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Mouse Ascites Sarcoma 180 Deoxythymidine Kinase. General Properties and Inhibition Studies†

Yung-Chi Cheng and William H. Prusoff*

ABSTRACT: Thymidine kinase derived from mouse Sarcoma 180 cells has been partially purified. Although only a ninefold purification has been achieved, the enzyme preparation is devoid of adenosine triphosphatase, deoxythymidine triphosphatase, thymidylate phosphatase, nucleoside diphosphokinase, thymidylate kinase, phosphodiesterase, nucleoside phosphotransferase, and DNA polymerase. The stability of the enzyme decreased as purity increased. Thymidine or rATP partially prevented inactivation; however, mercaptoethanol, in contrast to studies with this enzyme derived from other sources, markedly inactivated the enzyme. When the molar ratio of magnesium to rATP exceeded unity, inhibition of thymidine kinase activity resulted. Various nucleoside triphosphates were evaluated for phosphate donor capabilities and dTTP and 5-iodo- and 5-bromo-2′-deoxyuridine 5′-triphosphate were not only inactive but also prevented utilization of rATP. A comparison of rATP, dATP, and araATP showed no effect of the sugar moiety on their binding ability since the K_m for all three are identical; however, araATP relative to rATP and dATP was a poor phosphate donor. Various 5′ derivatives of thymidine were evaluated as inhibitors of thymidine kinase and the order of inhibition is $NH_2 > Br > H = Cl > F = I > PO_4 > CO_2H = NHCOCH_2Br$. Kinetic analysis showed 5′-amino-, fluoro-, or chlorothymidine to be competitive inhibitors of thymidine, the K_I for 5′-aminothymidine being 3 μM. Although subtle changes in the 2′ position of thymidine alter the binding affinity to the enzyme, considerable bulk tolerance is permitted in the 3′ but not the 5′ position.

Thymidine kinase (dThd-kinase) catalyzes the phosphorylation of dThd[1] to form dTMP in the presence of a nucleoside 5′-triphosphate donor such as ATP and a divalent cation such as Mg^{2+} (Ives *et al.*, 1963; Okazaki and Kornberg, 1964a,b). The activity of dThd-kinase appears in general to be closely related to the proliferative ability of the cell. Thus, the catalytic activity is elevated in regenerating liver (Bresnick *et al.*, 1967), DNA viral infected cells (Kit *et al.*, 1966; Kara and Weil, 1967; Sheinin, 1966), neoplastic tissues (Bresnick and Thompson, 1965; Hashimoto *et al.*, 1972), and cells entering the S phase of the cell cycle (Brent, 1971). Some

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[1] Abbreviations used are: dThd, deoxyribosylthymine (thymidine); dThd-kinase, thymidine kinase; dTMPase, deoxythymidylate phosphatase; aradThd, thymine arabinoside; 3′-NH_2-dThd, 2′-deoxy-3′-aminothymidine.

TABLE I: Purification of Deoxythymidine Kinase.

Fraction	Vol (ml)	Units	Protein (mg/ml)	Sp Act. (Units/mg)
I. Extract	49	27,440	15	37
II. Streptomycin	55	24,640	12	37
III. DEAE-cellulose	61	12,688	3.5	59
IV. Ammonium sulfate	7	8,960	6	213
V. Sephadex G-200	57	6,850	0.4	300
VI. Ammonium sulfate	5	4,500	3	320

properties of this enzyme have been studied with partially purified enzymes from a number of sources such as *Escherichia coli* (Okazaki and Kornberg, 1964a,b; Iwatsuki and Okazaki, 1967; Voytek *et al.*, 1971; Rohde and Lezius, 1971), Yoshida sarcoma (Hashimoto *et al.*, 1972), Walker carcinoma (Bresnick and Thompson, 1965), mouse embryo cells infected with polyoma virus (Sheinin, 1966), calf thymus (Her and Momparler, 1971), mitochondria of LA9 and LMTK$^-$ cells (Berk and Clayton, 1973), etc. The enzymic properties appear to vary dramatically with the source of origin. For instance, dThd-kinase from *E. coli* appears to be an allosteric protein involving protomer interactions as well as activation by dCDP (Okazaki and Kornberg, 1964a,b; Iwatsuki and Okazaki, 1967; Cysyk and Prusoff, 1972) or dCTP (Voytek *et al.*, 1971) and inhibition by dTTP (Okazaki and Kornberg, 1964a,b; Iwatsuki and Okazaki, 1967; Cysyk and Prusoff, 1972) through dimerization of the enzyme. However, the enzyme derived from calf thymus catalyzes the reaction following Michaelis–Menten type kinetics; furthermore, it is inhibited by dTTP but is not activated by deoxyribonucleotide derivatives of cytosine. The mode of inhibition of dThd-kinase by dTTP with respect to dThd is competitive with the enzyme derived from calf thymus (Her and Momparler, 1971), is noncompeitive with that derived from the Walker tumor (Bresnick and Thompson, 1965), and is either competitive or complex with the Ehrlich ascites enzyme depending on pH (Prusoff and Chang, 1970). In addition, there are other differences in kinetic properties, molecular weight (Bresnick and Thompson, 1965; Bresnick *et al.*, 1966; Toide *et al.*, 1970; Her and Momparler, 1971), etc. Thus, it has been difficult to generalize the properties of this enzyme.

