Methylenetetrahydrofolate Dehydrogenase of the Amethopterin-resistant Strain *Streptococcus faecium* var. *durans* A<sub>k</sub> and Its Repressibility by Serine

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Received for publication 31 January 1968

The methylenetetrahydrofolate dehydrogenase of the amethopterin-resistant strain *Streptococcus faecium* var. *durans* A<sub>k</sub> was purified 100-fold. Because it is extremely labile, this enzyme required protection by 1 mm nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) during purification; 0.01 mm EADP<sup>+</sup> with 0.1% bovine plasma albumin stabilized the purified enzyme during storage at -20 C. Although the enzyme has properties of sulfhydryl enzymes, thiol compounds were not stabilizers. Oxidation of methylenetetrahydrofolate, catalyzed by the purified enzyme preparation, is NADP<sup>+</sup>-specific and yields methenyltetrahydrofolate and the reduced pyridine nucleotide. K<sub>m</sub> values for NADP<sup>+</sup> and for 5,10-methylenetetrahydrofolate (prepared as the formaldehyde adduct of biologically synthesized 6,8-tetrahydrofolate) were calculated to be 0.021 and 0.026 mm, respectively. Neither pure bases and their derivatives nor serine inhibited the reaction. In growing cultures, the differential rate of synthesis of the methylenetetrahydrofolate dehydrogenase was dependent upon the composition of the medium. A medium which contained acid-hydrolyzed casein, and thus an exogenous source of serine, was repressive for this enzyme. In a serine-free, completely defined medium, the amount of folate added (for serine synthesis de novo) affected the duration of the initial exponential growth phase. At the termination of this phase, which primarily reflected the onset of a decreased rate of serine biosynthesis, synthesis of the methylenetetrahydrofolate dehydrogenase was derepressed. Exogenous serine in the completely defined medium prevented the derepression. Furthermore, physiological concentrations of L-serine were repressive not only for the dehydrogenase but also for the methenyltetrahydrofolate cyclohydrolase and the serine hydroxymethyltransferase. Concomitantly, the differential rate of synthesis of the formyltetrahydrofolate synthetase of *S. faecium* var. *durans* A<sub>k</sub> was increased. Apparently, serine regulates the differential rates of syntheses of these enzymes.

The coenzyme activity of folate in purine nucleotide, serine, and thymidylate biosynthesis resides in monomeric derivatives of tetrahydrofolate at oxidation levels of formate and formaldehyde (12, 13, 24). The interconversion (reaction 1), which is pyridine-nucleotide dependent, has been shown with purified preparations of the 5,10-methylenetetrahydrofolate dehydrogenase or 5,10-methylenetetrahydrofolate:nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>)-oxidoreductase (EC 1.5.1.5.) of avian and mammalian liver cells (14, 23), calf thymus (28), *Escherichia coli* (10), *Salmonella typhimurium* strain LT-2 (8), and yeast (26).

\[
5,10\text{-methylenetetrahydrofolate} + \text{NADP}^+ \rightleftharpoons \\
5,10\text{-methylenetetrahydrofolate}^+ \\
+ \text{NADPH} + \text{H}^+ 
\]

Presumably, in vivo, this enzyme maintains a physiological balance of the tetrahydrofolate coenzymes, as required and regulated by the metabolic needs of the cell. Accordingly, the mediation of this equilibrium might predispose the oxidoreductase to several regulatory mecha-
nisms. Dalal and Gots reported that purine nucleotides inhibit the activity of the *S. typhimurium* enzyme (7). A previous report from our laboratory noted the repressive action of serine on the biosynthesis of the enzyme from an amethopterin-resistant mutant strain, *Streptococcus faecium* var. *durans* A₅ (A. M. Albrecht, F. K. Pearce, and D. J. Hutchison, Bacteriol. Proc., p. 97, 1966).

This communication deals with the partial purification of the methylenetetrahydrofolate dehydrogenase of *S. faecium* var. *durans* A₅, its repressibility by serine or a derivative of serine, and the regulatory role of serine in folate metabolism.

**Materials and Methods**

**Bacterial strains.** *S. faecium* var. *durans* A₅ is an amethopterin-resistant mutant of *S. faecium* var. *durans* O (15, 16, 17), the strain of *S. faecium* var. *durans* used in our laboratory (5). Bacterial growth was followed turbidimetrically by determining the optical density (OD) of 2-ml portions of the cultures at 600 μm (Beckman spectrophotometer, model B) as described previously (6, 17). With this method, an OD of 0.20 reflected a viable count of approximately 2 x 10⁸ cells per ml of culture.

**Media.** The semidefined medium of Flynn et al. (11) was used without purine and pyrimidine supplementation. The serine-free minimal culture medium, completely defined (Table 1), was also devoid of purines and pyrimidines. The medium was sterilized by autoclaving at 121 C and a pressure of 15 psi for 10 min for volumes smaller than 1 liter and 15 min for 2-liter lots.

