THYMIDINE TRIPHOSPHATE SYNTHESIS IN
TETRAHYMENA

I. Studies on Thymidine Kinase

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ABSTRACT

The amount of thymidine-H3 converted to thymidine-H8 monophosphate in 30 min formed the basis for assays of thymidine kinase in cell extracts from Tetrahymena pyriformis. The optimal concentration of adenosine triphosphate is lower than that required by other cell types. Thymidine triphosphate does not exercise any feedback control of the enzyme. Other deoxypyrimidine nucleotides were tested, but these also failed to exhibit any feedback inhibition. At suboptimal adenosine triphosphate levels, thymidine triphosphate and other deoxypyrimidine nucleotides stimulate the reaction, suggesting that these nucleotides may act either directly or indirectly as phosphate donors in the crude enzyme preparations. This possibility was affirmed when thymidine triphosphate and deoxycytidine triphosphate were shown to be capable of limited phosphorylation of thymidine. Comparison of enzymatic activities in logarithmically growing culture and stationary phase culture, in which nuclear DNA synthesis has virtually ceased, reveals no change in enzymatic activity. The results suggest that thymidine kinase is a constitutive enzyme in Tetrahymena.

Thymidine triphosphate (TTP)1 has been reported (2, 5, 7, 11) to exercise feedback inhibition of the conversion of thymidine to thymidine monophosphate by thymidine kinase.

The control of TTP production in Tetrahymena is under investigation in this laboratory. This cell can make TTP from thymidine or from uracil. In the latter case the pathway is assumed to be through dUMP but this still remains to be demonstrated. This first report deals with the assay of thymidine kinase, attempts to demon-

1 Abbreviations used are: TTP, thymidine triphosphate; TDP, thymidine diphosphate; TMP, thymidine monophosphate; dUMP, deoxuryridine monophosphate; dCTP, deoxycytidine triphosphate; dCMP, deoxycytidine monophosphate.

strate feedback inhibition of this enzyme by TTP, TDP, dCTP, or dCMP, and measurements of enzyme activity in the presence and absence of DNA synthesis.

MATERIALS AND METHODS

Culture of Cells

Tetrahymena pyriformis, strain HSM, was cultured in 500 to 750 ml of synthetic medium (3) enriched with 0.04% (w/v) proteose peptone in Fernbach flasks at 24-25°C. Under these conditions and beginning with approximately 1 X 106 cells, growth is maintained for 7 or 8 days, and stationary phase is not reached until the cell count, as determined in a Coulter counter, is in excess of 200,000 cells per milliliter.
FIGURE 1 The effect of increasing ATP concentrations on thymidine kinase activity. The ratio of Mg++ concentration to ATP concentration was maintained at 1.6:1. Other conditions are given under Materials and Methods.

Preparation of Enzyme

The cells were harvested by centrifugation, resuspended, and washed twice in 0.154 M KCl and finally lysed at 0°C in fifteen times the packed cell volume of 0.2% Triton X-100 in 0.01 M Tris-Cl and 0.154 M KCl, pH 8.4.

Complete lysis of the cells requires 2 to 5 min. The lysate was then centrifuged at 35,000 g for 1 hr at 1°C, with the Servall RC-2 automatic refrigerated centrifuge.

Enzyme Assay

The usual reaction mixture to give optimal thymidine kinase activity contained the following substances at the concentrations indicated: ATP, 3.0 mM; MgCl2, 4.8 mM; Tris-Cl, 12.0 mM, pH 8.4; thymidine-5P, 0.2 mM (1 × 10⁶ cpm per μmole); and 0.080 ml of supernatant diluted so that the reaction mixture contained 160 to 170 μg of protein in a final volume of 0.2 ml. Negligible substrate inhibition is observed over the range 0.2 mM to 2.0 mM thymidine. The reaction was not stimulated by 3-phosphoglycerate or by phosphocreatine plus phosphocreatine kinase.

Under the conditions of the assay the amount of thymidine converted to thymidine monophosphate increased linearly with time for at least 30 min and was also linear with respect to protein concentration over the range 25 to 200 μg per assay. The amount of thymidine-5P converted to TMP-5P in 30 min at 30°C per 100 μg of protein in the extract was used as the basis for comparison of enzyme activity under various conditions. In all cases the conversion of TMP to TDP and TTP was less than 1%.

The assay was carried out at 30°C in a shaking water bath (the optimum temperature for Tetrahymena growth is 29-30°C). All assay mixtures were preincubated for 5 min before the addition of protein. The reaction was stopped 30 min after the enzyme addition by adding 0.030 ml of 50% (w/v) trichloroacetic acid, and the tubes were then stored at -12°C until chromatographed. Control assays were treated with trichloroacetic acid prior to adding the protein.

Chromatography and Counting

The frozen samples were thawed and centrifuged. Twenty microliters of the clear supernatant were applied to Whatman DE-81 ion exchange paper. Thymidine and TMP were added to each spot as markers. Descending chromatography, using 4 N formic acid in 0.1 M ammonium formate as the solvent, was carried out for approximately 3½ hr. The TMP spot was located under UV light, cut out, and eluted onto planchets with 1M ammonium bicarbonate. The eluent was air-dried on the planchet, spread evenly with formic acid to reduce self absorption, and counted in a windowless gas flow counter.

RESULTS

ATP Concentration and Feedback Inhibition

Fig. 1 shows the effect of varying the ATP concentration on thymidine kinase activity. The
optimal ATP concentration is much lower than that found for the partially purified enzyme from *Escherichia coli* (11), for mouse fibroblasts (7), rat liver (2), and Novikoff hepatoma (5). The low level of ATP required for the crude enzyme preparation from *Tetrahymena* is perhaps more remarkable since no ATP regenerating system is present in the reaction.