As part of a program concerned with the design of drugs that either inhibit enzymes concerned with thymidine metabolism or sensitize them to radiation inactivation, an understanding of the properties of these enzymes is essential. Some properties of dTMP-kinase derived from mouse ascites sarcoma 180 cells have been reported (Cheng and Prusoff, 1973a). This article will present some characteristics of a partially purified dThd-kinase derived from mouse ascites sarcoma 180 cells. In an attempt to understand the molecular substituents required for interaction at the active site of the enzyme, because such knowledge is important in designing active site directed drugs, a number of thymidine analogs with substitution by various groups either on the pyrimidine moiety or the 2′-, 3′-, and 5′-carbons of the sugar moiety were investigated as potential inhibitors of the dThd-kinase catalyzed reaction.

Experimental Section

Materials. [^{14}C]dThd was obtained from New England Nuclear, Inc. DE52 cellulose and DE81 chromatography paper were purchased from Whatman; ara-dThd was kindly provided by Ms. Iris Wempen. Dr. P. Langen generously provided our initial supply of 5′-F-dThd, and this plus all other 5′-substituent analogs of dThd were kindly provided by Drs. J. P. Neenan and W. Rohde. 3′-NH_2-dThd and dThd-3′-(*p*-acetamidophenyl phosphate) were generously provided by Dr. Glinski. Other nucleoside or nucleotide derivatives were purchased from P-L Biochemicals, Inc. All the reagents used were reagent grade.

Assay Procedures. The regular reaction mixture contained in a 0.1 ml final volume: Tris-HCl buffer (0.12 M) at pH 7.8, ATP (3 mM), $MgCl_2$ (3 mM), [^{14}C]dThd (1.5 mM; 25 μCi/mmol) bovine serum albumin (1%), phosphocreatine (3 mM), creatine kinase (0.54 unit), and a preparation of dThd-kinase (not more than 150 units). The mixture was incubated at 37° for 10 min in our standard assay. The conversion of dThd to dTMP was measured as described previously (Voytek *et al.*, 1971). Proteins were determined according to Lowry *et al.* (1951). One unit of dThd kinase activity is defined as the amount of enzyme catalyzing the formation of 1 nmol of dTMP from dThd per 10 min at 37°. The reaction rate is linear, when a partially purified enzyme preparation after step IV (Table I) is used, even after one-third of dThd has been converted into dTMP.

Purification Procedure. Sarcoma 180 ascites cells were harvested 4 days after inoculation of female CD-1 mice obtained from Charles River. The ascitic fluid from about 50 mice was pooled and the procedure for washing the cells has been described previously (Cheng and Prusoff, 1973a). All the procedures were performed at 4°.

Extraction. To 25 ml of washed cell pellets, 25 ml of ice-cold buffer (Tris-HCl, pH 7.8, 50 mM) was added. The mixture was 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 600*g* for 10 min in a Sorvall centrifuge and the pellets were washed with an additional 20 ml of the Tris-HCl buffer. The combined supernatant fluid and wash were centrifuged at 39,000 rpm for 1 hr in a Spinco 50-Ti rotor and the supernatant fraction was collected.

Streptomycin Treatment. A 10% streptomycin sulfate solution was prepared in 20 mM Tris-HCl buffer, and the pH was adjusted to 7.8 with 1 M KOH. Six milliliters were added dropwise to 49 ml of the 100,000*g* supernatant fraction over a period of 30 min with stirring. The supernatant fraction was collected after centrifugation.

DE52 Fractionation. The supernatant (55 ml) was loaded onto a DEAE-cellulose column (3 × 15 cm) previously equilibrated with Tris-HCl buffer (pH 7.8, 20 mM). The column was washed with 50 ml of the same buffer, and the eluent which contained the enzyme activity was pooled.

