Feedback Inhibition of Thymidine Kinase by Thymidine Triphosphate*

DAVID H. IVES,† P. A. MORSE, JR., AND VAN R. POTTER

From the McArdle Memorial Laboratory, The Medical School, University of Wisconsin, Madison 6, Wisconsin

(Received for publication, October 15, 1962)

That the regulation of cell division and deoxyribonucleic acid synthesis is subject to some kind of chemical control in higher organisms is widely accepted (2–5). Davidson (3) has suggested that deoxyribonucleic acid synthesis takes place at critical concentrations of the deoxyribonucleoside triphosphates and that these compounds are components of a homeostatic mechanism.

There are now at least five negative feedback controls that have been demonstrated in the formation of the pyrimidine deoxyriboside triphosphates (Fig. 1). The first was reported by Reichard, Canellakis, and Canellakis (6), who reported that the conversion of cytidine diphosphate to deoxycytidine diphosphate through unknown intermediates (7) is strongly inhibited by deoxyguanosine triphosphate and deoxyadenosine triphosphate and to a lesser extent by thymidine triphosphate but not by deoxycytidine triphosphate (labeled nfb1 in Fig. 1). This feedback suggests an explanation for the inhibition of tissue cultures by high levels of thymidine and also explains how deoxycytidine could relieve the inhibition (8). The Reichard mechanism would not prevent dTTP from accumulating if large amounts of thymidine were available, and it was thus of interest to find a second feedback mechanism (nfb2 in Fig. 1) in which dTTP prevents its own synthesis by inhibiting thymidine kinase (1).

This inhibition has been confirmed by Maley and Maley (9), who in addition reported inhibition of deoxyuridine kinase by dTTP, deoxycytidine kinase inhibition by dCTP, and deoxyuridine deaminase inhibition by dTMP (nfb3, nfb4, and nfb5 in Fig. 1). The available evidence indicates that deoxycytidine kinase is inhibited only by its distal product dCTP and not by other deoxyriboside triphosphates (9), and similarly, that thymidine kinase is inhibited only by its distal product dTTP and not by other deoxyriboside triphosphates, as reported below. The inhibition of thymidine kinase by dTTP, called nfb2 in Fig. 1, is the subject of this paper. Evidence is presented that the degree of inhibition is strongly modulated by the concentrations of the substrates, thymidine and adenosine triphosphate.

Fig. 1 indicates the well known alternative precursors of dTTP: CDP (7), deoxycytidine, deoxyuridine, and thymidine. In addition, formation of deoxyuridine phosphate from uridine diphosphate (7) and formation of dTMP from 5-methyl dCMP (10–12) must be considered as possible alternative pathways leading to dTTP.

EXPERIMENTAL PROCEDURE

Preparation of Supernatant Extracts—Novikoff hepatoma cells (strain N1-S1) from suspension tissue cultures in logarithmic growth were harvested in batches of 10 to 12 ml of packed cells, resuspended in 4 volumes of 0.154 M KCl, and homogenized 5 ml at a time in small Potter-Elvehjem homogenizers with tightly fitting, solid glass pestles 7 mm in diameter. The upper portion of the homogenizer tube was flared into a reservoir to accommodate the suspension. Homogenates were centrifuged in a Spinco model L preparative ultracentrifuge at 104,000 × g for 60 minutes.

Aliquots of the clear supernatants (S3) were stored at −20° in a series of small screw-capped vials, so that comparable experiments could be performed on enzyme preparations frozen and thawed only once. Both thymidine and thymidylate kinase activities remained stable for several months in the frozen state.

Partial purification of thymidine kinase and removal or inactivation of thymidylate kinase in extracts of the tissue culture cells were achieved by adjusting the pH to 4.3 with 1 N acetic acid (13), then centrifuging the turbid suspension at 104,000 × g for 10 minutes, followed by neutralization of the supernatant to pH 7.5 with KOH. This supernatant fraction retained 66 to 80% of the thymidine kinase, but practically no thymidylate kinase activity, in contrast to the previously reported results (13).

