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Effects of Mercury (II) Compounds on the Activity of dUTPases from Various Sources

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SUMMARY

The deoxyuridine triphosphate nucleotidohydrolases (dUTPases, EC 3.6.1.23) from *Escherichia coli* K-12-, *Acholeplasma laidlawii* B-PG9-, human KB cell-, and the herpes simplex virus (HSV) type 1- and 2-induced dUTPases were purified and used to determine the effect of various mercury (II) compounds on their activities. Mercuric acetate, 5-mercuri-dUTP (HgdUTP), and 5-mercuri-dCTP (HgdCTP) acted as irreversible active site-directed inhibitors of the dUTPases purified from eukaryotic organisms but not those from prokaryotic organisms. The inhibition constants (K_i) were estimated for the KB, HSV-1, and HSV-2 dUTPases to be 8 ± 2, 12 ± 3, and 9 ± 2 μM for mercuric acetate, 204 ± 25, 121 ± 15, and 111 ± 10 μM for HgdUTP, and 775 ± 25 and 651 ± 23 μM for HgdCTP, respectively. The conversion of HgdUTP to its mercurithio-derivative resulted in a decrease in the affinity of the derivative for the eukaryotic dUTPases. The 5-mercurithioethylene glycol derivative of dUTP did not act as a substrate for the KB dUTPase but it did act as a substrate for the HSV-1- and HSV-2-induced dUTPases with K_i values of 526 ± 47 and 483 ± 32 μM, respectively. These results demonstrate that the eukaryotic dUTPases can be distinguished based upon differences in their affinities for the mercurithio-derivatives of dUTP and suggest that there are differences in the steric binding properties of the nucleotide-binding site of these enzymes.

dUTP can act as a substrate for a number of DNA polymerases (1–3), but it is not normally incorporated into DNA *in vivo*. This is due to the action of dUTPase (EC 3.6.1.23), which specifically hydrolyzes dUTP to dUMP and pyrophosphate (4, 5). Although dUTPases have been purified from both prokaryotic (4, 6) and eukaryotic (5, 7, 8) sources, little is known concerning the structure-activity relationship of this enzyme with various synthetic substrates. The elucidation of this relationship is important since a number of pyrimidine nucleoside analogues have been developed for use in both antiviral and cancer chemotherapy (9–11) and it is not known what role the dUTPases may have in regulating the chemotherapeutic efficiency of these compounds. Furthermore, by elucidating differences in the structure-activity relationship of the cellular and virus-induced dUTPases, it may be possible to develop a specific class of antiviral agents based upon the activity of the dUTPases.

The data obtained in this study demonstrate that mercuric acetate, HgdUTP, and HgdCTP act as irreversible active site-directed inhibitors of eukaryotic dUTPases but not prokaryotic dUTPases. The results also demonstrate that differences exist in the steric binding properties of the nucleotide-binding site of the eukaryotic dUTPases since they can be distinguished based upon differences in their affinities for MEdUTP.

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Materials and Methods

Nonradioactive dUTP, dCTP, and the various mercaptans were purchased from Sigma Chemical Co. (St. Louis, MO). Radiolabeled [5-^{3}H]dUTP (11 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). HgdUTP and HgdCTP were purchased from PL Biochemicals (Milwaukee, WI). Dulbecco's modified Eagle's medium and bovine serum were purchased from Flow Laboratories (McLean, VA).

Extraction and purification of dUTPases. The dUTPases used in this study were purified from KB cells, KB cells infected with HSV type 1 (strain KOS) or type 2 (strain HG-52), *Acholeplasma laidlawii* B-PG9, and *Escherichia coli* K-12. The detailed procedures for the purification of the KB-, HSV-1-, and HSV-2-induced and *A. laidlawii* dUTPases have been described previously (6, 8).

E. coli K-12 was grown at 37° in LB medium until mid-exponential phase and was harvested by centrifugation. The pellet (2.5 g) was suspended in 20 ml of 10 mM Tris-Cl, pH 7.5, containing 1 mM 2-mercaptoethanol and 10% (v/v) glycerol, and lysed by sonic oscillation using a Branson Sonifier, model 350. The crude homogenate was heated to 70° for 10 min and then centrifuged at 4° for 30 min at 12,000 × *g*, and the precipitate was discarded. The pH of the supernatant was adjusted to 5 by the addition of cold 1 M acetic acid and the precipitate was removed by centrifugation. The pH of the supernatant was then

ABBREVIATIONS: dUTPase, deoxyuridine triphosphate nucleotidohydrolase (EC 3.6.1.23); HgdUTP, 5-mercuri-dUTP; HgdCTP, 5-mercuri-dCTP; MEdUTP, the 5-mercurithioethylene glycol derivative of dUTP; HSV, herpes simplex virus.

adjusted to 10 by the addition of 1 M KOH and allowed to sit on ice for 10 min. The pH of the solution was then adjusted to 7.5 by the addition of HCl, and the precipitate was removed by centrifugation. The dUTPase activity was then purified from the supernatant using the procedure described by Williams and Pollack (6).

Protein determination. Protein was estimated using the Coomassie blue dye-binding assay as described by Bio-Rad Laboratories using bovine serum albumin as the standard.