Ammonium Sulfate Fractionation. Ammonium sulfate (21 g/100 ml) was added slowly to the above pooled solution, and the pH was maintained at pH 7.8 by the addition of 1 M KOH. The solution was stirred for 2 hr. The formed precipitate was collected by centrifugation and dissolved in 7.5 ml of stabilizing buffer (Tris-HCl, 20 mM, pH 7.5; 90 μM dThd; 10% glycerol). The solution was dialyzed overnight against the same buffer, and the insoluble material formed was removed by centrifugation.

G-200 Column Chromatography. Portions (2.5 ml) of the supernatant fraction from the previous step were dialyzed against the stabilizing buffer supplemented with 0.1 M KCl for 4 hr before being loaded onto a G-200 column (3 × 40 cm) equilibrated previously with the KCl modified stabilizing

buffer. The enzyme appeared in the void volume, and the active fractions were pooled.

Ammonium Sulfate Reprecipitation. Ammonium sulfate was added to the above pooled solution (21 g/100 ml). The formed precipitate was collected, dissolved in 5 ml of stabilizing buffer, and dialyzed overnight at 4° against the same buffer.

Results

Comments on Enzyme Purification. Table I is a summary of the purification procedure. There is approximately a ninefold increase in specific activity. The extent of purification may indeed be higher but is masked due to the instability of the enzyme during the purification. The enzyme (step VI) will lose about 30% activity when stored at −20° in the presence of 100 μM dThd within a period of a week. The enzyme preparation after the second ammonium sulfate precipitation (step VI) is devoid of ATPase, dTTPase, dTMPase, NDP-kinase, dTMP-kinase, phosphodiesterase, nucleoside phosphotransferase, and DNA polymerase, and the studies in this paper, unless specified, were performed with this preparation.

The DE52 step is useful in purifying the enzyme only when used in the first few steps; otherwise, the enzyme is inactivated by passage through the column. This is consistent with observations of others (Bresnick and Thompson, 1965; Her and Momparler, 1971). When the preparation after streptomycin treatment was used for this step, about 20–25% of the activity was always retarded on the column, and could be eluted with a solution of KCl (0.3 M) and Tris-HCl buffer (pH 7.8, 0.02 M). The properties of this fraction are under investigation. This usually is related to the presence of more than one form of dThd-kinase in the cells (Taylor *et al.*, 1972; Berk and Clayton, 1973; Hashimoto *et al.*, 1972). In this communication the properties of only the major enzyme fraction which is eluted in the void volume of DE52 are discussed.

When fraction IV (Table I) was subjected to polyacrylamide gel electrophoresis by the method previously described (Voytek *et al.*, 1971), only a single peak of activity was observed, and this was confined to near or at the origin.

When the concentration of KCl of the elution buffer is increased to 1 M in the Sephadex G-200 column chromatography step, there is no shift of the position in which the enzyme is eluted. This finding is different from that observed with the enzyme prepared from Walker carcinoma cells (Bresnick *et al.*, 1966). Furthermore, the enzyme is not activated by phospholipase C as described for rat and human intestinal tissues (Salser and Balis, 1973). The recovery of the enzyme activity from the Sephadex G-200 column is lower with 1 M KCl as the elution buffer relative to that obtained with 0.1 M KCl. This is consistent with the observation of Rohde and Lezius (1971).

The electrofocusing technique was not useful for purifying this enzyme because the enzyme became inactivated during this procedure. Affinity chromatography used for purifying dThd-kinase from *E. coli* (Rohde and Lezius, 1971) was not successful for possible reasons that will be explained later.

Stability of the Enzyme. The crude preparation of the enzyme is reasonably stable in the absence of any additive below 4°. About 50% of the enzymic activity is lost within a period of 50 hr at 4°. However, when 2-mercaptoethanol (5 mM) is added, as others have done during purification of this enzyme from other sources (Okazaki and Kornberg, 1964a; Salser and Balis, 1973; Brent, 1971; Taylor *et al.*, 1972; Her and Momparler, 1971), the rate of inactivation increased. With increasing purity of the enzyme, the stability decreased; however, dThd (100 μM) will decrease the rate of inactivation.

TABLE II: Stability of Deoxythymidine Kinase in the Presence of Various Agents at 37°.[a]

Additive	% Orig Act. for Incubation Period (min)			
	10	20	30	50
None	31	16	13	7
Glycerol, 10%	39	22	16	9
EDTA, 0.3 mM	36		14	6
Mg^{2+}, 1.3 mM	42	28	20	12
Mercaptoethanol, 5 mM	4	1	0	0
dThd, 66 μM	48	42	38	30
Mercaptoethanol, 5 mM plus dThd, 66 μM	22	17	3	1
ATP, 1.3 mM plus EDTA, 0.3 mM	65	61	55	43
ATP, 1.3 mM plus Mg^{2+}, 1.3 mM	64	59	57	
Glycerol, 10% plus dThd, 66 μM	50	38		28

[a] The enzyme was preincubated under various conditions at 37°. The activity of the enzyme was assayed at each time interval by the standard assay procedure described in the Experimental Section.