Enzyme Assays—the rates of enzymatic conversion of thymidine-2-C14 to the products dTMP and dTTP provided a convenient means of determining the activities of thymidine kinase and thymidylate kinase, respectively. Two batches of thymidine-2-C14 (New England Nuclear Corporation) had specific activities of 6.29 and 25.69 μc per μmole. In conical centrifuge tubes, the standard assay mixture contained the following components: thymidine 2-C14, 0.16 mM; ATP, 0.0 mM; MgCl2, 9.0 mM; potassium 3-phosphoglycerate, 2.75 mM; Tris-HCl, pH 7.75, 0.65 mM; KCl, 0.154 M 0.15 ml; and 0.125 ml of the supernatant enzyme mixture, giving a total volume of 0.50 ml. At appropriate intervals, 0.1-ml aliquots were withdrawn, placed into centrifuge tubes, and heated for 2 minutes in a boiling water bath. The heated samples were stored at −20° until chromatographic separations could be made.

* This work was supported in part by a grant (No. C-646) from the National Cancer Institute, Public Health Service, United States Department of Health, Education and Welfare. A preliminary report was given to the American Society of Biological Chemists (1).

† United States Public Health Service Postdoctoral Fellow, 1960–1962. Present address, Department of Agricultural Biochemistry, Ohio State University, Columbus 10, Ohio.

This experiment has been confirmed in this laboratory with the use of Novikoff hepatoma cells in tissue culture (P. A. Morse, Jr., unpublished work).
The activity of phosphatase capable of hydrolyzing dTMP to thymidine was measured by means of C³⁰-labeled dTTP synthesized enzymatically from thymidine-2-C³⁰ and purified by column chromatography in the formic acid and ammonium formate systems of Haribert, Schmitz, Brumm, and Potter (14). Components of the standard reaction mixture were used, except that the ATP-phosphoglycerate energy-generating components were omitted. This procedure would be expected to determine the upper limits of phosphatase activities, since their magnesium requirements would be satisfied by free magnesium ions, unchelated by ATP.

Chromatographic and Counting Procedures—Frozen samples were thawed and centrifuged, and 25 to 50 μl of supernatant was applied as a narrow band 3 inches from one end of a 1-inch strip cut from the long axis of a sheet of DEAE-cellulose paper (Whatman No. DE20) (182 x 22 inches). Strips were attached to Kurz-Miramon glass frames (Kensington Scientific Corporation) with polyethylene clips and developed approximately 4 hours by descending chromatography, with 4 N formic acid, 0.10 M in ammonium formate. The atmosphere of the chromatography tank was not preequilibrated with the solvent, but the strips must be dried in inches from the ends of the strips; then the glass frames were saturated with solvent before beginning descending chromatography. The negative feedback "loops" are indicated by double bars. The feedback loops are labeled nfb1, nfb2, etc., in the order of their discovery (1, 6, 8, 9). The double bar on nfb2 and arrow labeled ATP indicates the block of nfb2 by ATP as shown in the present report. Standard abbreviations for phosphorylated compounds; deoxyribosides of cytosine, uracil, and thymine abbreviated as Cdr, Udr, and Tdr, respectively.

The dried strips were then counted on a radioactivity scanner (15). Thymidine moved freely with the solvent front, while dTTP was completely immobile being displaced from the origin by the salt in proportion to the salts in the assay mixture. dTMP and dTDP gave average Rf values of 0.58 and 0.22, respectively. Phenol red provided a convenient visual marker for dTDP, which moved just behind it. The radioactivity in each peak was expressed as a percentage of the total counts on the individual strip. The distribution of radioactive fractions on each strip then served as a time point on the kinetic plot illustrated in Fig. 2A (control experiment), a typical example of the standard assay for thymidine kinase in extracts of Novikoff cultures. dTDP accumulated only after the initial production of dTTP, and none was seen at 60 minutes or at earlier time points (cf. Bianchi et al. [10]). It seemed valid to assume, in any case, that both fractions were ultimately products of thymidylate kinase, so that dTTP plus any small amount of dTDP formed, was plotted as "dTTP." In experiments in which complete separation of dTTP and dTDP was essential, it was possible to effect such separation by extending the chromatographic development time to 6 hours, allowing any thymidine to drip off the end of the strip along with the solvent front. However, with the tracing of higher specific activity which permitted counting of smaller aliquots of the reaction mixture, complete separation of the four components, thymidine dTMP, dTTP, and dTTP, was possible in the 4 hours before the solvent reached the end of the strip. Careful separation of dTDP from dTTP was essential to show that the failure of dTTP to appear in detectable amounts could be explained by an extremely rapid conversion of dTDP to dTTP. The present study was greatly facilitated by the

![Diagram of negative feedback in biosynthesis of dTTP](https://example.com/diagram.png)

