Mouse Ascites Sarcoma 180 Thymidylate Kinase. General Properties, Kinetic Analysis, and Inhibition Studies†

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ABSTRACT: Thymidylate (dTMP) kinase derived from mouse Sacroma 180 ascites cells has been purified partially (14-fold), and shown to be free of ATPase, thymidylate phosphatase, nucleoside diphosphokinase, thymidine kinase, and nucleoside phosphotransferase. The enzyme is very unstable, in agreement with that reported for dTMP-kinase derived from other sources and may be stabilized in the presence of mercaptoethanol with dTMP, dTDP, or ADP but not with ATP, ATP-Mg^{2+}, or mercaptoethanol alone. Zone sedimentation in a sucrose density gradient indicated a molecular weight of 33,000. High levels of ATP will not inhibit the enzyme provided a stoichiometric or molar excess of Mg^{2+} is maintained. ATP or dATP but not GTP, CTP, UTP, dGTP, dCTP, or dTTP functions as a phosphate donor. The apparent V_{max} and K_m for ATP-Mg^{2+} and dATP-Mg^{2+} are the same. The activation energy was calculated from an Arrhenius plot to be 10.4 kcal mol^{-1} when log K_m of dTMP was plotted, and to be 15.6 kcal mol^{-1} when log V_{max} was plotted, observations in accord with the rate-limiting step not being the binding of either substrate to the enzyme.

Kinetic properties (initial velocity and product inhibition) are described and found to be compatible with a rapid equilibrium random mechanism. Competitive inhibition was observed by ADP *vs.* ATP-Mg^{2+} or by ADP *vs.* dTMP, and by dTDP *vs.* ATP-Mg^{2+} or by TDP *vs.* dTMP. Michaelis and dissociation constants were determined. dTTP is a potent competitive feedback inhibitor of dTMP with a K_i of 27 μM, under conditions of negligible degradation of dTTP to dTDP. Thymidine, in agreement with previous reports, is also a competitive inhibitor of dTMP, and has a K_i of 50 μM. In agreement with the studies of Langen and coworkers, 5′-halogenated thymidine derivatives are competitive inhibitors of dTMP-kinase. 5′-Carboxyl- and 5′-chlorothymidine were observed to be competitive inhibitors of dTMP. Inhibition of dTMP, presumably competitive, was also observed with 5′-iodo- and 5′-aminothymidine. The relative inhibitory effects of various 5′-substituted derivatives of thymidine are in the order of $-Cl > -$(pyro)triphosphate $> -$(pyro)diphosphate $> -CO_2H > -I = -OH > -NH_2$. No direct correlation was observed between the affinity of these thymidine derivatives for dTMP-kinase and their ability to stabilize the enzyme from inactivation.

Thymidylate kinase (dTMP-kinase)[1] is a key enzyme in the biosynthesis of dTTP, the immediate precursor of DNA-thymine, and catalyzes the reaction: ATP + dTMP → ADP + dTDP for which divalent cations are required (Grav and Smellie, 1965; Ives *et al.*, 1963; Kielley, 1970; Nelson and Carter, 1969). The enzyme has been considered to be very unstable; however, in the presence of dTMP and mercaptoethanol, stabilization is achieved (Bojarski and Hiatt, 1960; Grav and Smellie, 1965; Kielley, 1970; Nelson and Carter, 1969). Extensive purification of the enzyme from three different sources has been reported: *Escherichia coli* (Nelson and Carter, 1969), mouse hepatoma (Kielley, 1970), and Landshutz ascites tumor cells (Grav and Smellie, 1965), and marked differences have been observed in certain properties. Thus the molecular weight of the enzyme prepared from *E. coli* is 65,000 (Nelson and Carter, 1969), whereas that from mouse hepatoma is 35,000 (Kielley, 1970). Furthermore the microbial enzyme has less specificity than the mammalian enzyme for the nucleoside triphosphate as a phosphate donor.

As part of a program concerned with the design of compounds that either inhibit dTMP-kinase or sensitize it to radiation inactivation, it is essential to initially obtain an understanding of the properties of this enzyme. In this communication, a report of some properties of the partially purified dTMP-kinase derived from mouse ascites Sarcoma 180 cells is presented. Although a few kinetic properties of this enzyme have been described (Kielley, 1970; Langen and Kowollik, 1968; Nelson and Carter, 1970), kinetic properties such as initial velocity and product inhibition have not been reported. The present paper describes such properties of a partially purified enzyme derived from mouse Sarcoma 180 ascites cells, and suggests a possible mechanism of the enzymatic reaction.

dTMP-kinase has been reported to be inhibited by dTTP (Eker, 1968; Nelson and Carter, 1969), dThd (Langen and Kowollik, 1968), 5′-deoxythymidine (Kara and Duschinsky, 1969), 5′-halogenated dThd (Langen and Kowollik, 1968; Langen *et al.*, 1969a,b), and pyrimidines substituted in the 5 position (Chae *et al.*, 1972). Some of these compounds also serve as stabilizing reagents. Neither the mode of inhibition of dTMP-kinase nor the K_I of either dTTP or dThd has been reported. The mode of inhibition by 5′-halogenated dThd seems to be competitive with respect to dTMP, and I_{50} values of various 5′-halogenated dThd have been reported (Langen *et al.*, 1969a,b). A study of the mode of the inhibitory action by 5′-substituted dThd may be helpful for understanding those parameters at the active site of the enzyme that are susceptible to attack. Several previously unstudied 5′-substituted dThd compounds have been synthesized and evaluated as potential thymidylate kinase inhibitors. These studies are also presented in this communication.

Experimental Section

Materials. Nucleotides were products of the California Corp. for Biochemical Research or the Sigma Chemical Co.

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[1] Abbreviation used is: dTMP-kinase, thymidylate kinase.