The effect of various reagents was investigated on the stability of the purified enzyme when incubated at 37° for the indicated periods of time (Table II). The concentrations of ATP or dThd are at least sevenfold above their respective K_m values. The enzyme is partially stabilized by either ATP, dThd, or Mg^{2+}. The stabilizing effect of ATP is not dependent upon the presence of Mg^{2+} and is greater than that produced by dThd. The increased rate of inactivation in the presence of 5 mM 2-mercaptoethanol can be partially decreased by dThd. Glycerol (10%) may have exerted a small protection since these values are reproducible; however, in the presence of dThd no apparent effect was produced by glycerol. EDTA does not exert any significant effect on the stability of this enzyme. Since the enzyme preparation from Yoshida sarcoma forms a complex with RNA (Toide *et al.*, 1970), RNase or DNase was incubated with our enzyme preparation; however, neither activation, inactivation, nor alteration in enzyme stability was observed.

Effect of Mg^{2+} on Enzyme Activity. Since the enzyme stability is affected by either Mg^{2+}, ATP, or ATP–Mg^{2+}, the role of Mg^{2+} on the enzyme catalysis may be complex. Figure 1 shows the effect of varying concentrations of Mg^{2+}, with a fixed ATP concentration, on dThd-kinase activity. There is a definite requirement for Mg^{2+}; however, it will exert an inhibitory effect when the molar ratio of Mg^{2+} to ATP exceeds 1. This inhibitory effect is ATP dependent. As long as the molar concentration of Mg^{2+} does not exceed the molar concentration of ATP in the assay mixture, Mg^{2+} will not exert an inhibitory effect on the enzymatic reaction. When the concentration of Mg^{2+} is fixed but that of ATP varied, then no inhibition by ATP is observed even when the molar ratio of ATP to Mg^{2+} is as high as 25:1.

Specificity of the Triphosphate Nucleotide. Table III demonstrates that all the triphosphate nucleotides investigated, ex-

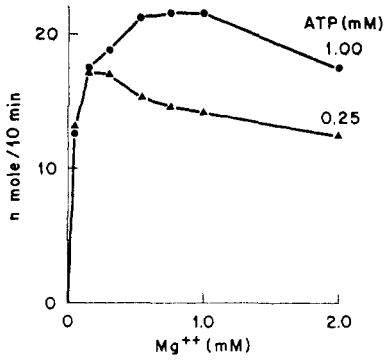

FIGURE 1: Effect of Mg^{2+} on dThd-kinase activity. The assay is described in the Experimental Section except for the concentrations of ATP and Mg^{2+} which are indicated in the figure.

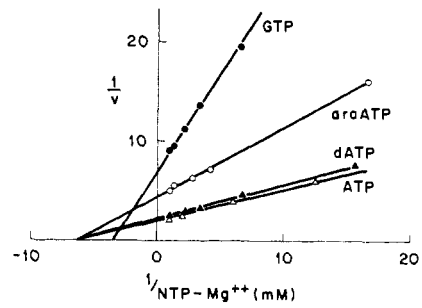

FIGURE 2: Double reciprocal plot of the dThd-kinase reaction rate *vs.* concentrations of various triphosphate nucleotides. The fixed concentration of dThd is 2 mM. The concentrations of various triphosphate nucleotides are indicated in the figure. Other experimental details are the same as described in the Experimental Section.

cept dTTP, I-dUTP, and Br-dUTP, serve as a phosphate donor in the dThd-kinase catalyzed reaction. The reaction rate catalyzed in the presence of the different nucleoside triphosphates varied regardless of concentration investigated. Various di- or monophosphate nucleotides studied were not able to serve as a phosphate donor. ATP and dATP were the most efficient phosphate donors. Kinetic studies shown in Figure 3B reveal that a concentration of ATP below 0.2 mM does not follow the simple Michaelis–Menten type kinetics. Figure 2 depicts a double reciprocal plot of ATP (0.2–1 mM) as the varying substrate and dThd as the fixed substrate and a linear line was obtained. The K_m of ATP calculated from this figure is 0.15 mM. The kinetic relationships observed when an equal amount of enzyme is used but with either dATP, araATP, or GTP replacing ATP as the phosphate donor, are also presented in Figure 2. A linear relationship of the double reciprocal plot is obtained in each case. dATP and araATP have the same K_m as ATP; however, the V_{max} of araATP is only half that of either ATP or dATP. The K_m of GTP is 0.30 mM which is higher than that of the other three nucleoside triphosphates tested, and the V_{max} is only 30% of that observed with ATP or dATP.

