PHOSPHATE DONOR SPECIFICITY AND SOME KINETIC PROPERTIES OF THYMIDINE KINASE PURIFIED FROM REGENERATING RAT LIVER BY A PROCEDURE WHICH INCLUDED AFFINITY-GEL CHROMATOGRAPHY

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An 8-step purification procedure was used to attain a 3,020-fold purification of dThd kinase from the liver of rats that had been partially hepatectomized 36 hr earlier. The procedure included the use of affinity chromatography on Sepharose columns that had been covalently linked to dThd-PP-hexanolamine. The dThd kinase preparation was not electrophoretically homogeneous; it gave 5 bands stainable with Coomassie Blue. The purified enzyme preparation utilized Ado-PPP or dAdo-PPP as phosphate donor, but reaction velocity was higher with Ado-PPP; Urd-PPP also supported phosphorylation but rates were very low. Phosphate transfer from either nucleoside triphosphate was noncompetitively inhibited by dThd-PPP or dCyd-PPP. The extent of inhibition by d-Thd-PPP was greater when the phosphate donor was Ado-PPP.**

INTRODUCTION

Thymidine kinase (E.C. 2.7.1.75) catalyzes the transfer of phosphate from Ado-PPP to dThd to form dThd-P and Ado-PP. The enzyme is inhibited by dThd-PPP (1-15). This inhibition is relieved by either substrate molecule, but the nature of the reversal is different for each substrate molecule and for enzymes from different sources. For instance, with the enzyme from *Escherichia coli* (3) or calf thymus (8), dThd-PPP inhibition was competitive with respect to the phosphate acceptor dThd, but, with respect to the phosphate donor, Ado-PPP, inhibition was either uncompetitive (3) or complex (8). With several tumors transplanted in rats, dThd-PPP inhibition was noncompetitive with respect to dThd, but complex with respect to Ado-PPP (15). In regenerating rat liver, dThd-PPP inhibition was reported as being noncompetitive with respect to Ado-PPP and complex with respect to dThd (11). From a study with a highly purified dThd kinase from regenerating rat liver, it was concluded that the enzyme displayed substrate cooperativity in the presence of dThd-PPP (11).

The principal phosphate donor for dThd kinase is Ado-PPP, but the enzyme from *E. coli* (3), calf thymus (8), human fetal liver (10), Yoshida sarcoma (9), Sarcoma 180 (12), and human acute myelocytic leukemia cells (14) also can utilize dAdo-PPP as a phosphate donor; however, except for the enzyme obtained from *E. coli* (3), and from two kinds of cancer cells (12, 14), dAdo-PPP is inferior to Ado-PPP as a phosphate donor. Nawata and Kamiya (13) reported that the kinase from regenerating rat liver utilized both Ado-PPP and dAdo-PPP, but no details as to the relative efficacy of dAdo-PPP were given.

Affinity chromatography using agarose covalently linked to ligands which resemble substrate, product, or effector molecules for various enzymes have proven effective in the purification of those enzymes, e.g., α -lactalbumin for the purification of lactose synthase (E.C. 2.4.1.22) (16) and Urd-PP-hexanolamine (P^1-(6-amino-1-hexyl)-P^2-(5'

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**The nucleosides deoxythymidine, adenosine, deoxyadenosine, guanosine, deoxyguanosine, uridine, cytidine, and deoxycytidine are indicated by dThd, Ado, dAdo, Guo, dGuo, Urd, Cyd, and dCyd, respectively; the extent of phosphorylation in the 5' position is indicated by -PP and -PPP for di- and triphosphates, respectively. dThd-PP-hexanolamine refers to P^1-(6-amino-1-hexyl) -P^2- (5'-deoxythymidine) - pyrophosphate.

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-uridine) pyrophosphate) for the purification of the portion of the lactose synthase complex that catalyzes the transfer of galactose from Urd-PP-galactose to *N*-acetylglucosamine (17). Rohde and Lezius (18) linked 5'-amino-2',5'-di-dThd to agarose and used affinity chromatography as part of the procedure to purify dThd kinase of *E. coli*. We have observed that dThd kinase from regenerating rat liver readily binds to columns of dThd-PP-hexanolamine-agarose, and can be eluted from these columns by Ado-PPP.

Our purpose in the present experiments with dThd kinase from regenerating rat liver was twofold; to determine (a) whether the inclusion of affinity chromatography in a purification procedure would yield a more homogeneous dThd kinase preparation than hitherto obtained, and (b) the efficacy of dAdo-PPP as a phosphate donor.