[^{14}C]dTMP was obtained from Schwarz/Mann BioResearch Inc. Calcium phosphate gel was kindly supplied by Dr. Cha and Dr. Baccanari from Brown University. Alumina Cγ gel was purchased from Calbiochem, DEAE-Sephadex A-50 from Pharmacia Fine Chemicals Inc., and MN-Polygram Cel 300 PEI/UV 254 thin-layer plates from Brinkman Instruments, Inc. 5′-Cl-dThd was kindly provided by Dr. Langen. 5′-CO_2H-dThd, 5′-IdThd, and 5′-NH_2-dThd were kindly prepared by Dr. Chang and Dr. Sciarini. All the reagents used were reagent grade.

Assay Procedures. The assay mixture contained Tris-HCl buffer (0.1 M, pH 7.5), ATP (8 mM), $MgCl_2$ (10 mM), [^{14}C]dTMP (77 μM; 2 Ci/mol), mercaptoethanol (0.3 mM), bovine serum albumin (1%), phosphocreatine (3 mM), creatine kinase (0.54 unit), and not more than 7 units of dTMP-kinase in a total volume of 0.3 ml. The mixture was incubated at 37° for 10 min in our standard assay. The reaction was stopped by heating the mixture at 100° for 3 min. An aliquot portion (15 μl) of the heated mixture was spotted on a thin-layer MN-polygram cell 300 PEI/UV 254 plate previously spotted with a marker mixture composed of dTTP, dTDP, dTMP, and dThd and separated using a solution of 0.5 M LiCl and 2 N acetic acid run to a distance of 16 cm. The areas containing dTTP, dTDP, dTMP, and dThd were located under ultraviolet light, and each area was cut and the powder was removed and placed in a scintillation vial (10 ml of 1,4-bis[2-(5-phenyloxyazolyl)]benzene in toluene). The amount of radioactivity was determined in a Packard liquid scintillation spectrometer.

One unit of dTMP-kinase activity is defined as the amount of enzyme catalyzing the formation of 1 nmol of dTDP from dTMP/min at 37° under our standard assay conditions. It is very important to have an ATP generating system (creatine kinase and phosphocreatine) in the assay mixture, especially when a crude cell extract, which may contain a significant amount of ATPase activity, is assayed for activity. The reaction rate is linear even when after one-quarter of the dTMP has been converted into dTDP.

Protein Determination. The method of Lowry and coworkers (1951) was used for protein determinations. Solutions of bovine serum albumin served as the standard, and 0.1 ml of the sample solution was used for each assay.

Purification Procedure. Sarcoma 180 ascites cells were harvested 7 days after inoculation of female CD-1 mice obtained from Charles River. The ascitic fluid from about 100 mice was pooled. After centrifugation, the contaminating erythrocytes were removed by resuspension in two volumes of distilled water for 30 sec with subsequent reconstitution to isotonicity by addition of an equal volume of a solution of 1.8% NaCl. The cell suspension was centrifuged and this procedure was repeated until all red blood cells had lysed. The pellets were washed three times with 0.3% NaC slolution and kept overnight at −70°. All succeeding steps were performed at 4°.

Extraction. The washed cell pellets (30 ml) were mixed with 60 ml of ice-cold stabilizing buffer (Tris-HCl, pH 7.5, 5 mM; dTMP, 40 μM; mercaptoethanol, 5 mM) and then frozen and thawed three times prior to sonication for 150 sec (30-sec interval; probe intensity, 45) with a Bronwell Biosonik II ultrasonicator. The homogenate was centrifuged at 39,000 rpm for 1 hr in a Spinco 50-Ti rotor and the supernatant fraction was collected.

Calcium Phosphate Gel Fractionation. To 65 ml of the supernatant fraction from the previous step, 20 ml (43 mg/ml) of calcium phosphate gel was added. After 30 min the gel suspension was centrifuged at 10,000 rpm for 5 min in a Sorvall centrifuge, and the gel was washed twice with a total volume of 15 ml of stabilizing buffer. The supernatant and washes were combined yielding a total volume of 86 ml.

Alumina Cγ Gel Fractionation. Alumina Cγ gel (5 g) was added to the combined solution from the previous step. After 20 min, the gel suspension was centrifuged and the gel pellet was washed twice with a total volume of 15 ml of stabilizing buffer. The combined supernatant and washes were concentrated using a 250-ml Amicon concentrator with a PM 10 membrane. When the solution had been concentrated to approximately 10 ml, 40 ml of 2.5 mM phosphate buffer (pH 7.5) containing 60 μM of dTMP and 5 mM mercaptoethanol was added, and again concentrated to 10 ml. This procedure was repeated twice. The volume of the final solution is 10 ml.

DEAE-Sephadex A-50 Fractionation. The concentrated enzyme solution from the previous step was loaded onto a DEAE-Sephadex A-50 column (2 × 5 cm) previously equilibrated with a buffered solution composed of potassium phosphate (2.5 mM, pH 7.5), dTMP (60 μM), and mercaptoethanol (5 mM). The column was washed with 40 ml of the same buffer and the eluent was concentrated to 10 ml.

Zone Sedimentation in Sucrose Density Gradients. Linear 6–15% (w/v) sucrose gradients were prepared according to Martin and Ames (1961). The 12-ml gradients contained Tris-HCl buffer (0.1 M, pH 7.5), 10 mM mercaptoethanol, and 40 μM dTMP. One milliliter of a solution of enzyme or protein marker was added to the top of each gradient. After centrifugation at 5° in a Spinco SW-40 rotor at 39,000 rpm for 40 hr, the bottoms of the tubes were punctured and 16 drop fractions were collected. dTMP-kinase activity and protein concentration were measured as described above.