Fig. 1. Negative feedback inhibitions in the biosynthesis of dTTP. Each kinase is represented by a bent arrow to indicate participation of ATP as cosubstrate and to distinguish the kinase from the phosphatase, which drives the reaction in the reverse direction. The negative feedback "loops" are indicated by dashed lines, and the blocked reactions are indicated by double bars. The feedback loops are labeled nfb1, nfb2, etc., in the order of their discovery (1, 6, 8, 9). The double bar on nfb2 and arrow labeled ATP indicates the block of nfb2 by ATP as shown in the present report. Standard abbreviations for phosphorylated compounds; deoxyribosides of cytosine, uracil, and thymine abbreviated as Cdr, Udr, and Tdr, respectively.

![Diagram of consecutive activities of thymidine kinase and thymidylate (dTTP) kinase](https://example.com/diagram.png)

Fig. 2. A. Consecutive activities of thymidine kinase and thymidylate (dTTP) kinase. Thymidine disappearance and products of thymidine are shown as a function of time with a high speed, soluble supernatant fraction (S₂) obtained from Novikoff cell line grown in tissue culture and incubated under the standard conditions described in "Experimental Procedure," except that the ATP-Mg concentration was 4.5 mM instead of 9.0 mM. The relative percentage of radioactivity in each compound was independent of the total radioactivity applied to the strip, but at least 5000 counts (as recorded) were always present at zero time and throughout the incubation period (see "Counting Procedures"). Since dTDP and dTTP were often incompletely resolved in terms of the threshold-activated triggering events to the digital recorder and printer, they were plotted as "dTTP" even though some dTDP was present at later time points (as seen by the inkwriter). The increase in thymidine after the 30-minute time point is the resultant effect of phosphatase action on thymidylate and the inhibition of thymidine kinase by dTTP. Compare with Fig. 9A at same ATP concentration.

B. Consecutive activities of thymidine kinase and thymidylate kinase in the presence of added dTTP. Conditions as in A except for added nonradioactive dTTP (0.1 mM). The added dTTP did not affect the measurement of the radioactive dTTP formed in the reaction and inhibited the conversion of thymidine to dTMP.

---

3 We are indebted to Dr. F. J. Bollum for suggesting the application of anion exchange paper to the separation of thymidine nucleotides.

---

Vol. 238, No. 4
fact that the cells employed show little or no tendency to convert thymidine to thymine or thymine to dihydrothymine and subsequent products involving loss of radioactive CO₂ from the thymidine-2-C³⁷. Both of these reactions occur in normal and regenerating liver, and can lead to decreases in the total radioactivity in the reaction mixture. With extracts from the Novikoff cell strain, the total radioactivity remained constant and small errors in pipetting aliquots of the reaction mixture for paper chromatography were eliminated by plotting the data from individual radioactivity spots or "peaks" as a percentage of the total on the strip.

When initial reaction rates were to be plotted, kinetic plots similar to Fig. 2A were drawn, and the reaction rates for thymidine kinase and thymidylate kinase were calculated from the slopes of the best approximations of straight lines for thymidine utilization and dTTP production, respectively. In general, linear kinetics were most nearly approximated at 30 minutes. All reaction rates are expressed as millimicromoles per hour per ml of reaction mixture.

Fig. 2B shows the inhibition of thymidine kinase by added dTTP and will be discussed in "Results."

**RESULTS**

**General Properties of Thymidine Kinase and Thymidylate Kinase**—Thymidine kinase and thymidylate kinase cooperate in a reaction sequence resulting in the formation of thymidine triphosphate (dTTP) from thymidine. Despite the similarity of their roles in the phosphorylative process, these two enzymes exhibit many points of difference in their properties. Fig. 2A (control experiment) suggests that thymidine kinase and thymidylate kinase have differing affinities for their substrates, thymidine and thymidylate, respectively. Thymidine kinase, as reflected by the descending thymidine content in the reaction mixture, typically showed linear and complete utilization of its substrate. Further indication of the affinity of thymidine kinase for thymidine is shown in Fig. 3 by the curve labeled control. The peak rate of dTTP formation shown in Fig. 2A (control curves) suggests that thymidylate kinase may require considerably higher concentrations of dTMP for saturation than does thymidine kinase for its substrate. The rate of dTTP + dTDP formation, as indicated by the slope of the broken line, was always maximal during the period when the dTMP concentration in the reaction mixture was greatest. After the dTMP maximum was reached and its concentration began to fall, the rate of dTTP + dTTP formation diminished rapidly.