MATERIALS AND METHODS

Animals. Male or female Holtzman rats, weighing 150 to 250 g, were housed in a room having 12-hr light and dark cycles and were fed Teklad Rat and Mouse Diet (Mogul Corporation, Winfield, IA.) *ad libitum*. Since hepatic thymidine kinase activity is low in rats of this age group but increases markedly during liver regeneration (19-22), rats were subjected to partial hepatectomy (23) at the beginning of the dark cycle and were killed 36 hr later.

Purification Procedures. All but two of the purification steps employed in the current study were used previously (11); however, since two new steps were added and the order in which some of the previous ones were applied was changed, the purification procedure is described in some detail.

Step 1: Regenerating hepatic remnants from 50 rats were homogenized in Tris buffer (50 m*M* Tris, pH 8.0) which also contained 250 m*M* sucrose, 25 m*M* KCl and 5 m*M* $MgCl_2$. Homogenates were centrifuged 30 min at 105,000 × *g*; the supernatant was Step 1 enzyme.

Step 2: Proteins were precipitated by addition of crystalline ammonium sulfate in amounts equivalent to 25% saturation at 25° (24).

Step 3: The precipitate from Step 2 was dissolved in Tris buffer (50 m*M* Tris, pH 8.0) which also contained 0.1 m*M* dThd; sufficient 2 *M* acetic acid was added to bring the solution to pH 5; after centrifugation, precipitated proteins were discarded and the supernatant was adjusted to pH 8 by the addition of 2 *M* ammonium hydroxide.

Step 4: The solution from the previous step was heated to 60° and held 4 min at this temperature. After rapid cooling in ice, precipitated proteins were discarded.

Step 5: Calcium phosphate gel was added (2 mg dry weight calcium phosphate/mg protein) to the ice-cold solution from Step 4: the slurry was stirred for 15 min, and the gel was collected by centrifugation and discarded.

Step 6: Crystalline ammonium sulfate was added to the supernatant solution from Step 5 exactly as described in Step 2; the precipitate was dissolved in Tris buffer (10 m*M* Tris, pH 8.0) to give a protein concentration of about 5 mg/ml, and the solution was stored at -80°. The 6-step procedure was repeated 6 times using regenerating livers from about 300 rats, so that about 50 mg of this enzyme preparation was accumulated.

Step 7: Approximately 50 mg of Step 6 protein was applied to a 0.9 × 10 cm dThd-PP-hexanolamine-Sepharose column; the column was washed with 20 ml of Tris buffer (10 m*M* Tris, pH 8.0); dThd kinase was eluted with the same buffer except that it was 1 m*M* in ATP. The elution of protein and dThd kinase activity from the affinity column is illustrated in Figure 1. A small amount of enzyme was eluted with other proteins during the buffer wash. Although this may have been due to overloading of the column, we believe it was associated with binding of Ado-PPP to some enzyme molecules. In preliminary studies with affinity columns we observed that despite extensive dialysis, passage of dThd kinase through affinity columns a second time resulted in elution of appreciable amounts of enzyme in the buffer wash. The eluate from the affinity column was concentrated (Amicon ultrafiltration apparatus) to about 2 mg protein/ml.

Step 8: Enzyme from the previous Step was applied to a DEAE-Sephadex column and was eluted with buffer of linearly increased ionic strength (from 10 m*M* to 300 m*M*

Tris, pH 8.0, containing 1 m*M* ATP). Steps 7 and 8 were performed twice, i.e., enzyme from the regenerating livers of 734 rats was purified. The purification achieved at each of the 8 steps in the procedure is shown in Table 1.

dThd Kinase Assay. Enzyme activity was assayed in reaction mixtures containing 400 μM dThd, 1 μCi [*methyl*-^3H] dThd (specific radioactivity was 6.7 Ci/mmole), 20 m*M* Ado-PPP, 20 m*M* $MgCl_2$, 6 m*M* mercaptoethanol, and 2 mg/ml of crystalline bovine serum albumin. In the formulation of this reaction mixture, we were guided primarily by the experience of others (25), however, the use of mercaptoethanol and bovine serum albumin was an outgrowth of our own experience (11). The extent of formation of dThd-P during a 15-min incubation at 37° was the basis for evaluating kinase activity. A unit of enzyme activity was arbitrarily defined as nmoles dThd-P formed/mg protein/15 min. dThd-P formation was estimated by the procedure of Breitman (1) exactly as described previously (11).