TABLE III: Activity of Various Triphosphate Nucleotides as Phosphate Donors in the Deoxythymidine Kinase Reaction.[a]

Compd	dTMP (nmol)/10 min	
	0.5 mM	1.0 mM
ATP	680	725
dATP	640	725
ara-ATP	320	400
GTP	114	164
dGTP	104	157
CTP	102	198
dCTP	92	97
UTP	70	135
dTTP	0	0
I-dUTP	0	0
Br-dUTP	0	0

[a] The assay was performed under the conditions as described in the Experimental Section with the exception that ATP was replaced by the various compounds listed. The Mg^{2+} concentration was identical with that of the triphosphate nucleotides. Two concentrations of triphosphate nucleotides were used: 1.0 and 0.5 mM.

Effect of dTTP. The effect of various triphosphate nucleotides on the phosphorylation of dThd by ATP is shown in Table IV. Since many of the triphosphate nucleotides, except for dTTP, BrdUTP, and IdUTP, serve as an alternative substrate but have different K_m and V_{max} values, it is not unexpected that some inhibition is observed. dTTP, I-dUTP, and Br-dUTP are not substitute phosphate donors, but completely inhibit the reaction. The type of inhibition dTTP exerts is demonstrated in Figures 3A and 4. Figure 3A indicates that dTTP will enlarge the sigmoidal nature of the curve when velocity *vs.* concentration of ATP is plotted with a fixed amount of dThd. When the ATP concentration is high, the inhibition caused by dTTP is prevented. Our unpublished data suggest that dTTP will compete with ATP and behave as a competitive inhibitor. dTTP acts as a noncompetitive inhibitor when dThd is the varied substrate and ATP is the fixed substrate as shown in Figure 4. The K_m of dTTP based on the data in Figure 4 in the presence or absence of dTTP (8.5 μM) is estimated to be 6 μM. The K_m of dThd is 9 μM.

Effect of the Thymidine Derivatives. Various 5′ derivatives of dThd were evaluated as potential inhibitors of dThd-kinase activity. The results using two different molar ratios of analog to thymidine are shown in Table V. The effect of these 5′ substitutions in the order of increasing inhibition is NH_2 > Br > H = Cl > F = I > PO_4 > CO_2H or $NHCOCH_2Br$.

TABLE IV: Effect of Various Triphosphate Nucleotides on Deoxythymidine Kinase Catalysis.[a]

Additive	% dTMP Formed
	100
ATP	108
dATP	105
ara-ATP	70
GTP	86
dGTP	75
CTP	95
dCTP	83
UTP	94
TTP	2.5
I-dUTP	2.3

[a] The assay was performed under the conditions described in the Experimental Section with the exception that ATP was 2 mM and the various additives were 1.8 mM. The Mg^{2+} concentration was identical with that of the triphosphate nucleotides.

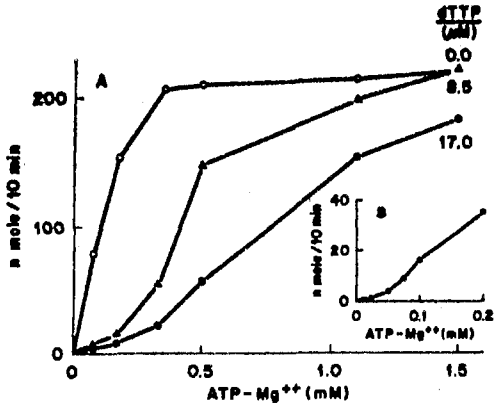

FIGURE 3: The effect of dTTP on dThd-kinase activity with ATP-Mg^{2+} as the variable substrate. The fixed concentration of dThd is 2 mM. The concentrations of dTTP and ATP-Mg^{2+} are indicated in the figure. Other experimental details are the same as described in the Experimental Section.