**Chemicals.** Dihydrofolate was prepared as described previously (1, 2). dl,riages of folate were obtained from Sigma Chemical Co. (St. Louis, Mo.) and General Biochemicals (Chagrin Falls, Ohio); for K₅₀ value determinations, it was purified by ion-exchange chromatography (1). dl-Tetrahydrofolate was prepared enzymatically with a 40-fold purified preparation of dihydrofolate reductase of *S. faecium* var. *durans* A₅ (2, 21). Chromatographically purified preparations of *S. faecium* var. *durans* were obtained, according to published procedures (18, 28), from reaction mixtures of formaldehyde and the enzymatically formed tetrahydrofolate. Yeh and Greenberg (28) demonstrated this biologically active diastereoisomer to be the dextrotoratory form. The preparation of 5,10-methylenetetrahydrofolate has been described elsewhere (3).

Nicotinamide adenine dinucleotide (NAD⁺), reduced nicotinamide adenine dinucleotide (NADH), NADP⁺, reduced nicotinamide adenine dinucleotide phosphate (NADPH), formaldehyde, diethylaminoethyl (DEAE)-cellulose, and the calcium phosphate gel were obtained from commercial sources (2, 3). The standard buffer was a solution of 0.01 M potassium phosphate and 1 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.35 to 7.40.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amt¹</th>
<th>Component</th>
<th>Amt²</th>
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<tr>
<td>Glucose</td>
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<td>Arginine</td>
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</tr>
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<td>NH₄Cl</td>
<td>2.0*</td>
<td>Cysteine (free base)</td>
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<td>KH₂PO₄</td>
<td>2.5*</td>
<td>Glycine</td>
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<tr>
<td>K₂HPO₄</td>
<td>7.0*</td>
<td>Glutamic acid</td>
<td>500</td>
</tr>
<tr>
<td>Na citrate</td>
<td>1.0*</td>
<td>Histidine-HCl</td>
<td>20</td>
</tr>
<tr>
<td>2H₂O</td>
<td>50.0</td>
<td>Isoleucine</td>
<td>100</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>5.0</td>
<td>Leucine</td>
<td>100</td>
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<td>FeSO₄·7H₂O</td>
<td>6.0</td>
<td>Lysine- HCl</td>
<td>200</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>5.0</td>
<td>Methionine</td>
<td>10</td>
</tr>
<tr>
<td>Ca pantothe-nate</td>
<td>0.2</td>
<td>Phenylala-nine</td>
<td>20</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.4</td>
<td>Threonine</td>
<td>50</td>
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<td>Pyridoxal-HCl</td>
<td>0.1</td>
<td>Tryptophan</td>
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<td>Biotin</td>
<td>0.01</td>
<td>Tyrosine</td>
<td>20</td>
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<tr>
<td>Tween 80</td>
<td>50.0</td>
<td>Valine</td>
<td>100</td>
</tr>
<tr>
<td>Folic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The pH was 6.8 to 7.0.  
* Components added as milligrams per liter.  
* Component additions were made as grams per liter.  
* Supplement, described in text.  
* With the exception of DL-valine, L-amino acids were used.

**Cell extracts for enzyme purification.** The bacteria were grown at 37°C in 6-liter lots of minimal medium supplemented with 25 μg of adenine and 5 μg of folate per ml, to a culture density of 0.6. They were harvested at 27,000 × g with the continuous-flow system of the Sorvall RC2 centrifuge. The cells from each harvest were washed by resuspension in 280 ml of standard buffer, subsequently centrifuged (12,000 × g), resuspended in 20 ml of standard buffer, and stored at −20°C. Extracts were prepared from thawed suspensions to which NADP⁺ (1 mM) was added. Cell disruption (80%) was achieved by subjecting 3-ml portions of the cell suspension to ultrasonic vibration for 3 min. The disintegrator (Measuring and Scientific Equipment, Ltd., London, England) was used with an alcohol-ice cooling mixture (−10°C). The cell extract was the supernatant solution of the combined 3-ml portions after two consecutive centrifugations (27,000 × g, 15 min).

**Experimental conditions for studies of enzyme synthesis.** The general methods for growing, harvesting, and extracting cells for these experiments have been described (1). In presenting the results as "differential plots" (22), the enzyme units were plotted versus total protein formed per milliliter of growing culture.

**Assay of 5,10-methylenetetrahydrofolate dehydrogenase.** To follow the purification of the enzyme, a method based upon that of Whiteley (27) was used.
Reaction mixtures contained 50 μmoles of potassium phosphate (pH 7.5), 30 μmoles of 2-mercaptoethanol, 0.4 μ mole of 5,10-methylenetetrahydrofolate, 1 μmole of formaldehyde, and 0.5 μmole of NADP+. This mixture was preincubated at 37 C for 5 min. Enzyme preparations were then added in 0.1 ml of standard buffer to yield a final volume of 1.0 ml. After incubation for 15 min, perchloric acid (2 ml, 25%) was added. The protein which precipitated on acidification was removed by centrifugation.

