 3 months at −15°C. This loss of activity was increased by repeated freezing and thawing. FAD partially protected the 205-2 enzyme activity against decay during the purification procedure and storage. Addition of FAD also provided partial protection of enzyme activity when the temperature of the solution was raised to 50°C. The protective action of FAD on the enzyme is mentioned here because of its practical applicability in the purification procedures. There is not sufficient evidence from these or other experiments to determine whether the flavin compound is a cofactor for this enzyme.

Recombination of Partially Purified Enzyme Fractions—Recombination of the three partially purified enzyme fractions resulted in a high rate of methionine synthesis when all the recognized cofactors were present (Table V). The time course of methionine synthesis by the purified enzymatic system is illustrated in Fig. 2. A lag period was observed when the system contained low, rate-limiting amounts of B12 enzyme. With larger amounts of B12 enzyme such a lag period was not evident, and a nearly proportional rate of methionine synthesis was observed over a 3- to 4-hour period.

Individual Reaction Steps in Methyl Group Formation—N⁵, N⁴⁰ Methylene tetrahydrofolic acid, made chemically, is able to substitute for tetrahydrofolic acid, pyridoxal phosphate, and serine hydroxymethylase in the standard incubation mixture. Moreover, a compound with the same ultraviolet absorption spectrum and susceptibility to reaction with Dimedon as the synthetic N⁵, N⁴⁰-methylene tetrahydrofolic acid can be isolated from an incubation mixture containing bicarbonate buffer, 85 mmoles, pH 9.3; tetrahydrofolic acid, 20 mmoles; pyridoxal phosphate, 5 mmoles; serine, 20 mmoles; and 60 µg of serine hydroxymethylase. These components are incubated under nitrogen for 2 hours at 37°C and the product is isolated by the method of Osborn, Talbert, and Huennekens (18). This biosynthetic material, presumably N⁵, N⁴⁰-methylene tetrahydrofolic acid, is an effective substrate for methionine synthesis in the presence of the 205-2 enzyme and the B12 enzyme. It would be expected that the enzymatically prepared material is entirely the naturally occurring stereoisomer, whereas the chemically prepared substance is probably a D,L mixture in regard to the stereoisomerism at carbon atom 6 of the pteridine ring.

The reaction steps catalyzed by the 205-2 enzyme and the B12 enzyme are now being studied. Two compounds were available which on theoretical grounds might have been considered as intermediates in the reactions of methionine biosynthesis. S-Adenosylhomocysteine, provided through the kindness of Dr. W. Sakami, was found to inhibit methionine synthesis by 25% when added to a complete system at a concentration of 1 × 10⁻⁴ M. The compound had practically no capacity to serve as an intermediate in the system, i.e. to replace homocysteine or to replace ATP. S-Adenosylmethionine received some support as an intermediate in methionine formation in animal systems from

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**Table V**

Recombination of purified enzyme fractions

<table>
<thead>
<tr>
<th>Enzyme content</th>
<th>Methionine synthesized (µmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>61</td>
</tr>
<tr>
<td>Omit serine hydroxymethylase</td>
<td>14</td>
</tr>
<tr>
<td>Omit B12 enzyme</td>
<td>0</td>
</tr>
<tr>
<td>Omit 205-2 enzyme</td>
<td>4</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Time course of methionine biosynthesis with purified enzyme fractions. Standard incubation conditions were employed with serine hydroxymethylase, 14 µg per ml, and 205-2 enzyme, 66 µg per ml. Amounts of B12 enzyme (in µg per ml) are indicated by numbers adjacent to each curve.
data published by Nakao and Greenberg (6), although none from that of Stevens and Sakami (5). In the system derived from E. coli which is herein reported, S-adenosylmethionine increased methionine synthesis slightly when added to a complete system. However, the compound was entirely unable to replace the normal substrates.

**DISCUSSION**

Our initial investigations have shown that two methionine-requiring mutants of E. coli (113-3 and 205-2 of Davis) are deficient under certain conditions in two different enzyme activities of the methionine synthetic pathway. Extracts of strain 113-3 cultured on methionine could be used to assay one of these enzymes (B12 enzyme). Likewise, extracts of strain 205-2 cultured on B12 plus methionine could be used to assay the other enzyme (205-2 enzyme). Both the enzymes were present in extracts of strain 113-3 cultured on B12 plus methionine. Despite the fact that the biochemical functions of these enzymes have not yet been established, it was possible to separate them by fractionation and to purify each partially in a manner similar to that used for enzymes of a known mode of action. By this procedure it has been possible to establish a biochemical system of coenzymes and of partially purified enzymatic components for study of the pathway of methionine biosynthesis.

In most of our studies using this complex enzymatic system, serine was the donor and homocysteine the acceptor of the 1-carbon unit appearing ultimately in the methyl group. With these substrates at least six cofactors and three enzymes are required for synthesis of methionine.

The initial step is the serine hydroxymethylase reaction which, with the participation of pyridoxal-P, results in transfer of the 1-carbon unit to the hydroxymethyl level of oxidation to tetrahydrofolic acid. The 1-carbon unit is subsequently reduced to the methyl level of oxidation and transferred to the ultimate acceptor, homocysteine. The reducing agent appears to be a reduced flavin nucleotide, which is regenerated by transfer of hydrogen from DPNH. The involvement of a flavin coenzyme in methionine biosynthesis had not been previously recognized.

The role of ATP in methionine synthesis remains an enigma. Present experimental evidence indicates that the enzyme fractions are not yet sufficiently pure to permit definitive studies of this cofactor. The involvement of vitamin B12 in this enzymatic system is indicated by its participation as the prosthetic group of one of the essential enzymes (28, 35). However, there is no evidence yet available concerning the mechanism of action of this substance.

**SUMMARY**

A system of three partially purified enzyme fractions and six organic cofactors has been established for the study of the biosynthesis of the methyl group of methionine from serine and homocysteine. The enzyme fractions were isolated from a soluble extract of an Escherichia coli mutant (strain 113-3 of B. D. Davis) which requires either vitamin B12 or methionine for growth. The organism was cultured on both B12 and methionine for isolation of the methionine-synthesizing system. The enzyme fractions were: (a) serine hydroxymethylase, (b) an enzyme which contains a derivative of vitamin B12 as a prosthetic group, and (c) a fraction containing a third enzyme which is lacking in a methionine-requiring mutant strain of E. coli (strain 205-2 of Davis). The latter two enzymes had not previously been recognized as essential components of the methionine-synthesizing system.

A combination of the three enzyme fractions in the presence of required cofactors and substrates carried out methionine synthesis under anaerobic conditions. Absolute requirements were demonstrated for serine, homocysteine, pyridoxal phosphate, tetrahydrofolic acid, acenozinium triphosphate, reduced diposphopyridine nucleotide, and flavin adenine dinucleotide or flavin mononucleotide. N\textsuperscript{5},N\textsuperscript{10}-Methylene tetrahydrofolic acid, either chemically or biosynthetically prepared, may serve as a substrate in the synthesis of the methyl group of methionine.

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