Comments on Enzyme Purification. The enzyme is precipitated completely with ammonium sulfate between 35 and 55% saturation. Additional subfractionation of the 35–55% ammonium sulfate fraction, even with adjustment of the pH to 7.5 with NaOH, not only did not increase the specific activity but also resulted in poor recovery. Similar observations have been reported (Grav and Smellie, 1965).

The enzyme preparation after the DEAE-Sephadex A-50 fractionation step is devoid of NDP-kinase, nucleoside phosphotransferase, dTMP-phosphatase, ATPase, and thymidine kinase activities. The studies in this paper were carried out with this preparation.

Table I is a summary of the purification procedure. Although only a 14-fold purification is reported, the probability is that a more extensive purification had been obtained which, however, is masked due to the instability of the enzyme thereby providing inactive protein which would affect specific activity. The enzyme was stored at −70° for 1 week without any significant loss of activity.

Results

Stability of the Enzyme. Figure 1 depicts the stability of the enzyme, (E), in the presence of various additives and mercaptoethanol at 37°. When there was no additive, the activity decreased to 50% within 6 min. dTMP stabilizes the enzyme, and full protection against inactivation is observed at a concentration of 40 μM. However, the other substrate, ATP–Mg^{2+}, does not stabilize the enzyme even when present at 6 mM. The enhanced inactivation by ATP–Mg^{2+} could be due to the removal by phosphorylation of the residue 2 μM dTMP present in the control (no additive); however,

TABLE I: Purification of dTMP-kinase.

Fraction	Vol (ml)	Act. (Units/ml)	Protein (mg/ml)	Sp Act. (Units/mg)	Purifcn fold	Recov (%)
I. Extract	65	14	8.2	1.7	1	100
II. Calcium phosphate gel	86	9.5	2.8	3.4	2	90
III. Alumina Cγ gel	10	62	4.4	14	8	68
IV. DEAE-Sephadex A-50	10	49	2.0	24	14	54

it seems more likely that the ATP–E complex or the ATP–Mg^{2+}–E complex is more unstable than that of the native enzyme. A similar observation has been reported (Kielley, 1970).

Both products of the thymidylate kinase reaction, ADP and dTDP, stabilize the enzyme, too. The concentration of either product used was at least a few times above the K_I value. The extent of the enzyme stabilization by ADP and dTDP however is different. The E–dTDP complex appears to be more stable than that of the E–ADP complex. Figure 1 also indicates that at least three of the reactants (ADP, dTDP, dTMP) can bind to the enzyme in the absence of each other.

Triphosphate Nucleotide Specificity. Two concentrations of various nucleoside triphosphates were evaluated as phosphate donors, and Table II shows only ATP and dATP could serve as substrates of dTMP-kinase; GTP, CTP, UTP, dGTP, dCTP, and dTTP could not. The apparent V_{max} for ATP- and dATP–magnesium complex is the same, however, the apparent K_m for ATP–Mg^{2+} is 1.5 mM and that for dATP–Mg^{2+} 1.1 mM.

Effect of the Ratio of ATP to Mg^{2+} on dTMP-Kinase Activity. When the molar concentration of ATP is greater than that of Mg^{2+} an inhibitory effect is observed (Figure 2A). Maximal activity is observed when the ratio of ATP to Mg^{2+} is in the neighborhood of 1. This relationship was verified as shown in Figure 2B. With a fixed concentration of ATP and varying concentrations of Mg^{2+}, the activity followed a sigmoid curve, and the activity in the presence of the lower concentrations of magnesium and 5 mM ATP is less than that observed with 2.5 mM ATP. The relative activities in the presence of these two concentrations of ATP reverse at a concentration of 4 mM Mg^{2+}. At this point the ratio of ATP to Mg^{2+} is 1.2 and 0.6 when the concentration of ATP is 5 and 2.5 mM, respectively. Although it is most likely that the active substrate is ATP–Mg^{2+}, ATP *per se* can still bind to the enzyme and hence when present in excess of the magnesium concentration an inhibition is observed. However, as shown in Figure 2B, an excess of Mg^{2+} relative to ATP does not appear to have an adverse effect on the enzymatic activity.

Effect of Temperature on Enzymatic Reaction. The apparent K_m of dTMP and V_{max} at each temperature shown in Figure 3A,B were obtained from Lineweaver–Burk plots with varying concentrations of dTMP (20–100 μM) and a fixed concentration of ATP–Mg^{2+} (8 mM). The enzyme under the conditions assayed was stable over the incubation period of 10 min with the exception of that studied at 40.5°, at which temperature the inactivation of the enzyme occurred when the dTMP concentration was below 50 μM. In both Figure 3A,B, a linear slope was obtained in the Arrhenius plot when either the log apparent K_m of dTMP as shown in Figure 3A or log V_{max} as shown in Figure 3B was used *vs.* the reciprocal of the absolute temperature. The activation energy was calculated by multiplying the slope by $-2.303R$ (Dixon and Webb, 1964). The activation energy obtained from Figure 3A was calculated to be 10.5 kcal mol^{-1}, and that from Figure 3B 15 kcal mol^{-1}.