The affinities of thymidine kinase and thymidylate kinase for ATP, the cosubstrate in each reaction, also differ markedly. As shown in Fig. 4, thymidine kinase activity was quite independent of the ATP-Mg concentration and exhibited zero order kinetics over a 16-fold range of concentrations of this cosubstrate. Thymidylate kinase, on the other hand, showed first order dependency on ATP-Mg concentration to 4.5 mM, or above. The optimal concentration appeared to be approximately 9.0 mM, while the complete omission of either ATP-Mg or of ATP from the reaction mixture resulted in the loss of all thymidylate kinase activity.

If, as has been supposed, the cosubstrate for kinase-catalyzed phosphorylative reactions is the ATP-Mg complex, it would be of interest to examine the effects of variation of the magnesium concentration on thymidine kinase and thymidylate kinase. In the experiment depicted in Fig. 5, the ATP concentration was fixed at 9 mM, the optimum for thymidylate kinase (see Fig. 4), and the magnesium concentration was varied. The net rate of thymidine phosphorylation was found to be optimal at 4.5 to 6.75 mM while the optimum for thymidylate kinase lay between 9 and 18 mM. The rather sharp decrease in activity of the ki-
Table I

Metal requirements of kinases

<table>
<thead>
<tr>
<th></th>
<th>Thymidine kinase</th>
<th>Thymidylate kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Undialyzed S1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>246</td>
<td>80</td>
</tr>
<tr>
<td>Complete - Mg</td>
<td>149</td>
<td>0</td>
</tr>
<tr>
<td>Complete - Mg, + Mn</td>
<td>150</td>
<td>88</td>
</tr>
<tr>
<td>Complete - Mg, + Ca</td>
<td>125</td>
<td>0</td>
</tr>
<tr>
<td>Complete - Mg, + Versene</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Complete + Versene</td>
<td>177</td>
<td>0</td>
</tr>
<tr>
<td><strong>Dialyzed S2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>238</td>
<td>13</td>
</tr>
<tr>
<td>Complete - Mg</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>Complete - Mg, + Mn</td>
<td>171</td>
<td>14</td>
</tr>
<tr>
<td>Complete - Mg, + Ca</td>
<td>133</td>
<td>0</td>
</tr>
<tr>
<td>Complete - Mg, + Versene</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Complete + Versene</td>
<td>136</td>
<td>0</td>
</tr>
</tbody>
</table>

*All rates were calculated at 30-minute time points of kinetic plots. The standard assay conditions described in “Experimental Procedure” were used; when metal ions were added their concentration was 0.05 mM, equal to that of the ATP. Where indicated, 10 mM Versene was added. The S1 was dialyzed for 24 hours at 4 °C in a medium containing: 2 m-Meraptoethanol, 1.0 mM thymidine, 0.1 mM Tris-HCl, 10 mM, pH 7.8; and KCl, 0.15 M. Appropriate calculations were made to take into account the dilution of the thymidine-2-C14 by the unlabeled thymidine in the enzyme preparation.

**nases with higher levels of magnesium may be due to the availability of unchelated Mg2+ to the nucleotide phosphatases.**

The species, as well as the concentration of divalent metallic cations required by thymidine kinase, also appears to differ from the requirement exhibited by thymidylate kinase. In Table I, it may be seen that, as predicted by Fig. 5, omission of added magnesium from the reaction mixture containing undialyzed enzyme resulted in loss of all thymidylate kinase activity, while more than half of the thymidine kinase activity was still present. Surprisingly, not even prolonged dialysis resulted in demonstration of an absolute metal requirement by thymidine kinase. However, the addition of 0.01 M Versene completely eliminated all thymidine kinase activity from both the dialyzed and undialyzed preparation lacking added magnesium. The effect of divalent cations other than magnesium on thymidine kinase and thymidylate kinase is of interest. Manganese only partly restored the thymidine kinase activity of the dialyzed preparation, but fully restored thymidylate kinase in both the undialyzed and dialyzed preparations. Calcium ions did not have any activating effect on thymidylate kinase, but did activate thymidine kinase to some extent in the dialyzed preparation. However, when some magnesium was present, as in the undialyzed enzyme, the calcium appeared to exert a slightly antagonistic effect on thymidine kinase.