The effect of TTP, TDP, dCTP and dCMP on the conversion of thymidine to TMP were investigated as possible feedback inhibitors.

Fig. 2 a and b shows the effect of adding these components directly to the assay mixture. In all cases no inhibition was observed, and the reaction was apparently stimulated.

Since the feedback inhibition may be modulated by the ATP concentration, experiments were carried out using suboptimal ATP concentrations. The results of these experiments are shown in Fig. 3 a and b. An even greater stimulation in the production of TMP is observed, suggesting that these nucleotides may also act as phosphate donors. This possibility was confirmed by the

**Figure 3.** The effect of increasing concentrations of TDP, TTP, dCMP, and dCTP at suboptimal concentrations of ATP on thymidine kinase activity. ATP = 0.3 mM; Mg$^{++}$ = 0.48 mM. Other conditions are given under Materials and Methods.
The utilization of TTP and dCTP as phosphate donors for thymidine kinase activity in the absence of ATP. Conditions are given under Materials and Methods.

formation of small amounts of TMP in the absence of ATP, using TTP and dCTP as phosphate donors (Fig. 4). This limited phosphorylation of thymidine by TTP and dCTP could explain the greater stimulation of TMP formation observed at low ATP concentration (Fig. 3 a and b).

The possibility that high thymidine concentration may reverse the feedback inhibition was considered. Attempts to demonstrate feedback inhibition with increasing concentrations of TTP at lowered thymidine concentration proved negative (Fig. 5 a). Lowering both ATP and thymidine concentrations to suboptimal levels and adding TTP again shows slight stimulation (Fig. 5 b).

All the above experiments were performed with an enzyme preparation obtained from logarithmically growing cells.

Stationary vs. Log Cultures

A comparison was made of the activities of thymidine kinase in logarithmically growing cultures and stationary phase cultures. To measure the level of DNA synthesis in stationary cultures, aliquots of the cultures were removed, prior to the enzyme assay, and inoculated with thymidine-H3. Smears were made of these cells after 5 hr of inoculation, and the percentage of labeled nuclei was determined by radioautography. In stationary cultures, 0 to 2% of the cells showed DNA synthesis. Under similar conditions, approximately 99% of the logarithmically growing cells has heavily labeled DNA. The results of the enzyme assays are given in Table I.

The data show that a 97 to 99% reduction in the percentage of cells making DNA is not accompanied by any reduction in thymidine kinase activity per unit protein. This indicates that there is probably abundant thymidine kinase already present in logarithmically growing cells.
TABLE I
Comparison of Log Phase and Stationary Phase Cultures

<table>
<thead>
<tr>
<th>Experiment</th>
<th>mmoles TMP formed per 100 μg protein</th>
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<tr>
<td></td>
<td>Log cultures (μ) Stationary cultures (μ)</td>
</tr>
<tr>
<td>1</td>
<td>4.0 4.7</td>
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<tr>
<td>2</td>
<td>7.7 6.0</td>
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<tr>
<td>3</td>
<td>5.7 6.1</td>
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<tr>
<td>4</td>
<td>5.6 6.5</td>
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<tr>
<td>5</td>
<td>11.2 13.2</td>
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<tr>
<td>6</td>
<td>13.0 12.6</td>
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* Experiments 1 to 4: ATP = 0.3 mM; Mg++ = 0.480 mM. Experiments 5 to 6: ATP = 3.0 mM; Mg++ = 4.80 mM. Other conditions are given under Material and Methods.
† Cultures contained between 71,000 and 120,000 cells per milliliter.
§ Cultures contained between 213,000 and 250,000 cells per milliliter that had been in stationary phase from 2 to 8 days.

It is tentatively concluded that thymidine kinase is a constitutive enzyme in *Tetrahymena*, although this appears to be contrary to the findings in other cell types (1, 4, 8, 9, 13). However, the constitutive nature of the enzyme in *Tetrahymena* is supported by two findings. First, in stationary phase cultures where nuclear DNA synthesis has dropped to a minimum, the amount of activity per unit protein is the same in extracts of stationary phase cells as in logarithmic phase cells. Perhaps a stronger indication of the constitutive nature of the enzyme comes from the finding that the level of enzyme activity in extracts from cells grown in medium containing thymidine is the same as the level of activity in extracts of cells grown without exogenous thymidine. The latter argument is not conclusive, in view of the evidence that thymidine kinase is not completely specific (10). Apparently, the enzyme also catalyzes the conversion of deoxyuridine to dUMP.

Finally, the demonstration of thymidine kinase in cells which have ceased to make DNA appears to be at odds with the previous, in vivo, radioautographic work that failed to detect conversion of thymidine to TMP in *Tetrahymena* that were not engaged in DNA synthesis (12). The radioautographic evidence is somewhat indirect, however, and the apparent conflict should not be regarded too seriously at this point. Perhaps the failure to detect thymidine kinase action in vivo may mean that an inhibition has been established during non-DNA synthetic periods. Disruption of such a postulated inhibition could occur in cell extracts.

As a continuation of this study of the control of TTP formation, we are now trying to define the pathway(s) by which TTP is derived from uracil. Secondly, we are attempting to identify the two unknown thymidine derivatives in *Tetrahymena* and their relations to the thymidine—TMP pathway.

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REFERENCES

3. Elliott, A. M., Hogg, J. R., Slater, J. B., and...