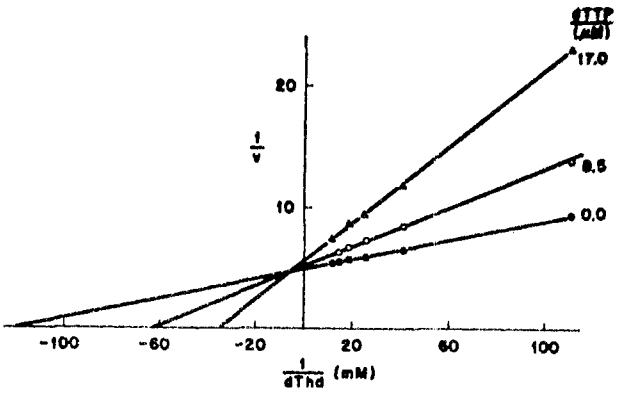

FIGURE 4: Double reciprocal plot of the effect of dTTP on dThd-kinase activity with dThd as the variable substrate. The fixed concentration of ATP-Mg^{2+} is 2 mM. The concentrations of dTTP and dThd are indicated in the figure. Other experimental details are the same as described in the Experimental Section.

A kinetic analysis was performed only with the thymidine derivatives substituted in the 5′ position with NH_2, F, or Cl. They all behaved as competitive inhibitors with respect to dThd. Figure 5 depicts the data obtained with 5′-NH_2-dThd at varying concentrations of dThd and a fixed concentration of ATP-Mg^{2+}. Table VI shows the inhibition constants (K_I) of several 5′-thymidine derivatives. The binding affinity of 5′-NH_2-dThd (K_I = 3 μM) to dThd-kinase is threefold larger than that of dThd (K_m = 9 μM). Preincubation of the enzyme with the various 5′ derivatives showed only dThd-5′-NHCO-CH_2Br and 5′-I-dThd to enhance the inactivation of the enzyme; the other compounds behaved only as reversible inhibitors.

The results of a study of the effect of several analogs of dThd with a free 5′-OH group on dTMP formation from dThd are presented in Table VII. Compounds such as I-dUrd, CF_3-dUrd, F-dUrd, and 6-aza-dThd act as alternative substrates in competing with dThd. No attempt has been made to determine the type of inhibition exerted by the other compounds listed in Table VII. Preincubation experiments have demonstrated that these compounds do not inactivate thymidine kinase.

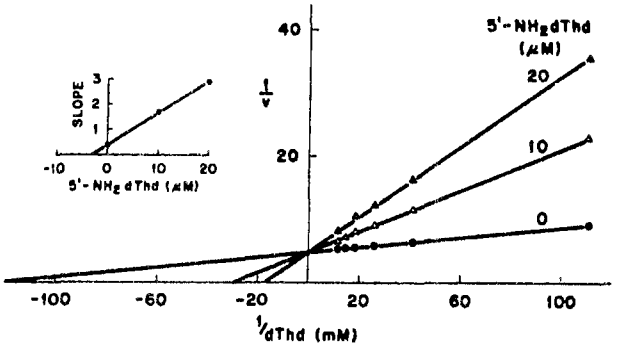

FIGURE 5: Double reciprocal plot of the effect of 5′-NH_2-dThd on dThd-kinase activity with dThd as the variable substrate. The fixed concentration of ATP-Mg^{2+} is 2 mM. The concentrations of dThd and 5′-NH_2-dThd are indicated in the figure. Other experimental details are the same as described in the Experimental Section.

TABLE V: Inhibition of Deoxythymidine Kinase by Various 5′ Derivatives of Thymidine.[a]

5′ Substituent	% Inhibn for Molar Ratio of Analog 1.3	2.5
NH_2	68	84
$NHCOCH_2Br$	0	7
CO_2H	2	5
H	17	28
F	9	16
Cl	16	25
Br	29	53
I	8	17
PO_4H	6	14
p-Bromoacetamidophenyl phosphate	3	17

[a] The assays were carried out with dThd (0.2 mM), ATP-Mg^{2+} (2 mM), and two concentrations of 5′-substituted dThd derivatives as indicated in the table. The other experimental details are described in the Experimental Section.

Discussion

When crude preparations of dThd-kinase from mouse ascites sarcoma 180 tumor cells were loaded onto a DEAE column, 50% of the activity appeared in the void volume and about 20% of the activity was retained on the column. This latter enzymic activity could be eluted with a high salt elution

TABLE VI: Inhibition Constants for Deoxythymidine Kinase of Several 5′-Thymidine Derivatives.[a]

Inhibitor	Inhibition Constant (μM)
dTTP[b]	6
5′-F-dThd	120
5′-Cl-dThd	90
5′-Br-dThd	27
5′-I-dThd[c]	120
5′-NH_2-dThd	3

[a] The inhibition constants were obtained from the double reciprocal plot of the effect of each inhibitor on dThd-kinase activity with dThd as the variable substrate and ATP-Mg^{2+} (2 mM) as the fixed substrate. [b] Obtained from Figure 4. [c] Calculated from Table V.