The absorbance (350 nm) of the clear supernatant solution arising from the product (5,10-methylenetetrahydrofolate) was determined. Reference mixtures contained all of the reactants except formaldehyde. When purified tetrahydrofolate was employed in the assay, tetrahydrofolate addition was decreased to 0.3 μmole, and tris(hydroxymethyl)aminomethane (Tris) buffer (50 μmole) was used under otherwise standard conditions.

One unit of activity was defined as the amount of activity required to yield an absorbance reading of 1.0 in 1 hr under the conditions employed.

Other enzyme assays. A method for measuring serine hydroxymethyltransferase activity, i.e., in the direction of serine formation from glycine, tetrahydrofolate, and formaldehyde, has been described (3). With cell extracts or fractions containing substantial amounts of 5,10-methylenetetrahydrofolate dehydrogenase, the activity of serine hydroxymethyltransferase was coupled to the dehydrogenase. In the presence of excess amounts of purified dehydrogenase and serine in place of formaldehyde, absorbance at 350 nm was proportional to the activity of serine hydroxymethyltransferase in catalyzing the formation of the substrate for the dehydrogenase, 5,10-methylenetetrahydrofolate, from serine and tetrahydrofolate. Procedures for measuring 5,10-methylenetetrahydrofolate cyclohydrolase and formyltetrahydrofolate synthetase were described previously (3).

Protein determination. The method of Lowry et al. (20) was followed.

RESULTS

Partial purification of 5,10-methylenetetrahydrofolate dehydrogenase. Portions (20 or 25 ml) of cell extract were used for each preparation (at 0 to 4 C) of purified dehydrogenase. The initial purification steps of protamine sulfate and ammonium sulfate precipitation were similar in the following sequential details: slow stirring during addition of precipitant, continued stirring for 15 min after each addition, and centrifugation (12,000 × g) to separate from the supernatant solution the precipitated material as a pellet. Protamine sulfate, as a 3% solution in standard buffer with 1 mM NADP+ (pH 7.0), was added dropwise to the extract (1 mg of protamine sulfate per 6.2 mg of protein). After protamine treatment, the first addition of freshly ground solid ammonium sulfate (0.22 g of salt per ml of the supernatant solution) formed a precipitate which was discarded. The pellet resulting from the addition of another portion of salt (0.25 g per ml of original volume of protamine sulfate-treated extract) was dissolved in 10 ml of standard buffer, 1 mM in NADP+, and was dialyzed for two successive 0.5-hr periods against two 500-ml portions of standard buffer, 0.1 mM in NADP+. After dialysis, NADP+ was replenished to 1 mM, and this fraction (Am S1) was stored at -20 C.

Ammonium sulfate was added to a thawed portion of fraction Am S1. The precipitate resulting from the addition of 0.32 g per ml was discarded. The pellet obtained after the addition of 0.11 g more per ml was dissolved in a volume of standard buffer (1 mM in NADP+) equal to one-half of the volume of fraction Am S1 treated. The resulting solution was dialyzed and replenished in respect to NADP+, as in the case of fraction Am S1, to yield fraction Am S2.

The procedure for further purification with calcium phosphate gel adsorption was similar to that used for the formyltetrahydrofolate synthetase (3); portions of fraction Am S2 were treated with an aqueous suspension of the gel to adsorb the dehydrogenase to the gel. The aqueous suspension of calcium phosphate, the 2-mercaptoethanol, and the eluting solutions each contained 1 mM NADP+. Successive treatment of the resultant gel pellet with 2 ml of 0.1 M mercaptoethanol, 5 ml of 0.05 M, 2 ml of 0.1 M, and 6 ml of 0.25 M potassium phosphate, pH 7.4, 1 mM in EDTA and NADP+, increased the specific activity of the dehydrogenase eluted by the 0.25 M buffer 14-fold (the calcium phosphate gel fraction). This fraction was dialyzed with stirring for two successive 0.5-hr periods against two 300-ml portions of standard buffer, 0.1 mM in NADP+. The dialyzed solution was then replenished with NADP+ (to 1 mM) and was stored at -20 C.

A 6-ml portion of the calcium phosphate gel fraction was applied to a column (1 × 15 cm) of DEAE-cellulose, equilibrated with standard buffer. The last 50 ml of the buffer contained 0.01 mM NADP+. Elution was stepwise with 10 ml of each of a series of potassium phosphate solutions (1 mM in EDTA and 0.01 mM in NADP+, pH 7.4) which increased by 0.03 M from 0.03 M to 0.3 M. Fractions of 3.5 ml were collected. The DEAE-cellulose eluates which contained the dehydrogenase of highest purification (100-fold) were 0.09 to 0.10 M in phosphate. Immediately before the DEAE-fractions were frozen, bovine plasma albumin was added (1 mg per ml) to stabilize the dehydrogenase. Although serine hydroxymethyltransferase activity was essentially absent, 5,10-methylenetetrahydrofolate cyclohydrolase was present in these purified preparations.