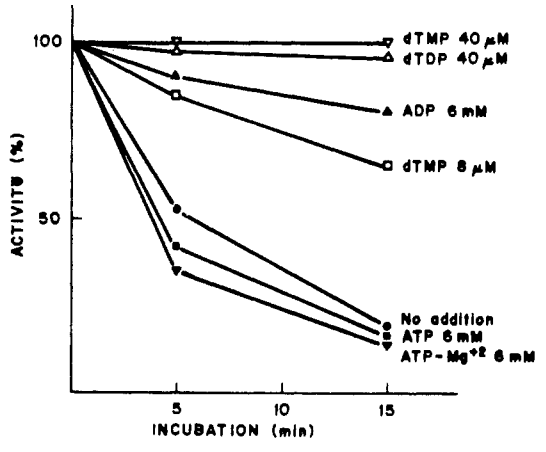


FIGURE 1: Effects of the reaction substrates and products on the stability of dTMP-kinase. The preliminary incubation mixture (0.5 ml) consists of the enzyme (49 units/ml of fraction IV diluted 20-fold) in 0.2 mM Tris-HCl at pH 7.5, 5 mM mercaptoethanol, 1% bovine serum albumin, and the various nucleotides with their concentrations indicated in the figure. After preliminary incubation at 37° for the designated time intervals, 25 μl was withdrawn and assayed for enzymatic activity in a total volume of 0.3-ml reaction mixture as described in the Experimental Section. Since the additives such as ADP or dTDP will have an inhibitory effect, zero time activity was assayed for each curve.

TABLE II: Activity of Various Triphosphate Nucleotides as Phosphate Donors in the dTMP-kinase Reaction.[a]

Compound	dTDP (nmol)/10 min	
	0.5 mM	3 mM
ATP	3.2	8.7
GTP	0.1	0.5
CTP	0.1	0.1
UTP	0.2	0.3
dATP	3.3	9.3
dGTP	0.1	0.5
dCTP	0.2	0.5
dTTP	0	0

[a] The assay was performed under the condition as described in the Experimental Section with the exception that ATP was replaced by the various compounds listed. Two concentrations of triphosphate nucleotides were used: 3 and 0.5 mM. The Mg^{2+} concentration was identical with that of the triphosphate nucleotide.

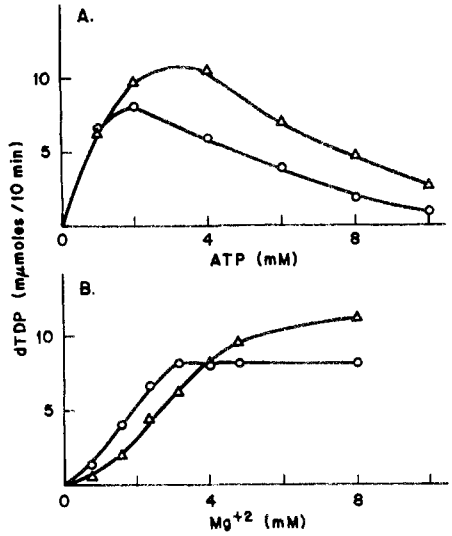


FIGURE 2: Effect of the ratio of ATP to Mg^{2+} on dTMP-kinase activity. The assay is described in the Experimental Section except for the concentration of ATP and Mg^{2+}. In A, △ with 4 mM Mg^{2+}; ○ with 2 mM Mg^{2+}. In B, △ with 5.0 mM ATP; ○ with 2.5 mM ATP.

Estimation of the Molecular Weight of dTMP-Kinase. Partially purified enzyme (fraction IV) was layered onto a 6–15% (w/v) sucrose gradient, and after centrifugation for 40 hr at 39,000 rpm in a Spinco SW-40 rotor the solution was collected dropwise from the bottom of the tube. Figure 4 shows the distribution of protein and dTMP-kinase. A single peak of dTMP-kinase activity was obtained, however, with only 20% recovery of the enzymatic activity. When fractions 9–12 were pooled, the specific activity of the enzyme did not increase presumably because of enzyme inactivation during the centrifugation. The molecular weight estimated by mobility relative to hemoglobin, ovalbumin, and myoglobin in a sucrose density gradient is 33,000.

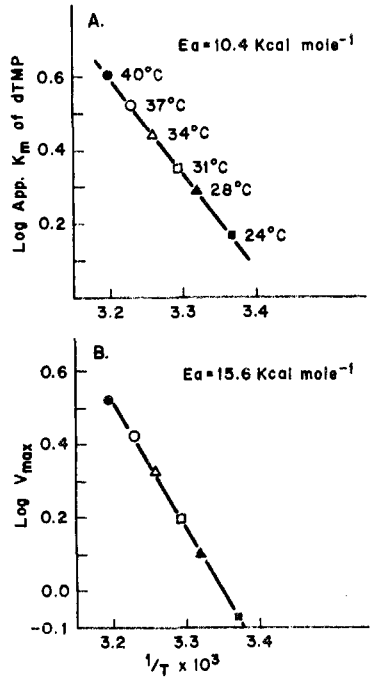


FIGURE 3: Effect of temperature on kinetic parameters of dTMP-kinase. The reaction mixtures contained varying concentrations of dTMP (25–100 μM) and a fixed concentration of ATP–Mg^{2+} (8 mM), and were incubated at the indicated temperature. After temperature equilibration, each reaction was initiated by addition of the enzyme. V_{max} and apparent K_m of dTMP were obtained from Lineweaver–Burk plots at each temperature.

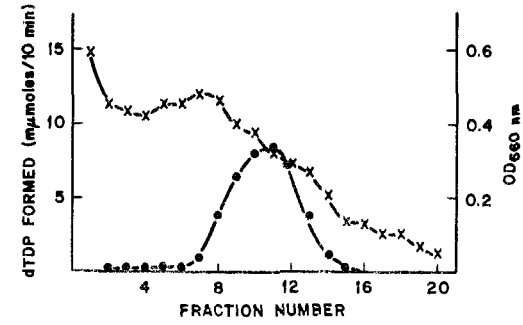


FIGURE 4: Thymidylate kinase activity and protein profile after sucrose density gradient centrifugation. One-half milliliter of enzyme solution (fraction IV, 49 U/ml) was layered on a sucrose gradient (6–14%, w/v) prepared as described in the Experimental Section and centrifuged for 40 hr at 38,000 rpm in a Spinco SW-40 rotor. Fractions (0.6 ml) were collected from the bottom of the tube and analyzed for dTMP-kinase activity and for protein: (×) protein; (●) enzyme activity.