TABLE VII: Effect of the Various Analogs of Thymidine on dThd-Kinase Catalysis.[a]

Compound (750 μM)	% Inhibition
5-I-dUrd	81
5-CF_3-dUrd	80
5-F-dUrd	48
5-NH_2-dUrd	12
5-$NHCOCH_2Cl$-dUrd	7
5-$NHCH_3$-dUrd	19
5,6-Dihydro-dThd	20
N-3-Me-I-dUrd	20
6-Aza-dThd	41
Thymine ribonucleoside	12
Thymine arabinoside	8
Thymidine 3′-phosphate	20
Thymidine 3′-acetamidophenylphosphate	21
Thymidine 3′-NH_2	45
2′,3′-Dideoxy-dThd	31

[a] The experiments were performed as described in Table V except the concentration of the compound tested is 0.75 mM.

buffer. Since multiple species of dThd-kinase were indicated to exist in a number of mammalian sources, such as Yoshida sarcoma (Hashimoto *et al.*, 1972) and human fetal tissue (Taylor *et al.*, 1972), it is important to point out that only the nonadsorbed enzyme fraction (being the major fraction) from the DEAE column was used for further purification and characterization. Although the enzyme was purified only ninefold, those enzymes which could interfere with these studies had been removed.

A number of laboratories have used mercaptoethanol routinely for stabilizing this enzyme prepared from various sources (Okazaki and Kornberg, 1964a; Salser and Balis, 1973; Brent, 1971; Taylor *et al.*, 1972; Her and Momparler, 1971). However, we have found that mercaptoethanol (5 mM) either at 4 or 37° enchanced the inactivation of the enzyme derived from mouse S-180 tumor cells. An involvement of a disulfide bridge in the maintenance of the enzyme activity is indicated. This enhancement of inactivation by mercaptoethanol could be partially reversed by dThd, which seems to bind to the enzyme in the absence of the other substrate (ATP). ATP or ATP-Mg^{2+} can also bind to the enzyme in the absence of dThd.

The catalytic reaction requires Mg^{2+}; however, the role of Mg^{2+} in this reaction is not clear. Ives *et al.* (1963) have observed an inhibition of the reaction by a high concentration of Mg^{2+}, which they attributed to an activation of dTMPase present in their preparation derived from Novikoff hepatoma cells (Ives *et al.*, 1963); however, there is no dTMPase activity in our preparation. We have observed that the inhibition by high concentrations of Mg^{2+} could be reversed by ATP which presumably chelates the Mg^{2+} ions. The observation that Mg^{2+} alone can stabilize the mouse sarcoma 180 enzyme against heat inactivation, as well as decrease the ultraviolet (uv) sensitivity of *E. coli* dThd-kinase (Cysyk and Prusoff, 1972), suggests that Mg^{2+} can exert an effect directly on the enzyme. Thus, the requirement for Mg^{2+} may be complex. Although the formation of the Mg^{2+}-ATP complex in a 1:1 molar ratio is required for optimal activity, Mg^{2+} is not required for ATP to exert a maximal stabilization of the mouse sarcoma 180 dThd-kinase against heat inactivation.

The enzyme from *E. coli* is an allosteric enzyme (Okazaki and Kornberg, 1964b). The enzyme derived from various mammalian cells follows Michaelis–Menten type kinetics with respect to ATP (Bresnick *et al.*, 1966; Her and Momparler, 1971). Within the concentration range of 0.2–2 mM ATP, the reaction velocities of mouse S180 dThd-kinase follow the simple Michaelis–Menten equation (Figure 2). However, when the concentration of ATP is below 0.2 mM, a sigmoid curve is resolved when the velocity is plotted against the concentration of ATP (Figure 3B). The sigmoid nature of the curve is enlarged in the presence of the feedback inhibitor dTTP, and ATP prevents completely the inhibition caused by dTTP. The binding affinity of dTTP to the enzyme is about 40 times higher than that of ATP ($K_m^{ATP}/K_I^{dTTP} \approx 40$). dTTP will behave as a noncompetitive inhibitor when dThd is the varied substrate and ATP the fixed substrate. These observations are similar to those made by Bresnick *et al.* (1966), but different from that of Her and Momparler (1971) who observed competitive inhibition. One possible explanation for such a difference is that when the ratio of ATP to dTTP is high, ATP will saturate the high affinity binding site (allosteric site) where dThd does not bind. dTTP will then only affect the catalytic site where dThd can compete with dTTP for the binding site. Therefore, with different ratios of ATP to dTTP, the type of inhibition exerted by dTTP with respect to dThd may vary.