A summary of the purification is presented in Table 2.
Calcium phosphate extract

DEAE-fraction

1782

J.

3 ................ 3.5

DEAE-fraction 1

Fraction Am S1

Fraction Am S2

Calcium phosphate gel fraction

DEAE-fraction 1

DEAE-fraction 2

DEAE-fraction 3

---

19.0 625.8 9.7 6,070 100

15.2 376.7 13.3 5,010 83

7.3 81.5 27.5 2,241 37

6.8 8.7 134.6 1,171 19

3.5 0.32 750 240 4

3.5 0.28 923 258 4

3.5 0.19 1078 214 4

a Recovery of total protein and enzyme activity was computed on the basis of volume of the extract (20 ml).

**Stability and protection of enzyme in cell extracts.** Although the 5,10-methylenetetrahydrofolate dehydrogenase retains activity within intact cells frozen at -20 C for several months, a single freezing and thawing inactivated the enzyme in cell extracts (Table 3). Glutathione (1 and 10 mM), cysteine (10 mM), and 2-mercaptoethanol (10 mM) had little, if any, stabilizing effect. NADP+ (1 mM) was most effective in maintaining activity throughout freezing and thawing, and also in stabilizing the enzyme during preparation of the extracts.

**Stability of the purified enzyme.** The activity of the Am S1, Am S2, and calcium phosphate gel fractions was effectively stabilized by 1 mM NADP+. The DEAE-fractions were stored at -20 C with 0.01 mM NADP+ and thus required the additional protection afforded by 0.1% bovine plasma albumin. Preparations doubly protected with albumin and 0.1 mM NADP+ retained activity for 3 days at 4 C and indefinitely when frozen. With 0.01 mM NADP+ and albumin, DEAE-fractions gradually lost 50% of the activity within 3 days at 4 C; the activity of frozen fractions was decreased slightly by repeated freezing and thawing. Glycerol (25%) alone or 0.1 mM NADP+ alone was ineffective in protecting activity. When combined, they were less efficient than bovine plasma albumin and 0.01 mM NADP+.

The effect of temperature on the stability of the dehydrogenase was studied with the Am S2 fraction in standard buffer, 0.1 mM in NADP+. Full activity was retained for 30 min at 30 and 37 C, whereas activity began to decline after 20 min at 45 and 55 C; 33 and 51% loss occurred after 3 and 10 min, respectively.

**Reactants for the 5,10-methylenetetrahydrofolate dehydrogenase reaction.** The maximal activity of 5,10-methylenetetrahydrofolate dehydrogenase in the standard assay depended upon tetrahydrofolate, formaldehyde, and NADP+

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<table>
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<tr>
<th>Enzyme fraction</th>
<th>Vol</th>
<th>Total protein yieldb</th>
<th>Specific activity</th>
<th>Overall yieldb</th>
</tr>
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<tbody>
<tr>
<td>Cell extract</td>
<td>20.0</td>
<td>625.8</td>
<td>9.7</td>
<td>6,070</td>
</tr>
<tr>
<td>Fraction Am S1</td>
<td>15.2</td>
<td>376.7</td>
<td>13.3</td>
<td>5,010</td>
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<tr>
<td>Fraction Am S2</td>
<td>7.3</td>
<td>81.5</td>
<td>27.5</td>
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<tr>
<td>Calcium phosphate gel fraction</td>
<td>6.8</td>
<td>8.7</td>
<td>134.6</td>
<td>1,171</td>
</tr>
<tr>
<td>DEAE-fraction 1</td>
<td>3.5</td>
<td>0.32</td>
<td>750</td>
<td>240</td>
</tr>
<tr>
<td>DEAE-fraction 2</td>
<td>3.5</td>
<td>0.28</td>
<td>923</td>
<td>258</td>
</tr>
<tr>
<td>DEAE-fraction 3</td>
<td>3.5</td>
<td>0.19</td>
<td>1078</td>
<td>214</td>
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</tbody>
</table>

a Recovery of total protein and enzyme activity was computed on the basis of volume of the extract (20 ml).

**Table 2. Summary of purification of 5,10-methylenetetrahydrofolate dehydrogenase**

<table>
<thead>
<tr>
<th>NADP+</th>
<th>Specific activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Before freezingb</td>
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<tr>
<td>0.0</td>
<td>2.3</td>
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<tr>
<td>0.1</td>
<td>4.8</td>
</tr>
<tr>
<td>1.0</td>
<td>5.6</td>
</tr>
</tbody>
</table>

a A culture in the semidefined medium (3 mg of folate per ml) was harvested during the exponential phase. Cells were washed and resuspended in the standard buffer (1). NADP+, as indicated, was added to portions of the cell suspension prior to ultrasonic disruption.

b Frozen storage at -20 C.

c Slow thawing (at 25 C) of extracts which had been stored at -20 C.