Initial Velocity Analysis. It was found that the reaction followed the Michaelis–Menten equation over the concentration range of dTMP and ATP–Mg^{2+} studied, and no substrate inhibition was observed. ATP–Mg^{2+} is the active substrate of the enzyme and not the ATP alone.

When dTMP was the variable substrate with ATP–Mg^{2+} as the fixed substrate, the Lineweaver–Burk plots as shown in Figure 5 revealed a crossing pattern in which all the lines intersected in a point. Similar patterns were obtained when ATP–Mg^{2+} was the variable substrate with dTMP as the fixed substrate as shown in Figure 6. The results indicate that the reaction followed a sequential mechanism (Cleland, 1963), *i.e.*, both substrates must add to the enzyme before any product is released. The initial velocity is described by: $v = V(A)(B)/[K_{ia}K_b + K_a(B) + K_b(A) + (A)(B)]$ (Friedkin and Malcker, 1961), where A and B could be either dTMP or ATP–Mg^{2+} if the random mechanism applied. The true Michaelis constants (K_a and K_b) of the two substrates were

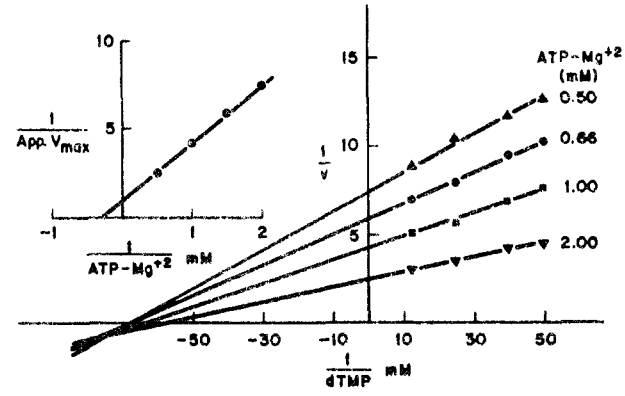


FIGURE 5: Double-reciprocal plot of the effect of ATP–Mg^{2+} on thymidylate kinase activity with dTMP as the variable substrate. The assay mixture contained Tris-HCl buffer (0.1 M, pH 7.5), mercaptoethanol (2 mM), bovine serum albumin (1%), phosphocreatine (3 mM), creatine kinase (1.8 units/ml), and a variable amount of dTMP and ATP–Mg^{2+} as indicated in the figure. The reaction was started by addition of enzyme, and then incubated at 37° for 10 min. Kinetic parameters were estimated from this plot and the replot (left corner).

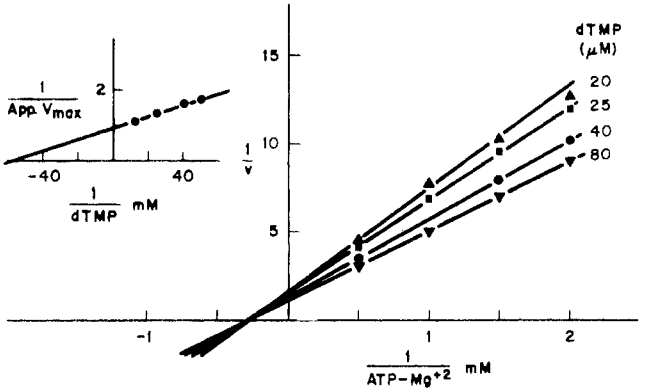


FIGURE 6: Double-reciprocal plot of the effect of dTMP on thymidylate kinase activity with ATP-Mg^{2+} as the variable substrate. The concentration of dTMP and ATP-Mg^{2+} are indicated in the figure. The experimental details were as described in Figure 5.

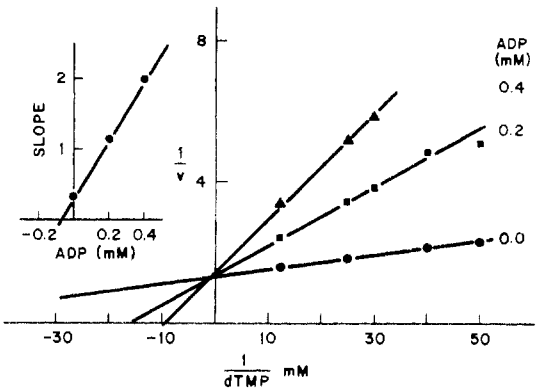


FIGURE 7: Double-reciprocal plot of the effect of ADP, a product inhibitor, on thymidylate kinase activity with dTMP as the variable substrate. The fixed concentration of ATP-Mg^{2+} is 2 mM. The concentrations of dTMP and ADP are indicated in the figure. No ATP regeneration system was used in the reaction mixture. Other experimental details are the same as described in Figure 5. The apparent K_I was obtained by replotting as shown in the left corner of the figure.

determined by replotting as shown in the left corner of Figures 5 and 6. Dissociation constants (K_{ia}; K_{ib}) of the two substrates are those concentrations at which the lines intersect the abscissa (Cleland, 1963). The kinetic parameters are presented in Table III. For the random mechanism, $K_{ia}K_b = K_aK_{ib}$ (Dixon and Webb, 1964) and the experimental data obtained followed this relationship.