In agreement with observations reported by Bianchi et al. (16) and by Bojarski and Hiatt (17), thymidine kinase was found to be fairly stable to dialysis, while thymidylate kinase was completely lost unless the dialyzing medium contained 0.1 mM thymidine which served to maintain at least part of the activity.

The pH optima of the two enzymes in the unfractionated soluble enzyme mixture proved to be strikingly different, with the two activities changing in a reciprocal relationship as the pH was altered. Thymidine kinase had a broad optimum at pH 8.25 to 8.50, while thymidylate kinase was virtually inactive above that range. As the pH was decreased toward 7.0, thymidine kinase activity decreased approximately 25% whereas thymidylate kinase increased to a plateau. These optima represent the most favorable balance between phosphorylation and dephosphorylation in the complex supernatant enzyme mixture, and the pH optima of the purified kinase may prove to be somewhat different from those presented here. Parenthetically, it should be emphasized that in experiments where Tris buffers are employed, it is necessary that pH measurements be made directly on the reaction mixtures, since the pH of this buffer system is highly sensitive both to ionic changes and to temperature changes (18). In our hands, the pH of Tris-HCl buffers prepared at room temperature routinely dropped 0.25 unit when added to the reaction mixtures and warmed to 37 °C.

**Evidence for Feedback Inhibition of Thymidine Kinase—**In following the time courses of the thymidine thymidylate kinase reaction system (Fig. 2A (control)), the complete utilization of thymidine, immediately followed by its reappearance, raised an interesting question. If thymidine kinase was sufficiently active to phosphorylate completely all the thymidine at a rate almost linear with time, why could it not continue to phosphorylate the thymidine that was hydrolized from the dTMP by phosphatase? It seemed unlikely that enzyme denaturation could have occurred so quickly, since thymidine kinase is generally fairly stable in the absence of its substrate, so the explanation seemed to be that a product was inhibiting the forward reaction at the low substrate concentrations while allowing the phosphatase reaction to continue. Preparations of supernatant enzyme which had been aged for 48 hours at 0 °C, resulting in the loss of thymidylate kinase, were capable of phosphorylating the thymidine quantitatively to dTMP, but without the "rebound" of thymidine seen in Fig. 2A (data not shown). This raised the possibility that the product of the dTMP kinase reaction, dTTP, could be inhibiting thymidine kinase, thereby preventing the complete dephosphorylation of thymidine released by phosphatase. To test this hypothesis, direct addition of 0.1 mM dTTP to the reaction mixture was effected, with the results seen in Fig. 2B. An immediate and progressive inhibition of thymidine utilization was observed, resulting in the incomplete utilization of the substrate. The progressive nature of the inhibition can be explained in part by the addition of endogenously produced dTTP to that present initially and in part by the increasing ratio of dTTP to thymidine.

The decreased rate of dTTP production in Fig. 2B must be explained in terms of thymidine kinase inhibition, rather than by direct inhibition of thymidylate kinase by its product since, as is seen in Fig. 6, dTTP has no direct effect on the rate of its own production from dTMP. Under the conditions of this ex-
no change whatsoever in the rate of labeled dTTP synthesis from C14-dTMP, but did affect the net rate of substrate utilization, since, in the control (Fig. 6A), thymidine kinase was able to rephosphorylate any thymidine produced by phosphatase action, while in the presence of dTTP thymidine kinase was inhibited, allowing thymidine to accumulate at the expense of dTMP. Moreover, the increased rate of thymidine production cannot be explained by a direct activation of thymidylate phosphatase, since when ATP and 3-phosphoglycerate were omitted from the experimental system described in Fig. 5, the phosphatase was completely unaffected by the addition of dTTP to the reaction mixture (not shown). The substitution of dTDP for dTTP in any experimental system involving thymidine kinase inhibition had exactly the same effect as that produced by dTTP, and auxiliary experiments showed that in the presence of ATP and phosphoglycerate the 0.11 mM dTDP was converted to dTTP within the 5-minute interval after the enzyme was added to the ice-cold reaction mixture, before placing it in the 37° water bath. It is likely, then, that this extremely active diphosphonucleoside kinase very rapidly converted dTDP to dTTP, so that the actual inhibition observed was caused solely by the triphosphate derivative of thymidine.