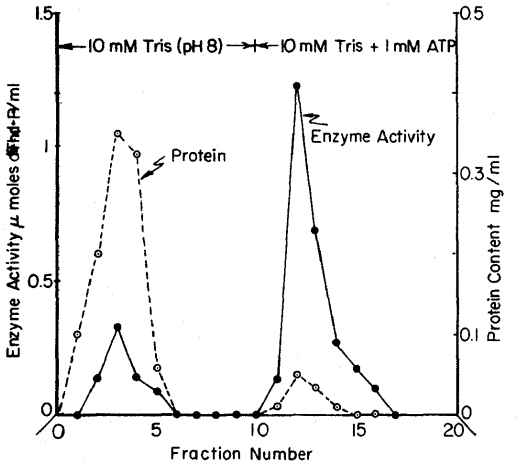

FIGURE 1. Chromatography of Step 6 dThd kinase on a dThd-PP-hexanolamine-Sepharose column as described in Step 7 of Methods.

Disc Gel Electrophoresis. We used a procedure described by Albrecht and Van Zyl (26) for treatment of purified dThd kinase with urea and sodium dodecyl sulfate (SDS) and for preparation of SDS-containing polyacrylamide gels. The SDS-containing buffer used during electrophoresis was described by Weber and Osborn (27). About 50 μg of Step 8 protein in 10% sucrose was applied to each gel column. After electrophoresis for 4 hr at 5 mA/column, protein bands were stained with Coomassie Blue (26).

Preparation of dThd-PP-Hexanolamine-Sepharose. dThd-PP-hexanolamine was synthesized by the imidazolide method in the manner employed for synthesis of Urd-P-hexanolamine (17). About 500 g of washed Sepharose 4B (Pharmacia) in 300 ml of water was stirred during addition of 150 to 200 mg of pulverized cyanogen bromide per g of gel (17). The mixture was brought to pH 11 ± 0.2 by addition of 4*N* NaOH; the temperature was held at 20° by addition of crushed ice (17). After about a 15-min period, when all the cyanogen bromide had dissolved, the reaction was stopped by addition of 400 to 500 ml of chipped ice and the mixture of ice and gel

TABLE 1. *Purification of dThd kinase from regenerating rat liver.*[a]

Step	Procedure	Total protein (mg/ml)	Enzyme activity (units)	Specific activity (units/ mg protein)	Fold purification	Recovery (%)
1	105,000 × *g* supernatant	156,650	1,397,000	9	--	--
2	$(NH_4)_2SO_4$ precipitation	14,040	820,070	58	6	59
3	pH 5 supernatant	3,580	624,140	174	19	45
4	Heat at 60°	1,580	554,100	350	39	40
5	$Ca_3(PO_4)_2$ treatment	553	520,210	940	104	37
6	$(NH_4)_2SO_4$ precipitation	95	270,790	2,850	317	19
7	Affinity gel column	10	155,020	15,500	1,720	11
8	DEAE-cellulose column	1.3	35,320	27,200	3,020	2.5

[a] Details for each step are given in Methods.

was filtered on a 2-liter coarse, sintered glass funnel (17). The gel was washed with 10 to 12 bed-volumes of ice-cold water and added to 200 to 300 ml of water containing 1 to 2 mmoles of dThd-PP-hexanolamine (17). The temperature of this mixture was maintained at 4°; the pH was adjusted to 10 and it was stirred 12 to 18 h (17). The gel then was packed in a column and washed with water at room temperature (17). After washing, columns contained 1 to 2 μ moles dThd/ml of settled gel.

Analytical Procedure. Protein content was estimated by the colorimetric procedure of Lowry *et al.* (28) using crystalline bovine serum albumin as a standard. Radioactivity was determined in a xylene-dioxane-Cellosolve phosphor solution (29) using either a Mark I, Nuclear Chicago or an LS-245 Beckman Instruments liquid scintillation system. Quenching was corrected by channels ratio (30).

RESULTS AND DISCUSSION

Purification of dThd Kinase, As shown in Table 1, the purification procedure increased the specific activity approximately 3,020-fold; thus, by this criterion, the procedure employed here was slightly superior to the one employed previously (11). When Step 8 enzyme was treated with urea and subjected to electrophoresis on polyacrylamide-SDS gels, 5 stained bands were readily detected; since 8 stained bands were observed previously (11), apparently better purification was achieved with the present procedure, but this advantage was accompanied by a poorer recovery of enzyme activity.