Product Inhibition Studies. Several enzyme mechanisms can be distinguished by studies of the effects of reaction products on the initial velocity of the reaction. ADP gave linear competitive inhibition with either dTMP (Figure 7) or ATP-Mg^{2+} as the variable substrate. dTDP also gave linear competitive inhibition with either dTMP (Figure 8) or ATP-Mg^{2+} as the variable substrate. The reaction velocity is given by: $v = V(A)(B)/[K_{ia}K_b(1 + (I)/K_I) + K_a(B) + K_b(A) + (A)(B)]$, where A and B could be either dTMP or ATP-Mg^{2+} and I could be either dTDP or ADP, since $K_{ia} = K_a$ and $K_{ib} = K_b$ as presented in Table III. The apparent inhibition constants were obtained by replotting the slope of each line *vs.* the concentration of the inhibitor. True K_I values (Cheng *et al.*, 1971) were calculated following the equation: apparent $K_I = K_I \times (K_B + (B))/K_B$, where B is the fixed substrate concentration used in each of the studies. True inhibition constants (K_I) of dTDP and ADP are present in Table III.

TABLE III: Kinetic Constants for dTMP-kinase Determined from Initial Velocity and Product Inhibition Studies.

Kinetic Constant	Init Vel	Product Inhibn
K (ATP-Mg^{2+})	3.3 mM	
K_i (ATP-Mg^{2+})	3.3 mM	
K (dTMP)	14.3 μM	
K_i (dTMP)	13.3 μM	
K (ADP)		43.5 μM[a]
		43.5 μM[b]
K (TDP)		7.35 μM[c]
		7.45 μM[d]

[a] Obtained from Figure 7. [b] Obtained from a double-reciprocal plot of the effect of ADP on thymidylate kinase activity with ATP-Mg^{2+} as the variable substrate. [c] Obtained from Figure 8. [d] Obtained from a double-reciprocal plot of the effect of dTDP on thymidylate kinase activity with ATP-Mg^{2+} as the variable substrate.

Inhibitory Effect of Various 5′-Substituted dThd Derivatives on dTMP Kinase Activity. Various concentrations of different 5′-substituted dThd derivatives were added to the reaction mixture and the inhibitory effects on dTMP kinase activity are presented in Figure 9. dTTP, a biological feedback inhibitor of several enzymes (dThd-kinase, dCMP-kinase, ribonucleoside diphosphoreductase) concerned with the biosynthesis of this compound, functions as an inhibitor of dTMP-kinase also. Of significance is the observation, in agreement with the report of Langen and Kowollik (1968), that dThd, the immediate exogenous precursor of dTMP, also exerts a potent inhibitory effect. The relative inhibitory effects of various 5′-substituted derivatives of dThd are in the order of -Cl > -(pyro)triphosphate > -(pyro)diphosphate > $-CO_2H$ > -I = -OH > $-NH_2$.

Stabilizing Effect of Various 5′-Substituted dThd Deriva-

TABLE IV: Stabilizing Effect of Various 5′-Substituted dThd Derivatives on dTMP-kinase.[a]

Addn to the Preliminary Incubn System	dTMP-kinase Act. (%) Remaining after Preliminary Incubn	
	5 min	15 min
Control (no addition)	52	20
dThd	73	45
dTMP	100	99
dTDP	97	96
dTTP	100	98
5′-IdThd	65	33

[a] The incubation system consisted of the enzyme (49 units/ml of stock (fraction IV) enzyme diluted 20-fold) in 0.5 mM Tris-HCl at pH 7.5, 5 mM mercaptoethanol, 1% of bovine serum albumin, and 40 μM of dThd, and the various dThd derivatives. After incubation at 37° for the designed time interval 25 μl was withdrawn for the assay. The other experimental details are described in the Experimental Section. Activities at zero time for the various compounds were assayed separately.

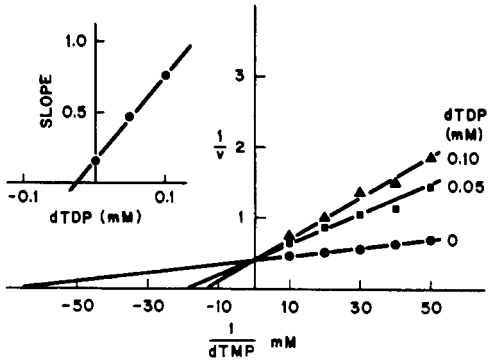


FIGURE 8: Double-reciprocal plot of the effect of dTDP, a product inhibitor, on thymidylate kinase activity with dTMP as the variable substrate. The fixed concentration of ATP-Mg^{2+} is 8 mM. The concentration of dTMP and dTDP are indicated in the figure. No ATP regenerating system was used in the reaction mixture. The other experimental details are the same as described in Figure 5. The apparent K_I was obtained by replotting as shown in the left corner of the figure.

tives on dTMP-Kinase. Table IV shows that dTTP, dThd, and 5′-IdThd also stabilize dTMP-kinase as do dTDP and dTMP. In spite of the fact that the inhibition constants of dThd and 5′-IdThd appear to be the same, 5′-IdThd has a significantly less stabilizing ability than dThd (Table IV).

Inhibition Studies. It is found that the inhibition of dTMP-kinase by dTTP, 5′-CO_2H-dThd and 5′-Cl-dThd are competitive. The K_I values of these derivatives were obtained by replotting the slope and concentration of inhibitor, and the inhibition constants are presented in Table V. These values are apparent inhibition constants with ATP-Mg^{2+} concentration at 8 mM.

Discussion

dTMP-kinase of mouse Sarcoma 180 ascites cells has been purified only 14-fold. However, since there is no ATPase, dTMP-phosphatase, NDP-kinase, thymidine kinase, or nucleoside phosphotransferase activities present in this partially purified preparation, it is possible to sudy some of the properties of the enzyme.