In separate experiments (not shown), it was found that a variety of other nucleoside triphosphates had no inhibitory effect on thymidine kinase. These included UTP, dCTP, dGTP, and dATP. It seems, then, that the inhibition by dTTP is rather specific, since no other nucleotide with that effect has been found. Since dTMP added to the reaction mixture had no effect, the inhibition of thymidine kinase by polyphosphate derivatives of thymidine cannot be explained merely by the dilution of the substrate pool by hydrolytic products of dTDP or dTTP. This conclusion was further substantiated by an experiment (not shown) in which C14-dTDP, 0.11 mM, was incubated without the ATP-regenerating system. In the absence of ATP and 3-phosphoglycerate, the dTDP was converted to dTMP at an extremely low rate, only approximately 5% being hydrolyzed to dTMP after 1 hour of incubation. Thus, it would seem that stepwise degradation of dTTP and dTDP could not possibly dilute the substrate pool enough to account for the inhibition of thymidine kinase caused by these compounds when using extracts of Novikoff cells.

Nature of Inhibition by dTTP—To obtain a clue as to the type of inhibition produced by dTTP on thymidine kinase, the effect of variations in amount of enzyme on the initial rates of thymidine utilization in the presence or absence of the inhibitor was observed (Fig. 7). Within the range of enzyme linearity, the amount of enzyme in the reaction mixture had no effect upon the degree of inhibition due to dTTP. Therefore, the inhibition may be presumed to be of the reversible or competitive class of inhibition (19), since an irreversibly bound inhibitor would be expected to titrate a finite quantity of enzyme, resulting in parallel lines, rather than the converging lines seen in Fig. 7.

Further evidence of the reversible nature of dTTP inhibition is shown in Fig. 3. At low thymidine concentrations, a fixed concentration of dTTP caused almost complete inhibition of the rate of conversion of thymidine to dTMP in a system incapable of converting dTMP to dTTP. As the substrate concentration was increased, the inhibition tended to be overcome. A salient feature of these data is the fact that nonclassical kinetics were exhibited by the control samples with a partially purified pH 4.5 supernatant enzyme fraction lacking thymidylate kinase activity. Even at concentrations of thymidine as low as 0.008 mM, first order dependence on substrate was not seen, while a rather gradual increase in thymidine kinase activity with increasing levels of substrate was observed. Although these data suggest that the inhibition of thymidine kinase by dTTP is competitive, application of the Lineweaver-Burk formulation (20) must await further purification of the enzyme.

If the substrate concentration was fixed at 0.16 mM and the inhibitor concentration varied, high concentrations of dTTP almost completely inhibited thymidine kinase (Fig. 8). Again, this experiment tends to indicate that a reversible competition exists between thymidine and thymidine triphosphate for a site on the enzyme thymidine kinase.

Since there appears to be competition between thymidine and
Feedback Inhibition of Thymidine Kinase by dTTP

Vol. 238, No. 4

FIG. 8. The effect of dTTP on thymidine kinase activity. Conditions as in Fig. 2A except with pH 4.5 supernatant fraction and dTTP varied as shown.

FIG. 9. A, the modulating effect of ATP-Mg on the inhibition of thymidine kinase by endogenous dTTP. No effect on thymidine disappearance is seen during 0 to 30 minutes, but after this time enough dTTP is produced (see Fig. 2A) to inhibit the reconversion of thymidine to dTMP when thymidine is produced by phosphatase action on dTMP. Conditions as in Fig. 2A except for variation in ATP-Mg as shown on the curves. In a similar experiment (not shown) with pH 4.5 supernatant fraction, no thymidine formation occurred in the 30 to 180-minute period because no dTTP was formed. (dTMP and dTTP were quantitatively measured but are not plotted in Fig. 9A or 9B. See Fig. 2A and 2B.) B, the modulating effect of ATP-Mg on the inhibition of thymidine kinase by exogenous dTTP (0.16 mM). The disappearance of thymidine was strongly inhibited at all time points when the ATP-Mg concentration was low, but weakly inhibited at high concentrations of ATP-Mg. Conditions as in Fig. 9A except for added dTTP.