**Table 3. Effect of NADP+ on the stability of 5,10-methylenetetrahydrofolate dehydrogenase**

**Table 4. Components of the 5,10-methylenetetrahydrofolate dehydrogenase reaction**

Component omitted from system | Absorbance at 350 nm
---|---
Complete system | 0.304
Formaldehyde | 0.063
Tetrahydrofolate | 0.027
NADP+ | 0.018

a Reaction conditions were standard except that reactants were omitted as indicated. Each mixture contained the DEAE-fraction (1.36 μg of protein) and bovine plasma albumin (50 μg). Appropriate corrections were made for the absorbance of mixtures incubated with enzyme which had been inactivated by heating at 100 C for 10 min.

(Table 4). An unidentified, nondialyzable, and heat-labile substance contributed to the absorbance at 350 nm. It thereby appeared to partially replace the formaldehyde requirement. Activity measurements of all preparations were corrected for absorbance caused by this apparent endog-
The effect of pH. Enzyme activity was essentially the same in Tris buffer as in potassium phosphate in the pH range of 6.5 to 7.5 under otherwise standard conditions. An optimum near pH 7.2 was observed with Tris buffer (Fig. 1), which was used for further studies. With the chromatographically purified 5,10-methylenetetrahydrofolate, the K_m value was calculated by the methods of Lineweaver and Burk (19). With the chromatographically purified diastereoisomer of 5,10-methylenetetrahydrofolate formed as the formaldehyde adduct of biologically synthesized L-tetrahydrofolate, the K_m value of 0.026 mM was 25% of that for dl-L-tetrahydrofolate in experiments with excess formaldehyde. In reactions with either formaldehyde and dl-L-tetrahydrofolate or preformed d,L-5,10-methylenetetrahydrofolate, the K_m for NADP+ was 0.021 mM.

Reaction products. The products of the enzymatic oxidation of 5,10-methylenetetrahydrofolate by the dehydrogenases from other sources have been shown to be NADPH and 5,10-methylenetetrahydrofolate (10, 26, 28). The spectrophotometric experiment summarized in Fig. 2 was designed to determine the products of the reaction carried out by the S. faecium var. durans Δκ enzyme. The spectrum of the substrate, 5,10-methylenetetrahydrofolate (A, curve 1), was recorded prior to the addition of enzyme to the reaction mixture. Upon enzyme addition, the reaction, as followed by the absorbance increase at 340 nm, reached a plateau within 10 min (B). At this point, the composite of absorption spectra (curve 2) suggested the presence of NADPH and 10-formyltetrahydrofolate. The decrease in the absorbance at 340 nm which occurred upon the addition of the S. faecium var. durans Δκ di-

![Fig. 1. Effect of pH on the oxidation of methylenetetrahydrofolate catalyzed by fraction Am S2 (38.6 μg of protein). The standard assay conditions were used except that each reaction mixture of experiment A (●) contained 0.3 μmole of chromatographically purified dl-L-tetrahydrofolate, 1 μmole of formaldehyde, and 80 μmoles of Tris buffer; each reaction mixture of experiment B (△) contained 0.15 μmole of chromatographically purified d,L-methylenetetrahydrofolate, 0.45 μmole of formaldehyde, and 50 μmoles of Tris buffer. The pH was varied as indicated and was determined with facsimile reaction mixtures which were not treated with perchloric acid.](http://jb.asm.org/)

### Table 5. K_m values for reactants in 5,10-methylenetetrahydrofolate oxidation

<table>
<thead>
<tr>
<th>Reactant</th>
<th>K_m value (mM)</th>
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<tbody>
<tr>
<td>Formaldehyde</td>
<td>0.18</td>
</tr>
<tr>
<td>dl-L-Tetrahydrofolate</td>
<td>0.108</td>
</tr>
<tr>
<td>d,L-5,10-Methylenetetrahydrofolate</td>
<td>0.026</td>
</tr>
<tr>
<td>NADP+</td>
<td>0.021</td>
</tr>
</tbody>
</table>