The enzyme derived from mouse Sarcoma 180 ascites cells is very unstable, and similar observations have been reported for the enzyme prepared from other sources. When suitable amounts of dTMP, dTDP, or ADP and mercaptoethanol are present the enzyme can be stabilized albeit to different extents. The report that ADP did not stabilize the enzyme derived from mouse hepatoma (Kielley, 1970) may be due to the use of an inadequate concentration of ADP. A number of studies of the activity of dTMP-kinase have been reported (Adelstein and Kohn, 1967; Bollum and Potter, 1959; Eker, 1968; Fausto and Lancker, 1965; Fiala and Fiala, 1970; Gordon *et al.*, 1968; Kara and Weil, 1967; Labow *et al.*, 1969; Nagano and Mano, 1968; Tanooka *et al.*, 1971; Valotaire and Duval, 1972; Weinstock and Dju, 1971); however, these preparations did not always contain stabilizing reagents, a factor of great importance for such activity studies. Another consideration, that is essential for activity studies using cell homogenates, is the requirement of an ATP regenerating system to compensate for the degradation of ATP by ATPase present in crude preparations. We have observed that the presence of presumably low levels of dTMP-phosphatase in the crude preparation will not significantly interfere in the activity assay, provided a sufficient excess of dTMP is present (unpublished results).

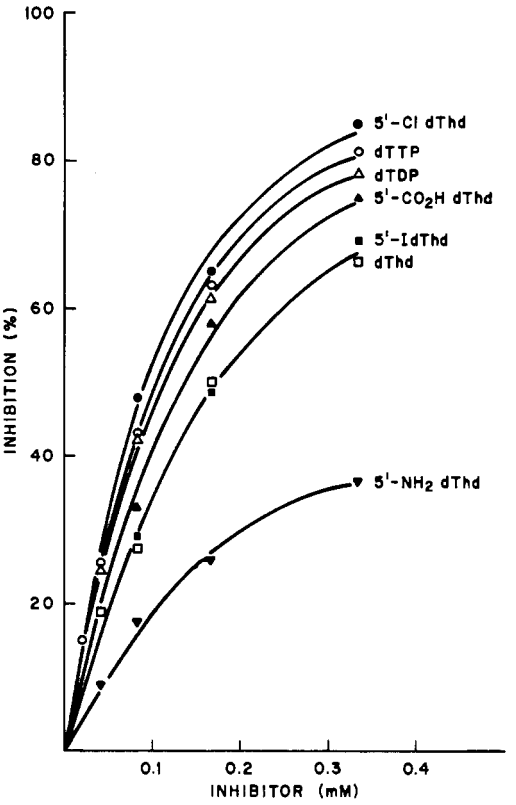


FIGURE 9: Inhibitory effects of various 5′-substituted derivatives of thymidine on dTMP-kinase. The assays were carried out with dTMP (60 μM), ATP-Mg^{2+} (8 mM), and various amounts of 5′-substituted dThd derivatives as indicated in the figure. The other experimental details are the same as described in Figure 5.

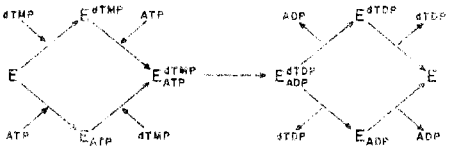
When a high concentration of substrate is used during the assay for enzyme activity, it is important to determine whether a substrate inhibition phenomenon is present. Thus, dTMP-kinase prepared from *E. coli* has been shown to be inhibited when the molar concentration of ATP is in excess of Mg^{2+}; however, this inhibitory effect could be removed by addition of Mg^{2+} to a stoichiometric relationship (Nelson and Carter, 1969). Our studies with dTMP-

TABLE V: Inhibition Constants for dTMP-kinase of Thymidine, Thymidine Nucleotides, and Several 5′-Thymidine Derivatives.[a]

Inhibitor	Inhibn Constant (μM)
dTDP	30
dTTP	27
dThd[b]	50
5′-CldThd	20
5′-CO_2H-dThd	30
5′-NH_2dThd[b]	130

[a] The inhibition constants were obtained from the double-reciprocal plot of the effect of each inhibitor on dTMP-kinase activity with dTMP as the variable substrate and ATP-Mg^{2+} (2 mM) as the fixed substrate. [b] Calculated from the concentration of inhibitor causing 50% inhibition from Figure 9.

SCHEME I



kinase derived from Sarcoma 180 cells indicate that when one maintains a molar Mg^{2+} concentration in excess of ATP, at least over the concentration range studied, then high levels of ATP will not inhibit the enzyme. Similarly Nelson and Carter (1969) observed that an excess of Mg^{2+} relative to ATP resulted in no inhibition. Our results also suggest the free ATP may compete with ATP-Mg^{2+}, presumably the real substrate, and act as an inhibitor.

The specificity of triphosphate nucleotides as a phosphate donor for dTMP phosphorylation by dTMP-kinase prepared from *E. coli* and mouse hepatoma is quite different (Kielley, 1970; Nelson and Carter, 1969). This may be due to the difference in the concentration of the triphosphate nucleotides investigated. In the present study two concentrations of triphosphate nucleotides were evaluated and only ATP and dATP could serve as triphosphate nucleotide substrates. The initial velocity studies indicated that the V_{max} of both ATP and dATP are the same; furthermore, the apparent K_m of ATP-Mg^{2+} and dATP-Mg^{2+} are similar. Consideration of these observations in conjunction with the calculation of the activation energy from the Arrhenius plot of log V_{max} *vs.* $1/T$ being greater than that determined from the plot of log K_m of dTMP *vs.* $1/T$, affords the conclusion that the rate-limiting step in this enzymatic reaction may not be the binding of either substrate to the enzyme (Dixon and Webb, 1964).

A consideration, in this study of the properties of the partially purified enzyme preparation of dTMP-kinase, is whether there is an isozyme pattern present as observed with NDP-kinase (Cheng *et al.*, 1971). Unfortunately subjection of Sarcoma 180 dTMP-kinase to electrophoresis resulted in a complete loss of the enzyme activity. However, consideration of (a) only one symmetrical enzymatic peak is obtained from sucrose density gradient centrifugation; (b) the Arrhenius plots being linear in both plots; and (c) the data from the kinetic study presented suggests that our enzyme preparation has only one species of dTMP-kinase with respect to these properties.