dTTP for sites on thymidine kinase, it was of interest to examine the effect of variations in the concentration of the cosubstrate, ATP, on the inhibition by dTTP. Accordingly, reaction mixtures with freshly prepared Novikoff cell Ss and additions of an equimolar mixture of ATP and magnesium, varied over a 16-fold range of concentrations, were prepared with and without dTTP. In the controls (Fig. 9A), the initial rate of thymidine utilization was almost completely independent of the ATP-Mg concentration, but the "rebound" of dephosphorylated thymidine was maximal at lower ATP-Mg concentrations, and completely absent at higher concentrations. However, in the presence of dTTP (Fig. 9B) the degree of inhibition of thymidine utilization was profoundly influenced by the ATP-Mg concentration. At low concentrations of ATP-Mg, dTTP equimolar to the substrate completely inhibited thymidine kinase, while at higher concentrations of ATP-Mg inhibition was nearly overcome. Therefore, some type of competition exists, not only between thymidine and dTTP, but also between ATP and dTTP. Thus, the distal product of the system, dTTP, competes with each of the substrates of thymidine kinase, for sites on the enzyme, while these two substrates, thymidine and ATP, do not appear to compete with each other. This then, would suggest that dTTP overlaps adjacent but somewhat independent affinity points of thymidine kinase normally binding thymidine and ATP. Because the reaction mixtures in the experiment depicted in Fig. 9 were actively producing dTTP endogenously, the variable degree of thymidine rebound seen in the controls may be explained by the reversal of inhibition from this endogenous dTTP by the higher levels of ATP. If this explanation is valid, then enzyme preparations lacking the ability to produce dTTP should exhibit no tendency toward thymidine rebound. With the partially fractionated thymidine kinase preparation devoid of thymidylate kinase activity, it was indeed observed that no such reappearance of thymidine occurred at any of the ATP-Mg concentrations shown in Fig. 9A (data not shown). Since this enzyme preparation had demonstrable phosphatase activity, it must be concluded that thymidine kinase remains active after the initial utilization of its substrate, and that the rebound seen in Fig. 9A was due to the inhibition of this enzyme by endogenously produced dTTP. When 0.16 mM dTTP was added in the absence of thymidylate kinase, the degree of inhibition of thymidine kinase caused thereby was powerfully affected by the concentration of ATP-Mg, just as it was in the experiment shown in Fig. 9B. In this case, it was observed that the inhibition had much less tendency to be progressive with time since the effect of endogenously accumulating dTTP was not being added to that present in the reaction mixture initially. The effect of ATP on the inhibition by dTTP is shown in Fig. 10, based on an experiment similar to that shown in Fig. 9B but in which no endogenous dTTP was formed.

While all of the data presented graphically in this paper were obtained from experiments employing extracts of Novikoff hepatoma cells from tissue culture, all indications suggest that...
the phenomenon of dTTP feedback on thymidine kinase is completely generalized throughout the biological realm. The thymi-
dine kinase in extracts from the following tissues and organisms was, without exception, strongly inhibited by direct additions of
dTTP to the reaction mixtures in amounts equimolar to the
substrate: regenerating rat liver, rat spleen, rat thymus, Morris
7800 hepatoma, *Escherichia coli*, and *Physarum polycephalum*
slime mold. To this list can be added the rat embryo as re-
ported in the confirming experiments by Maley and Maley (9).

**DISCUSSION**

Thymidine kinase appears to be the first kinase shown to be
subject to negative feedback by a distal product. The inhibi-
tion is brought about, not by the immediate product of the re-
action (dTMP), but by a more distal product (dTTP), a situa-
tion common in cases of feedback inhibition (21). Since an
exactly analogous feedback has been shown for deoxyctydine
kinase (9), it is possible that similar product inhibitions exist
among other kinases, but that the distal products thereof have
not been adequately tested.

Inhibition has been shown by the endogenous product of the
thymidine kinase-thymidylate kinase system, wherein accumu-
lations of dTTP in a reaction mixture appear to slow the phos-
phorylation of thymidine in the face of the opposing phosphatase
reaction, resulting in the reappearance of substrate after its
initial utilization (see Fig. 2A). On the other hand, exogenous
dTTP added to the reaction mixture has an instantaneous effect
on the rate of thymidine utilization.