*The experimental procedure was standard except for a 5-min incubation period. The composition of the reaction mixtures (1 ml, 0.03 mM in 2-mercaptoethanol and 0.05 to 0.06 mM in Tris, pH 7.3) was varied as was required to study the effect of concentration of individual reactants. With varied amounts of NADP+ (between 0.001 and 0.5 μmole), 0.08 μmole of d,L-methylenetetrahydrofolate and 1.1 enzyme units (DEAE-fraction) were used. For determination of other K_m values, the concentration of NADP+ was constant at 0.5 mM. With varied amounts of formaldehyde (between 0.03 and 0.3 μmole), 0.3 μmole of dl-L-tetrahydrofolate and 3.1 enzyme units (calcium phosphate gel fraction) were constant additions. The tetrahydrofolate value (in the range of 0.03 to 0.3 μmole) was determined with 1 μmole of formaldehyde and 2.7 enzyme units (DEAE-fraction); the methylenetetrahydrofolate value was determined with 0.35 μmole of formaldehyde and 1.7 enzyme units (DEAE-fraction). Standard correction of absorbance measurements at 350 nm was made in experiments dealing with the K_m values for formaldehyde and tetrahydrofolate. A buffered mixture without enzyme and a water blank were the reference cuvettes in the determination of the K_m values for methylenetetrahydrofolate and NADP+, respectively.*
hydrofolate reductase and dihydrofolate to one portion of the oxidation mixture (B) confirmed the presence of NADPH. That 10-formyl-tetrahydrofolate contributed to the absorbance in the regions of 300 to 260 μm (A, curve 2) was supported by the spectrum of 5,10-methenyl-tetrahydrofolate, the cyclized form of 10-formyl-tetrahydrofolate, obtained with an acidified portion of the reaction mixture (curve 3). Since the slightly alkaline reaction mixture (Tris buffer) favored the uncyclized form, 10-formyltetrahydrofolate (24), and since the dehydrogenase preparation was contaminated with the 5,10-methenyltetrahydrofolate cyclohydrolase, identification of the immediate oxidation product by direct observation was hindered.

In the experiment described above, 0.12 μmole of 5,10-methylenetetrahydrofolate, for which the molar extinction was assumed to be 32,000 at 294 μm (4), was present initially. As determined by the absorbance change at 340 μm, 0.093 μmole of NADPH was formed during the oxidation. After acidification, 0.084 μmole of 5,10-methenyltetrahydrofolate was present according to calculations based on its molar extinction of 22,000 at 355 μm (25). These calculations and those of several other similar experiments suggest that 70 to 75% of the methylene compound was oxidized at pH 7.4.

**Reverse reaction.** The rapid alkaline hydrolysis of 5,10-methylenetetrahydrofolate to 10-formyltetrahydrofolate in Tris buffer, pH 7.3, prevented study of the reaction between NADPH and 5,10-methylenetetrahydrofolate. Judged by the unchanged absorption spectrum of NADPH after incubation with 10-formyltetrahydrofolate and the dehydrogenase, NADPH could not react with the 10-formyl derivative.

**Effect of metals.** In the standard reaction with Tris buffer, K⁺, Na⁺, NH₄⁺, and Li⁺, tested as chloride salts, each at a concentration of 0.1 M, had no effect upon the reaction. With the divalent cation Mg²⁺, slight inhibition (5%) was observed. Ca²⁺ could not be tested because of precipitation in the reaction mixture.

**Effect of thiol compounds.** When reactions contained 0.5 μmole of mercaptoethanol, the additional amounts of 2.5 and 7.5 μmoles of mercaptoethanol increased the extent of the reaction 8 and 19%, respectively. 2,3-Dimercaptopropanol was more effective; 0.5 μmole gave a 17% increase. Reduced glutathione and cysteine at concentrations of 2.5 and 7.5 mM did not affect the reaction.

**Effect of sulfhydryl reagents**. Several sulfhydryl reagents were tested in the reaction with fraction Am S2. After a 10-min preincubation of the enzyme with the reagent in a buffered mixture...
(pH 7.4) containing 2,3-dimercaptopropanol (1 mM) and tetrahydrofolate (0.2 mM), NADP was added to initiate the reaction under otherwise standard conditions. Although 1 mM iodosobenzoate and 3 mM p-chloromercuribenzoate completely inhibited the reaction, 1 mM p-chloromercuribenzoate had no effect. N-ethylmaleimide at concentrations of 1 and 10 mM inhibited the reaction by 8 and 43%, respectively.

Effect of purine and pyrimidine bases and their derivatives. The observations of Dalal and Gots (7) that purine nucleotides inhibit the 5,10-methylene-tetrahydrofolate dehydrogenase of S. typhimurium strain LT-2 prompted the investigation of the effect of these compounds on the S. faecium var. durans A enzyme. The following compounds were tested at a concentration of 1 mM in the standard enzyme assay procedure with fraction Am S2: adenine, adenosine, adenyl acid, adenosine 5'-triphosphate, guanine, guanosine, guanosine 5'-triphosphate, xanthine, xanthosine, hypoxanthine, uracil, uridine, uridylic acid, deoxyxuridylic acid, and thymine. Only guanine interfered significantly with the reaction; a 48% inhibition was observed.

Culture conditions for optimal synthesis of 5,10-methylene-tetrahydrofolate dehydrogenase. Because the differential rate of synthesis of 5,10-methylene-tetrahydrofolate dehydrogenase depended upon the composition of the medium, the defined serine-free medium (Table 1) supplemented with 5 mg of folate and 25 g of adenine per ml was selected to grow cells for enzyme purification.