Since the Step 8 enzyme had 5 readily detectable protein bands following treatment with urea and electrophoresis on SDS-containing polyacrylamide gels, it is unlikely that this preparation represents a homogeneous protein. The molecular weight of dThd kinase from regenerating rat liver was estimated to be 70,000 (11) to 81,000 (31); that of the enzyme from *E. coli* was estimated to be 89,000 (32) to 93,000 (17). When first isolated from *E. coli,* dThd kinase existed in a monomeric form and had a molecular weight of 42,000, but in the presence of activator or inhibitor deoxynucleotides, e.g., dCyd-PP and dThd-PPP, respectively, it dimerized and then had a molecular weight of about 90,000 (32). Later, when the bacterial enzyme was purified to electrophoretic homogeneity, electrophoresis on SDS-containing polyacrylamide gels gave a single protein band (17). From these considerations, we expected a homogeneous preparation of dThd kinase from regenerating liver to show no more than 2 protein bands after treatment with urea and electrophoresis on SDS-containing polyacrylamide gels.

Rohde and Lezius (18) used affinity chromatography on agarose columns to which 5'-amino-2',5'-di-dThd was covalently attached as part of the purification for dThd kinase from *E. coli*. Enzyme was eluted by increasing the ionic strength of the buffer (18); in the present study, dThd kinase was eluted from dThd-PP-hexanolamine-Sepharose columns by adding Ado-PPP to the eluting buffer.

Kinetic Properties of dThd Kinase. Experiments to determine whether nucleoside triphosphates other than Ado-PPP could act as phosphate donors in the phosporylation of dThd by dThd kinase from regenerating rat liver are summarized in Table 2. Ado-PPP and dAdo-PPP readily served as phosphate donors, and, to a limited extent, Urd-PPP supported phosphorylation. Nowata and Kamiya (13) previously reported that dThd kinase from regenerating rat liver utilized Ado-PPP and dAdo-PPP as phosphate donors but the enzyme failed to use Cyd-PPP, Guo-PPP, or dGuo-PPP, apparently Urd-PPP was not tested. The enzymes isolated from *E. coli* (3), Yoshida sarcoma (9), and human leukemia cells (14) also utilized a limited array of nucleoside or deoxynucleoside triphosphates as phosphate donors, whereas the enzymes isolated from a transplantable mouse tumor (12), calf thymus (8), and human adult liver (10) readily utilized triphosphates of Urd, Guo, dGuo, Cyd, and dCyd.

Although both Ado-PPP and dAdo-PPP served as phosphate donors, higher reaction velocity was attained with Ado-PPP. This is illustrated in Figure 2. In the absence of dThd-PPP, dAdo-PPP produced a velocity of 9,140 units, whereas Ado-PPP supported a velocity of 13,630 units. dThd-PPP decreased enzyme activity. At the highest concentration of dThd-PPP used, however, the reaction velocity was essentially the same irrespective of the phosphate donor. Thus,

TABLE 2. *Phosphorylation of dThd by nucleoside and deoxynucleoside triphosphates catalyzed by dThd kinase from regenerating rat liver.*

Concentration of phosphate donor[a]	Enzyme activity (units/mg protein) and phosphate donor	
m*M*	Ado-PPP	dAdo-PPP
0.2	11,280 ± 410	7,720 ± 1130
0.4	12,390 ± 180	9,710 ± 910
1.2	17,270 ± 410	13,480 ± 140
2.4	17,890 ± 170	15,000 ± 320
	Guo-PPP	dGuo-PPP
0.2	5,820 ± 1260	1,080 ± 110
0.4	4,920 ± 20	1,080 ± 90
1.2	7,170 ± 1270	1,100 ± 90
2.4	7,100 ± 936	1,060 ± 100
	Cyd-PPP	dCyd-PPP
0.2	5,640 ± 270	3,300 ± 120
0.4	6,660 ± 180	3,530 ± 70
1.2	6,030 ± 380	3,050 ± 90
2.4	5,450 ± 290	3,050 ± 150
	Urd-PPP	dUrd-PPP
0.2	3,360 ± 530	430 ± 200
0.4	5,634 ± 420	620 ± 80
1.2	8,230 ± 900	420 ± 100
2.4	8,930 ± 1350	760 ± 110

[a] dThd was present at 400 μ M. Other conditions were as described in Materials and Methods.