Enzyme assay. The dUTPase assay employed was a modification of the assay described by Williams and Cheng (5). The standard reaction mixture contained in a total volume of 0.1 ml: 50 mM Tris-Cl, pH 8.0, 1 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin, 40 μM [5-^{3}H] dUTP (50 μCi/μmol), and the enzyme (0.03–0.05 unit, 0.01–0.1 μg of protein). Under these conditions, reactions were linear for at least 3 hr. The reactions were terminated by spotting 50 μl of the reaction mixture on a DE-81 filter disc and immediately washing the disc in a 4 M formic acid, 1 mM ammonium formate solution. The discs were then processed as described previously (5) and the radioactivity bound to the disc was determined by scintillation counting using a Beckman LS8100 scintillation counter. A unit of dUTPase activity was defined as the amount of enzyme which converted 1 nmol of dUTP to dUMP and pyrophosphate per min at 37°.

MEdUTP synthesis. HgdUTP was mixed with a 3-fold excess of 2-mercaptoethanol and allowed to incubate at 37° for 15 min. Conversion of HgdUTP to MEdUTP was monitored by electrophoresis on cellulose thin layer plates as described by Dale *et al.* (12).

Inhibition studies. In experiments to estimate the inhibition constants (K_i) of the test compounds, the purified enzyme was mixed with the inhibitor and incubated at 37° for various time periods. At specific intervals, an aliquot was removed and the reaction was terminated by the addition of 2-mercaptoethanol to a final concentration of 5 mM. Residual dUTPase activity was determined using the standard reaction mixture. The percentage of dUTPase activity was determined by comparing the residual dUTPase activity in the test sample to a control that lacked the inhibitor. The K_i was estimated using the procedure described by Plapp (13).

Results

Preliminary studies with various sulfhydryl inhibitors demonstrated that the KB- and HSV-induced dUTPases could be distinguished from the *E. coli* and *A. laidlawii* dUTPases based upon differences in their sensitivities to these compounds (Table 1). The activities of the eukaryotic dUTPases were inhibited significantly by mercuric acetate, *p*-hydroxymercuribenzoate, and HgdUTP and to a lesser extent by *N*-ethylmaleimide, whereas the activities of the prokaryotic enzymes were not inhibited by any of these compounds.

To examine further the mechanism of inhibition of the eukaryotic dUTPases by mercuric acetate and the 5-mercurideoxypyrimidine triphosphates, kinetic analyses were performed. Mercuric acetate irreversibly inhibited the KB-induced dUTPase in a concentration- and time-dependent manner (Fig. 1). The K_i of mercuric acetate for the KB-induced dUTPase was 8 ± 2 μM (Fig. 1, *inset*). The inhibitory effect of mercuric acetate for the enzyme was partially reversed by the addition of dUTP (80 μM) and completely reversed by the addition of 2-mercaptoethanol (2 mM) provided they were added simultaneously with mercuric acetate (data not shown). Similar results were obtained with the HSV-1 and HSV-2 dUTPases with K_i values of 12 ± 3 and 9 ± 2 μM, respectively (data not shown).

HgdUTP also irreversibly inhibited the eukaryotic dUTPases in a concentration and time dependent manner (Fig. 2). However, HgdUTP exhibited a greater affinity for the HSV-induced dUTPases. The K_i for the KB-induced dUTPase was estimated to be 204 ± 25 μM (Fig. 2A), whereas the K_i values for the HSV-1- and HSV-2-induced dUTPases were 121 ± 15 μM (Fig. 2B) and 111 ± 10 μM (Fig. 2C), respectively. Similar results were observed with HgdCTP. HgdCTP irreversibly inhibited the eukaryotic dUTPases in a concentration- and time-dependent manner (Fig. 3), with the HSV-induced dUTPases being more sensitive to inhibition than the KB enzyme. However, the affinity of HgdCTP for these dUTPases was 54–97 times less than that of mercuric acetate and 4–6 times less than HgdUTP. The K_i of HgdCTP for the KB-induced dUTPase was 775 ± 25 μM (Fig. 3A), whereas the K_i values for the HSV-1- and HSV-2-induced dUTPases were 651 ± 23 (Fig. 3B) and 525 ± 32 μM (Fig. 3C), respectively.

The inhibition of the KB dUTPase by both HgdUTP and HgdCTP was dependent upon the concentration of dUTP (Fig. 4). The addition of dUTP at a concentration of 80 μM reduced the inhibition caused by HgdUTP (50 μM) (Fig. 4A) and HgdCTP (120 μM) (Fig. 4B) by approximately 95%. dCTP at a concentration of 1 mM had no effect on the inhibition caused by HgdCTP (Fig. 4B). Similar results were obtained for the HSV-induced dUTPases (data not shown).

