Direct Spectrophotometric Evidence for the Oxidation of Tetrahydrofolate during the Enzymatic Synthesis of Thymidylate*

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Indirect evidence suggests that tetrahydrofolate participates in the enzymatic synthesis of thymidylate not only as a coenzyme for the transfer of a 1-carbon moiety but also as the hydrogen donor for the reduction of the 1-carbon moiety to a methyl group (1-7). Accordingly the over-all reaction may be written:

\[
dUMP + 5, 10\text{-methylene tetrahydrofolate} \xrightarrow{\text{Mg}^{++} \text{ enzyme}} dTMP + \text{dihydrofolate}
\]

A direct spectrophotometric method has been devised to follow the oxidation of tetrahydrofolate during the synthesis of thymidylate. It is based on the marked spectral change which occurs when 5,10-methylene tetrahydrofolate is converted to dihydrofolate. A nonenzymatic reaction between formaldehyde and tetrahydrofolate yields 5,10-methylene tetrahydrofolate (also called hydroxymethyltetrahydrofolate) (8, 9). The instability of tetrahydrofolate is well known; however, in the presence of an excess of formaldehyde and a high concentration of 2-mercaptoethanol (0.1 M) there is essentially no degradation of 5,10-methylene tetrahydrofolate for a period of 1 hour at room temperature. The spectra of tetrahydrofolate and dihydrofolate in the presence of formaldehyde and 2-mercaptoethanol are shown in Fig. 1. Of special note is the difference spectrum, Fig. 1 (Curve C), characterized by an increase in absorbancy at 338 nm (\(\Delta A = 6600\)), and a decrease in absorbancy at 303 nm (\(\Delta A = 9000\)). An identical difference spectrum was obtained with a reaction mixture consisting of deoxyuridylate, tetrahydrofolate, formaldehyde, Mg++, 2-mercaptoethanol, and purified *Escherichia coli* thymidylate synthetase (Fig. 2).

The spectral change which occurred during the enzymatic oxidation of 5,10-methylene tetrahydrofolate could be correlated with the synthesis of \(P^{32}\)-labeled thymidylate (Table I). For each mole of thymidylate formed, 1 mole of dihydrofolate appeared as measured by the increase in absorbancy at 340 nm.

The product of the enzymatic oxidation of tetrahydrofolate was adsorbed on DEAE-cellulose and recovered in excellent yield by gradient elution with Tris (13). Deoxyuridylate (0.94 \(\mu\) mole), \(dL\)-tetrahydrofolate (2.2 \(\mu\) moles) and *E. coli* thymidylate synthetase (2.2 mg) in 11 ml of Mixture I (Fig. 1) were incubated for 60 minutes at room temperature. The increase in absorbancy at 340 nm was 0.540, equivalent to the formation of 0.93 \(\mu\) mole of dihydrofolate. The reaction mixture was then poured through a DEAE-cellulose column (1.8 \(\times\) 10 cm) and elution started. The mixing chamber contained 200 ml of 0.005 M Tris, pH 7.0, and the reservoir, 400 ml of 1 M Tris, pH 7.0; both solutions were in 0.2 M mercaptoethanol. Tetrahydrofolate and protein appeared first in tubes 32 to 42 (3 ml fractions), followed by dihydrofolate in tubes 62 to 78. The same elution pattern was obtained with a known mixture of tetrahydrofolate and dihydrofolate. The isolated product was further identified...
The incubation mixture consisted of 0.040 μmole of dUMP (labeled with P32, 80,000 c.p.m.); 0.16 μmole of dl, l-tetrahydrofolate; and enzyme (0.2 mg of protein) in 1 ml of Mixture I (Fig. 1). The increase in absorbancy was measured at 340 μm (25°). The reference cuvette contained all components except dUMP. At intervals, 20 μl were transferred to a spot on Whatman No. 3 MM paper which contained 0.1 μmole each of dUMP and dTMP. After 16 hours of ascending chromatography with a solvent of 600 g of (NH4)2SO4, 1 liter of 0.1 M potassium phosphate buffer, pH 6.8; and 20 ml of n-propanol (10), dUMP (RF, 0.55) and dTMP (RF, 0.43) zones were cut out and direct counts made of their radioactivity with an end window detector.

The oxidation mixture consisting of 0.046 pmole of dUMP, 0.16 pmole of dl, l-tetrahydrofolate, and enzyme (0.2 mg of protein) in 1 ml of Mixture I (Fig. 1) was incubated at 25° for 40 minutes. The reference cuvette contained all components except dUMP.

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>Increase of absorbancy at 340 μm</th>
<th>Dihydrofolate formed*</th>
<th>Thymidylate formed†</th>
<th>Dihydrofolate Thymidylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td>μmole</td>
<td>μmole</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.100</td>
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<td>30</td>
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<tr>
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<td>36.7</td>
<td>40.0</td>
<td>0.92</td>
</tr>
</tbody>
</table>

* Calculated on the basis that Δ A at 340 μm is +4.400 when 1 μmole of 5,10-methylenetetrahydrofolate per ml is converted to dihydrofolate (Fig. 1). Different molar extinction coefficients have been reported for the folates. Blakley's values (9, 12) were selected because the calculated increase of extinction at 340 μm during thymidylate synthesis agreed with that obtained when a known quantity of deoxyuridylate was made limiting.

† Calculated from the specific activity of P32-labeled dUMP.

Fluorodeoxyuridylate, 5 X 10^-6 m. — Complete system plus 5-Fluorodeoxyuridylate, 5 X 10^-3 m. — Complete system with various nucleic acid derivatives* substituted one at a time for dUMP.

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