The enzyme can use only a triphosphate nucleotide as the phosphate donor. In agreement with other studies of mammalian enzymes, ATP and dATP were most active and equivalent as phosphate donors; however, ATP was more active than dATP for the *E. coli* enzyme (Okazaki and Kornberg, 1964b). Except for dTTP, I-dUTP, and Br-dUTP, the other nucleoside triphosphates studied all served as phosphate donors, however, with a lower V_{max} and higher K_m as demonstrated with GTP (Figure 2). ara-ATP can also serve as a phosphate donor, but with a lower V_{max} and an equivalent K_m relative to ATP or dATP. Thus, the nucleotide base appears to be more critical than the sugar moiety for binding of the triphosphate nucleotide to the enzyme, since the K_m values for ATP, dATP, and ara-ATP are identical. The sugar moiety of these triphosphate nucleotides, however, may have a greater determining effect on the transphosphorylation process.

Several 5′ derivatives of dThd are good reversible inhibitors of dTMP-kinase derived from mouse sarcoma 180 (Cheng and Prusoff, 1973a). Thus it was of interest to determine what effect they have on dThd-kinase derived from the same source. 5′-NH_2-dThd, a poor inhibitor of dTMP-kinase, was found to be a good inhibitor of dThd-kinase. The order of the inhibitory effect of each compound is similar to the I_{50} value reported by Neenan and Rohde (1973) for dThd-kinase derived from Walker 256 tumor cells. However, it is completely different from the order of inhibition of dTMP-kinase. Thus, 5′NH_2-dThd is at least 30-fold better than 5′-Cl-dThd as an inhibitor of dThd-kinase, whereas 5′-Cl-dThd is 6-fold better than 5′-NH_2-dThd as an inhibitor of dTMP-kinase. The sequence of 5′-halogenated derivatives of dThd as inhibitors of both enzymes is also different. For dTMP-kinase F > Cl > Br > I, whereas for dThd-kinase Br > Cl > F ≈ I. The effect on dThd-kinase could be due to a balance between a steric effect and an electronegative effect of the 5′ substituents. The bulk tolerance of the enzyme at this position seems to be poor (Baker and Neenan, 1972). This may explain in part why affinity chromatography with thymidine binding to the matrix at the 5′ position did not work for our preparation of sarcoma 180 dThd-kinase.

Since thymidine is phosphorylated at the 5′-OH position by dThd-kinase, a thymidine analog with a free 5′-OH group may act as an alternative substrate and hence behave as a competitive inhibitor in the dThd-kinase phosphorylation reaction. I-dUrd, Br-dUrd, F-dUrd, CF_3-dUrd, and 6-aza-dThd indeed act in this manner (Bresnick and Thompson, 1959; Bresnick and Williams, 1967; Heidelberger *et al.*, 1965; Okazaki and Kornberg, 1964a). However, this is not always true since *N*-3-Me-I-dUrd was found to act as an uncompetitive inhibitor of dThd-kinase derived from *E. coli* (Voytek *et al.*, 1972).

The binding of various compounds to dThd-kinase was investigated (Table VII). With the concentration of inhibitor and substrate used, a compound will show 50% inhibition with a K_I of about 40 μM if it behaves as a competitive inhibitor and with a K_I of about 750 μM if it behaves as either a noncompetitive or an uncompetitive inhibitor. The basis for these calculations has been reported (Cheng and Prusoff, 1973b). It is quite clear from the data in Table VII that a subtle change of the thymidine molecule will result in a dramatic change in its affinity to the enzyme. Thus, merely adding a hydroxyl group either cis or trans to the 3′-OH in the thymidine molecule (thymine ribonucleoside; thymine arabinoside) results in loss of almost all the binding affinity to the enzyme. Phosphorylation has been shown to be essential for many nucleosides such as I-dUrd or CF_3-dUrd in order for them to exert their biological effect. Thus, it is not surprising that thymine riboside or thymine arabinoside does not have significant pharmacological activity on neoplastic growth, because they appear to interact poorly if at all with thymidine kinase.

A substitution of the 5-methyl group on the pyrimidine moiety of dThd by NH_2 also results in a loss of considerable binding potential. The binding affinities of thymidine 3′-phosphate and thymidine 3′-(*p*-acetamidophenyl phosphate) were similar, although both have less binding affinity relative to dThd. Thus, the 3′ position of the sugar moiety of dThd allows more tolerance to bulk than the 5′ position and hence may afford a more successful preparation of an affinity column for purifying the sarcoma 180 thymidine kinase by attachment of the 3′ position to the matrix. This does not preclude the successful use of a column to which a thymidine analog is attached by the 5′ position for purification of enzymes derived from other sources.

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