The inhibition by dTTP is strongly affected by competition
with both the substrate (thymidine) and the cosubstrate (ATP).
Since UTP, dCTP, dGTP, and dATP do not inhibit, it may be
suggested that the enzyme has affinity points for moieties of
the thymine base (T), deoxyribose (dR), adenine (A), ribose (R),
and pyrophosphate (P-P) as follows:

<table>
<thead>
<tr>
<th>Affinity points on enzyme</th>
<th>T</th>
<th>dR</th>
<th>P-P</th>
<th>R</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>dTTP</td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

The substrate probably attaches to the first two and the ATP
to the last three sites listed. If dTTP attaches to the enzyme
at the thymine, deoxyribose, and pyrophosphate affinity points,
it would be expected to overlap sites binding both thymidine
and ATP, thereby entering into competition with them. Com-
ounds such as UTP, dCTP, dGTP, and dATP, however, would be
ineffective as inhibitors because they could react with only two
of the three affinity points used by dTTP. Moreover, the
proximal reaction product, dTMP, would not inhibit for the same
reason. Further studies to determine whether ATP can be
replaced by GTP, CTP, or UTP as cosubstrate would be of
interest, and the possible inhibitory actions of thymine riboside
(5-methyluridine) and deoxyuridine and their phosphorylated
derivatives should be studied to learn more about the active
centers on the enzyme.

The net effect of feedback by endogenously produced dTTP
is potentiated by conditions which provide free Mg2+ ions to
the phosphatase which hydrolyzes dTMP and thus opposes
thymidine kinase. Characteristically, magnesium in molar
excess over ATP resulted in an immediate decrease in the rate
of thymidine utilization. This was seen over a wide range of
ATP concentrations, while the original reaction rate was re-

stored when the concentration of ATP was increased to that of
magnesium (see Fig. 4), suggesting that the Mg:ATP ratio,
rather than their absolute concentrations, determines the bal-
ance between thymidine kinase and phosphatase. When 3-
phosphoglycerate (the ATP-regenerating system) was omitted,6
the initial rate of thymidine utilization was normal, but fell off
rapidly as the Mg:ATP ratio increased, while at the same time
thymidine kinase was being progressively inhibited by accumu-
lating dTTP.

**SUMMARY**

Thymidine kinase and thymidylate kinase in the high speed
supernatant fraction of Novikoff hepatoma cells from suspension
tissue culture were assayed in terms of products formed from
thymidine-2-C14 and adenosine triphosphate (ATP). These
products, including thymidylate (dTMP), thymidine dipho-
phosphate (dTDP), and thymidine triphosphate (dTTP), were
rapidly and conveniently separated from the labeled starting material
on a new system utilizing chromatography on diethylaminoethyl
cellulose anion exchange paper, followed by determination of
their relative radioactivities on an automatic strip scanner.

Thymidine kinase and thymidylate kinase, despite their sim-
ilarity of function were found to differ in a variety of properties,
including their affinities for substrates and metal cofactors, their
pH optima, and their susceptibility to product inhibition. Thy-
midine kinase, but not thymidylate kinase, was found to be
strongly inhibited by its distal product, dTTP, at concentrations
equal to that of the thymidine in the assay mixture. This inhibi-
tion could be demonstrated both by dTTP formed endog-
ously in the combined thymidine-thymidylate kinase system,
and by exogenous additions of dTTP. The degree of inhibition
was independent of enzyme concentration but was strongly
modulated by substrate concentration. The inhibition tended to
be reversed by elevated concentrations either of thymidine
or of ATP, suggesting that each of these cosubstrates compete
with dTTP for sites on the enzyme thymidine kinase.

The inhibition of thymidine kinase appeared to be highly
specific, since neither the proximal product, dTMP, nor a variety
of other nucleotides, including uridine triphosphate, deoxycyti-
dine triphosphate, deoxycytosine triphosphate, and dATP, were
found to inhibit the enzyme at concentrations equal to that of
the substrate. The inhibition was demonstrated in supernatant
extracts of a variety of tissues and organisms, including regen-
erating rat liver, rat spleen, rat thymus, Morris 7800 hepatoma,
*Escherichia coli*, and *Physarum polycephalum* slime mold.

**Acknowledgments**—We gratefully acknowledge the helpful pre-
liminary experiments by Miss Geraldine De Graaia, the radio-
activity measurements performed by Mrs. Nijole Saponara and Miss Mary Sloan, the *Escherichia coli* preparations furnished by
Drs. Roger Brethauer and Leon Marcus, the Morris 7800 hepa-
tomas provided by Dr. H. P. Morris, and the *Physarum* extracts
provided by Dr. W. Sachsenmaier.

**REFERENCES**


Feedback Inhibition of Thymidine Kinase by Thymidine Triphosphate

David H. Ives, P. A. Morse, Jr. and Van R. Potter


Access the most updated version of this article at http://www.jbc.org/content/238/4/1467.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/238/4/1467.citation.full.html#ref-list-1