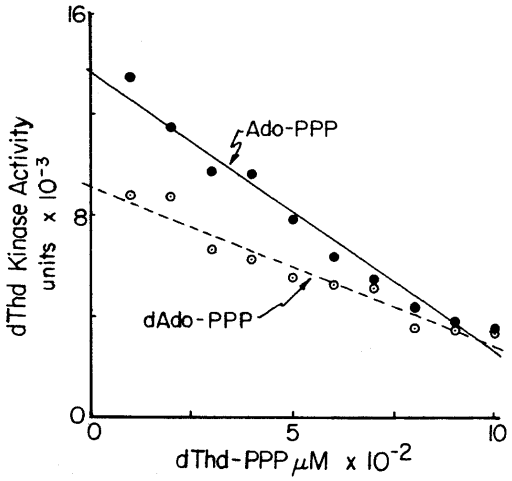

FIGURE 2. Inhibition of Step 8 dThd kinase activity by dThd-PPP with Ado-PPP or dAdo-PPP as the phosphate donor. Concentration of phosphate donors was 20 m*M*, and of the phosphate acceptor, dThd, was 400 μ M. Other conditions were as described in Methods. Correlation coefficients for the Ado-PPP and dAdo-PPP regression lines were 0.99 and 0.97, respectively.

dThd kinase appeared to be less sensitive to dThd-PPP inhibition when the phosphate donor was dAdo-PPP. This observation was supported by plots depicted in Figure 3. In the absence of dThd-PPP, velocity *versus* substrate concentrations plotted in the double reciprocal manner yielded regression lines with slopes that were $(10.97 \pm 0.23) \times 10^{-6}$ and $(6.49 \pm 0.17) \times 10^{-6}$ for dAdo-PPP and Ado-PPP, respectively; the ratio of these two slopes is 1.69 ± 0.06. In the presence of dThd-PPP, slopes of regression lines for dAdo-PPP and Ado-PPP were $(101.32 \pm 3.93) \times 10^{-6}$ and $(82.47 \pm 3.14) \times 10^{-6}$, respectively; the ratio here is 1.23 ± 0.07. Thus, the idea that phosphate trans-

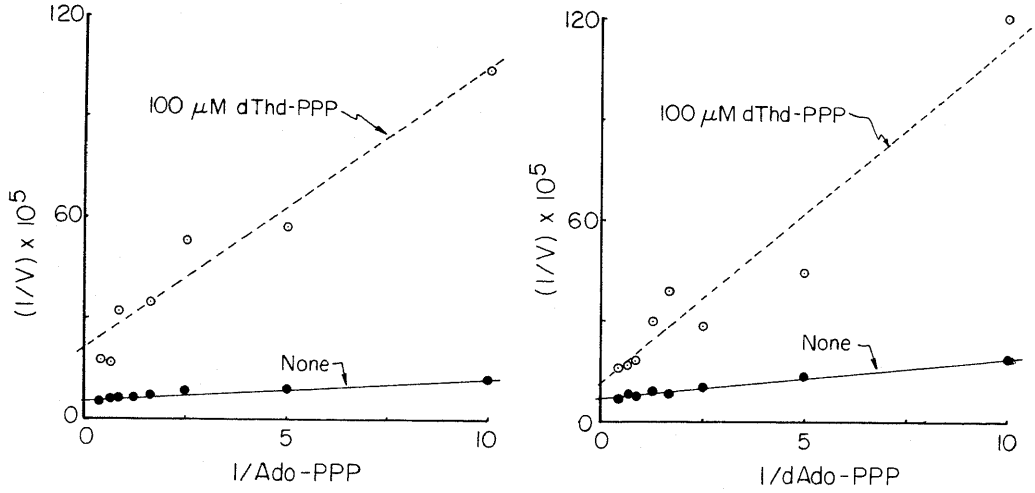

FIGURE 3. Double reciprocal plots of reaction velocity V *versus* phosphate donor concentrations for Step 8 enzyme with and without dThd-PPP. Concentrations of dThd and dThd-PPP were 400 and 100 μ *M*, respectively; other conditions were as described in Methods. Correlation coefficients for Ado-PPP and dAdo-PPP alone were 0.98 and 0.99, respectively, and for these nucleoside triphosphates in the presence of dThd-PPP were 0.97 and 0.97, respectively.