In a semidefined medium (11) containing acid-hydrolyzed casein as a source of serine, a supplement of 1 mg of folate per ml was adequate to mediate the biosynthesis of purines and thymine and thus yield the characteristic growth curve (Fig. 3, curve A). During growth in this medium, the dehydrogenase activity of S. faecium var. durans A was relatively constant (Table 6).

In the defined minimal medium which was also devoid of serine, growth comparable to curve A required a larger supplement of folate (10 mg/ml). This finding agrees with the data of Johnson and Hutchison (17). Under such conditions, the specific activity of the dehydrogenase of exponentially growing cells was equal to the constant differential rate of synthesis (considered the reference value) during growth in the semidefined medium (Table 6). Lower folate supplements altered the growth (Fig. 3, curves B and C); this primarily reflected a decreased rate of serine synthesis de novo. With 2 mg of folate per ml (curve B), the initial exponential phase was extremely short and the subsequent growth rate was markedly decreased; dehydrogenase activity during both growth phases was elevated above the reference value (Table 6). The greater supplement (5 mg) promoted a longer initial exponential phase (curve C). The onset of a slightly growth-limiting rate of serine biosynthesis was indicated

![Figure 3. Growth curves of Streptococcus faecium var. durans A in purine- and pyrimidine-free media: semidefined (with casein hydrolysate), supplemented with 1 mg of folate/ml (A); completely defined, serine-free with 2 and 5 mg of folate/ml (B and C, respectively); and completely defined with 100 mg of L-serine and 5 mg of folate/ml (D).](http://jb.asm.org/)
by the subtle deflection in the growth curve at an OD of 0.22. The differential plot of data (Fig. 4) for enzyme synthesis in this culture shows that dehydrogenase activity of cells harvested prior to the deflection resembled the reference activity. After the deflection, synthesis of the dehydrogenase proceeded at an increased differential rate; i.e., synthesis was derepressed. Addition of serine to the minimal medium promoted typical growth. In fact, the rate was stimulated (Fig. 3, curve D), but serine prevented the derepression (Fig. 4).

Thus, optimal yields of the dehydrogenase were obtained with cultures harvested from the serine-free medium (5 μg of folate per ml) soon after the maximal effects of derepression were expressed.

Since formyltetrahydrofolate synthetase and 5,10-methylenetetrahydrofolate dehydrogenase share several purification characteristics (3), it was advantageous to decrease the formyltetrahydrofolate synthetase of cells used for purification of the dehydrogenase by repressing the formation of the synthetase with exogenous adenine (1). Adenine supplementation reduced the synthetase activity to 25% of the level of cells cultivated in the absence of adenine without affecting the dehydrogenase (Table 7).  

Repressibility by serine. In view of the higher specific activity of cell extracts prepared with NADP+ (Table 3 and 6), the rates of formation of the enzyme in cultures grown in the presence and absence of serine were reassessed by measuring the activity of cell extracts prepared with 1 mM NADP+. The data refuted the possibility that cultures produced a less stable enzyme while utilizing exogenous serine. NADP protection increased the values for both derepressed and repressed synthesis. Under the repressive conditions, the differential rate of synthesis of the dehydrogenase was decreased 67%.

The effect of serine on the oxidation of methylenetetrahydrofolate was also tested. A serine hydroxymethyltransferase-free preparation of the dehydrogenase catalyzed the oxidation to the same extent in the absence and in the presence of 1 and 3 mM serine.

Regulation by physiological concentrations of serine. Additions of 5 and 15 μg of L-serine per ml of minimal medium also evoked the regulatory response. These amounts of L-serine extended the initial exponential growth phase of curve C (Fig. 3) approximately 30 and 45 min, respectively, and were repressive for the dehydrogenase (Fig. 5). The differential plots for enzyme synthesis during growth between culture densities of 0.2 and 0.6 (Fig. 5) show that serine also repressed the 5,10-methylenetetrahydrofolate cyclohydrolase and the serine hydroxymethyltransferase. Concomitantly, serine increased the differential rate for the formyltetrahydrofolate synthetase.

**DISCUSSION**

The enzyme 5,10-methylenetetrahydrofolate dehydrogenase is probably constitutive in all cells in which tetrahydrofolate cofactors mediate the biosynthesis of serine or of purines and thymine or of both. Knowledge of this enzyme among

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**TABLE 7. Differential effect of adenine on the formation of folate enzymes during growth of Streptococcus faecium var. durans A**

<table>
<thead>
<tr>
<th>OD of cultures</th>
<th>5,10-Methylenetetrahydrofolate dehydrogenase</th>
<th>Formyltetrahydrofolate synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td>With adenine</td>
<td>Without adenine</td>
<td>With adenine</td>
</tr>
<tr>
<td>0.2</td>
<td>9.3</td>
<td>7.4</td>
</tr>
<tr>
<td>0.4</td>
<td>10.2</td>
<td>10.4</td>
</tr>
<tr>
<td>0.6</td>
<td>11.3</td>
<td>11.7</td>
</tr>
</tbody>
</table>

* The minimal medium which was devoid of serine, thymine, and purine contained 5 μg of folate per ml; the adenine supplement was 25 μg per ml. Enzyme activity values are expressed as units per milligram of protein of extract, 1 mm in NADP+. 