Initial velocity studies have shown that the plots of reciprocal velocity *vs.* reciprocal substrate concentration at various fixed concentrations of the other substrate are linear and intersect at one point. This indicates that the mechanism is sequential, *i.e.*, both substrates must attach to the enzyme before any product is released. However, it is not possible to distinguish whether reactants can add in a random order or must follow an obligatory order of addition from these results. Product inhibition studies can be used to distinguish between various sequential mechanisms. The results indicate competitive inhibition by ADP *vs.* ATP-Mg^{2+} or dTMP, and by dTDP *vs.* ATP-Mg^{2+} or dTMP. These data suggest that the reaction follows a rapid-equilibrium random mechanism. Michaelis constants and dissociation constants of both substrates were determined experimentally. The relationship of $K_{ia}K_b = K_{ib}K_a$ applied, and this further supports the reaction mechanism to be random (Dixon and Webb, 1964) as shown in Scheme I.

The reports concerning the feedback inhibitory effect by dTTP on dTMP-kinase are controversial (Eker, 1968; Ives *et al.*, 1963; Nelson and Carter, 1969) and this may be related to different methods of preparations or source of the enzyme used. Our results indicate that dTTP is a potent competitive feedback inhibitor with an inhibition constant of 27 μM (Table V). An appropriate control experiment has been performed which indicates that the degradation of dTTP to dTDP under our assay conditions is negligible.

dThd was evaluated for a potential inhibitory effect on dTMP-kinase as was reported (Kara and Duschinsky, 1969; Langen and Kowollik, 1968). It was indeed found to be a competitive inhibitor with an inhibition constant of 50 μM (Table V), a concentration that is lower than the level of dThd reported to exert a toxic effect on cells (Bootsma *et al.*, 1964; Cleaver, 1967; Morris and Fischer, 1963).

Cooper and coworkers (1966) have observed with increasing concentrations of dThd that the increase in the dTMP pool derived from dThd was greater than the expansion of the dTDP and dTTP pools a finding that could be due to the inhibition of dTMP-kinase by the high concentration of dThd. It is conceivable that such an inhibition of dTMP-kinase could act as a safeguard by protecting the cell from accumulating an abnormal concentration of dTTP which is a potent feedback inhibitor of many reactions, particularly ribonucleoside diphosphate reductase.

All of the 5′-substituted dThd derivatives evaluated appear to have an inhibitory effect on dTMP kinase although the affinities of these compounds for the enzyme were different. This is consistent with the observations reported by Langen *et al.* (1968, 1969a,b) who studied the inhibitory effect of various 5′-halogenated dThd derivatives on dTMP-kinase. The 5′-F derivative of dThd was reported to be a competitive inhibitor, and we have found the 5′-Cl and 5′-CO_2H derivatives also behaved as competitive inhibitors. It is reasonable to postulate that 5′-I and the 5′-NH_2 derivatives also act in the same manner.

The stability experiment (Table IV) indicates that the high binding affinity of a compound need not always be the one that had a high efficacy in stabilizing the enzyme. It is well established in pharmacology that a marked difference may exist between the "affinity" and the "efficacy" of a drug. Although two substances may interact with an enzyme, a conformational change required to invoke either an inhibition or an activation may or may not be induced or may be partially so. Thus it is not unexpected that the binding constants are not directly related to the probable conformational change required to achieve stability of thymidylate kinase. We have (unpublished results) observed that 5-IdUrd and 5′-I-5-IdUrd also inhibit this enzyme. Chae *et al.* (1972) have reported that several 5-substituted pyrimidines were also inhibitors of dTMP-kinase. It thus appears that many substituents in the 5 or 5′ position of dThd do interfere with the binding of dTMP to the enzyme. An attempt is in progress to develop drugs by modifications of either 5 and/or 5′ position of dThd, to increase the binding affinity as well as their ability to inactivate dTMP-kinase.

Acknowledgment

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Fatty Acid Synthesis by the Liver Perfused with Deuterated and Tritiated Water†

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ABSTRACT: Fatty acid synthesis has been studied in livers of rats perfused with 10% D_2O and with >90% D_2O. The number of deuterium atoms incorporated in 100% D_2O is 22.3 and 24.9 per molecule of newly synthesized palmitate and stearate respectively. The result for palmitate agrees with the result obtained by Jungas with rat adipose tissue under quite different conditions ((1968), *Biochemistry 10*, 3717). Mass spectrometric measurements of deuterium content were used to measure the rate of fatty acid synthesis. The results so obtained agree well with results obtained by measuring tritium incorporation from ^3H_2O. Mass spectrometric examination of fatty acids synthesized in the presence of high concentrations of D_2O provides direct information concerning the extents to which a fatty acid is formed by *de novo* synthesis and by chain elongation of other fatty acids. Stearate is synthesized at about 40% the rate of palmitate. Of the stearate formed about 97% is made by *de novo* synthesis. (An alternative interpretation of the last result is that the chain elongation mechanism uses only palmitate synthesized *de novo* and little or no preexisting palmitate.)

Deuterium oxide was the first labeled precursor to be used for demonstrating the *de novo* synthesis of fatty acids (Rittenberg and Schoenheimer, 1937; Waelsch *et al.*, 1940; Stetten and Boxer, 1944). It was shown that deuterium from D_2O is incorporated into long-chain fatty acids, but this finding was not developed into a quantitatively reliable method for measuring rates of fatty acid synthesis. This application has come into use only recently, except that the use of tritium oxide is now preferred to deuterium oxide (Clark *et al.*, 1968; Fain *et al.*, 1965; Jungas, 1968; Lowenstein, 1971; Windmueller and

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