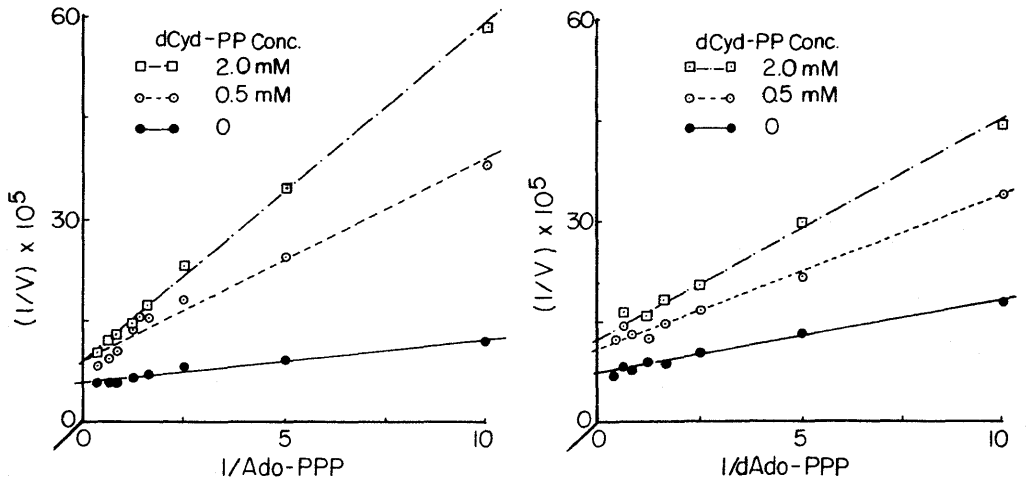

FIGURE 4. The influence of dCyd-PP on double reciprocal plots of reaction velocity for Step 8 enzyme *versus* phosphate donor concentrations. Conditions were as described in Figure 3. Correlation coefficients for Ado-PPP and dAdo-PPP were as listed in Figure 3; for these nucleoside triphosphates in the presence of 0.5 m*M* and 2 m*M* dCyd-PP, they were 0.99 in each instance.

fer from dAdo-PPP is less sensitive to the detrimental influence of dThd-PPP is supported. It was previously reported (11) that dThd-PPP was a noncompetitive inhibitor with respect to the phosphate donor Ado-PPP; as shown in Figure 3, this conclusion was corroborated and it is clear that dThd-PPP also is a noncompetitive inhibitor of phosphate transfer from dAdo-PPP.

The dThd kinase from *E. coli* was activated by dCyd-PP (3); later (32, 33) it was shown that dCyd-PP also influenced the temperature stability and the sedimentation coefficient of dThd kinase. For the most part, dCyd-PP or dCyd-PPP have not exerted efficacious influences on dThd kinase from eukaryotic sources (5, 10, 12). Bresnick *et al*. (4) reported that dCyd-PPP inhibited dThd kinase isolated from the liver of adult rats but exerted little influence on the activity of the enzyme from regenerating rat liver. As shown in Figure 4, irrespective of whether the phosphate donor was Ado-PPP or dAdo-PPP, dCyd-PP was a pronounced noncompetitive inhibitor of dThd kinase purified from regenerating rat liver. As noted previously, lines resulting from regression of reciprocals of velocity on reciprocals of dAdo-PPP concentration and Ado-PPP concentration had slopes whose ratio was 1.69 ± 0.06. In the presence of 2 m*M* dCyd-PP the ratio of slopes from these two regression lines was 1.53 ± 0.03. Thus, it appeared that interactions of dCyd-PP with dThd kinase were not appreciably changed when dAdo-PPP was used as the phosphate donor.

In our hands, dCyd-PP at 0.5 or 2 m*M* was a noncompetitive inhibitor of dThd kinase. Bresnick *et al*. (4) saw no appreciable inhibition of this same enzyme using dCyd-PPP at 0.08 m*M*. Nawata and Kamiya (13) separated the kinase from regenerating rat liver into two fractions on DEAE-cellulose columns; one fraction was inhibited slightly by 0.1 m*M* dCyd-PPP, while both fractions were inhibited by 1 m*M* dCyd-PPP. These observations suggest that dCyd-PP may be a more effective inhibitor than dCyd-PPP and the failure of Bresnick *et al*. (4) to observe inhibition probably was due to the low concentration of dCyd-PPP used.

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