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**FIG. 4.** Formation of 5,10-methylenetetrahydrofolate dehydrogenase in the absence (▲) and presence (■) of 100 μg of L-serine per ml of defined medium (5 μg of folate/ml). The reference value (●) expresses the differential rate of enzyme synthesis in the semidefined medium. Extracts were prepared in standard buffer from cells harvested at different points of growth in each medium. The amounts of enzyme activity and protein of each extract were then determined.
Several properties common to the *E. coli* and *S. typhimurium* enzymes differ from those of the yeast enzyme (26). Limited knowledge of the dehydrogenases, however, prevents any categorical or descriptive distinction of microbial properties as bacterial or yeastlike. The *S. faecium* var. *durans* A*<sub>k</sub> enzyme resembles the yeast enzyme in certain respects: it is not inhibited by phosphate, it has optimal activity near pH 7.2, and it is effectively stimulated by dimercaptoopropanol and to a smaller extent by mercaptoethanol; it is inhibited by iodosobenzoate, *p*-chloromercuribenzoate, and *N*-ethylmaleimide. But, the *S. faecium* var. *durans* A<sub>0</sub> enzyme resembles the *E. coli* and *Salmonella* enzyme that cations are ineffective in the oxidation of methylenetetrahydrofolate.

A pronounced difference between the *S. faecium* var. *durans* A<sub>0</sub> and *S. typhimurium* enzymes involves the inhibition by purine nucleotides of the reaction catalyzed by the *S. typhimurium* enzyme; 0.35 to 0.4 mM concentrations inhibit 50% of the reaction (7, 8). Dalal and Gots view the inhibition as a control mechanism which prevents the unnecessary accumulation of the 5,10-methenyl and 10-formyl derivatives of tetrahydrofolate, the coenzymes of purine biosynthesis. Neither 1 mM adenosine triphosphate nor 1 mM guanosine triphosphate has any effect on the reaction with the *S. faecium* var. *durans* A<sub>0</sub> dehydrogenase.

With *S. faecium* var. *durans* A<sub>0</sub>, the inhibition of dehydrogenase activity is probably unnecessary since regulation occurs at the biosynthetic level. Serine or a derivative of serine acts as a repressor in controlling the formation of methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate cyclohydrolase (Fig. 5). The kinetics of the observed repression suggest that, when serine is synthesized de novo in excess of its need for cell substance or is provided exogenously, it contributes the β-carbon to the active C-1 pool as 5,10-methylenetetrahydrofolate (Fig. 6). Thus, serine eases the drain on 10-formyltetrahydrofolate and diminishes the need for the “converting enzymes.” The minimal repression of serine hydroxymethyltransferase by serine implies continued need for this enzyme, especially for the frugal use of preformed serine as a monocarbon donor.

The physiological function of formyltetrahydrofolate synthetase to activate formate explains the obligatory formation of this enzyme for growth in minimal medium. Purines synthesized de novo may repress partially (1). Exogenous serine does not repress, but does indirectly increase the synthesis of this enzyme by causing a
more rapid exhaustion of purines. An alternative explanation for the inductive effect of serine on the formyltetrahydrofolate synthetase is that a probable decrease of 10-formyltetrahydrofolate results from the repressed level of cyclohydrolyase. The need for formyltetrahydrofolate for purine nucleotide biosynthesis, and perhaps for formation of methionyl-soluble ribonucleic acid (19), would accelerate the synthesis of the formate-activating enzyme.

It is noteworthy that the synthesis of the dehydrogenase and the cyclohydrolyase is not completely "turned-off" either by the endogenous serine formed during growth in medium heavily supplemented with folate (50 μg/ml) or by excessive amounts of serine (50 and 100 μg/ml). The repressibility limit, the sensitivity of the repression mechanism to small supplements (5 μg of serine/ml), and the derepression following the exhaustion of the endogenous repressor reflect the operational significance of this phenomenon in the metabolically controlled equilibrium of monocarbon coenzyme derivatives of tetrahydrofolate.

Because serine is a folate-dependent metabolite and an active C-1 donor (Fig. 6), it has a unique regulatory position within the economy of folate metabolism. During growth, the co-repressive action of exogenous serine is seen as the sparing of folate (17), because folate coenzymes are reserved for thymine and purine biosynthesis. Presumably, serine synthesized de novo is similarly repressive. Thus, a minimal excess of endogenous serine triggers the repression mechanism to maintain the physiological balance.

Acknowledgments

We thank William J. Suling for capable technical assistance.

This investigation was supported by grant CA-08748 from the National Cancer Institute and by grant T-107 from the American Cancer Society.

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