To determine the effects of mercaptans on the ability of HgdUTP to inhibit the activities of the various dUTPases, studies were conducted using 2-mercaptoethanol (0.2–2 mM) and a fixed concentration of HgdUTP (0.2 mM). The results are shown in Table 2. The greatest inhibition of the KB- and HSV-induced dUTPases occurred when the 2-mercaptoethanol/HgdUTP ratio was 1:1. We could not determine what effect lower concentrations of 2-mercaptoethanol had on the reaction, since the eukaryotic dUTPases were unstable under these conditions. The reversal of HgdUTP inhibition was not due to the reductive demercuration of HgdUTP but, rather, due to the formation of MEdUTP. Kinetic analyses demonstrated that

TABLE 1

Effect of mercury (II) compounds on the activity of various dUTPases

The reaction mixture contained, in addition to the components in the standard reaction mixture, 1 mM 2-mercaptoethanol and the additive. The concentrations of the additive per assay were 5, 3, 2, and 0.3 mM for *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, mercuric acetate, and HgdUTP, respectively. The amounts of purified enzyme used per assay were 0.04, 0.05, 0.03, 0.03, and 0.04 unit for the *E. coli*-, *A. laidlawii*-, KB-, HSV-1-, and HSV-2-induced dUTPases, respectively. The reaction mixtures were incubated at 37° for 60 min. The values represent the mean ± standard deviation for four experiments.

Additive	Enzyme source				
	E. coli	*A. laidlawii*	KB	HSV-1	HSV-2
			% activity remaining		
None	100 ± 2	100 ± 2	100 ± 1	100 ± 1	100 ± 3
N-Ethylmaleimide	98 ± 3	97 ± 4	87 ± 5	59 ± 3	52 ± 3
p-Hydroxymercuribenzoate	100 ± 2	98 ± 3	5 ± 1	1 ± 0.3	1 ± 0.6
Mercuric acetate	98 ± 3	99 ± 2	10 ± 1	7 ± 2	6 ± 2
HgdUTP	99 ± 1	100 ± 2	53 ± 2	27 ± 2	21 ± 2

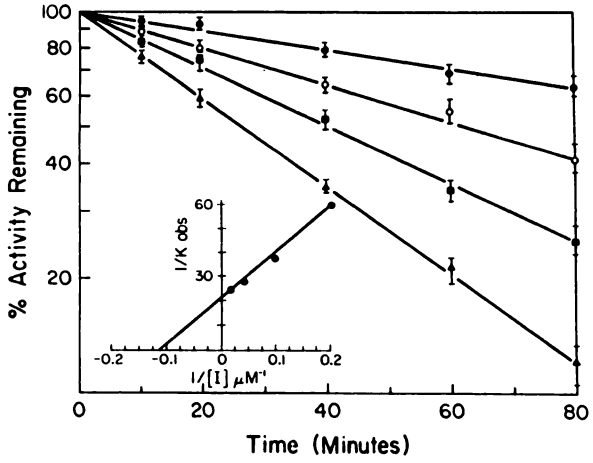

Fig. 1. Time- and dose-dependent inactivation of KB dUTPase by mercuric acetate. Inhibition studies were performed as described in Materials and Methods. Aliquots of the reaction mixture were removed at 15-min intervals and examined for residual dUTPase activity by further incubation at 37°. Approximately 0.045 unit of dUTPase activity was used per assay. *Inset*, K_i determination. ●, 5 μM; ○, 10 μM; ■, 25 μM; ▲, 50 μM.

MEdUTP did not act as a substrate for the KB dUTPase but that it did act as a substrate for the HSV-1- and HSV-2-induced dUTPases with K_i values of 526 ± 47 and 483 ± 32 μM, respectively (Fig. 5).

A further examination of a number of mercaptans demonstrated that, except for 2-thiouracil, all the mercaptans tested decreased the inhibitory effects of HgdUTP for the dUTPases (Table 3). Addition of the mercaptans essentially abolished the inhibitory effect of HgdUTP for the KB dUTPase. Conversely, whereas the addition of the mercaptans did reduce the inhibitory effects of HgdUTP for the HSV-induced dUTPases, there was still significant inhibition of the HSV-induced enzymes.

Discussion

Previous studies have demonstrated that mercury (II) compounds (14) as well as the mercurated pyrimidines (12, 15) irreversibly inhibit a number of enzymes that are sensitive to thio-modification by binding to an essential sulfhydryl group in the active site of the enzyme. The data presented in this study demonstrate that mercuric acetate, HgdUTP, HgdCTP, and MEdUTP act as irreversible active site-directed inhibitors of the dUTPases purified from eukaryotic organisms but not those purified from prokaryotic organisms. This suggests that there are major differences in the structural and steric binding properties of the nucleotide-binding site of these enzymes. Neither of the dUTPases purified from prokaryotic organisms were inhibited by any of the mercury (II) compounds examined, suggesting that either these dUTPases do not have a sulfhydryl group in the active site or that it is protected in some manner. Although there is no information concerning the amino acid sequence of the dUTPases from *A. laidlawii* or the eukaryotic organisms, the amino acid sequence of the *E. coli* dUTPase is known and it does not contain a cysteine in the proposed active site of the enzyme (16). Differences in the steric binding properties of the eukaryotic dUTPases are further demonstrated by the fact that, whereas the conversion of HgdUTP to MEdUTP results in a decreased binding affinity of the derivative for the eukaryotic dUTPases when compared to HgdUTP, it increases

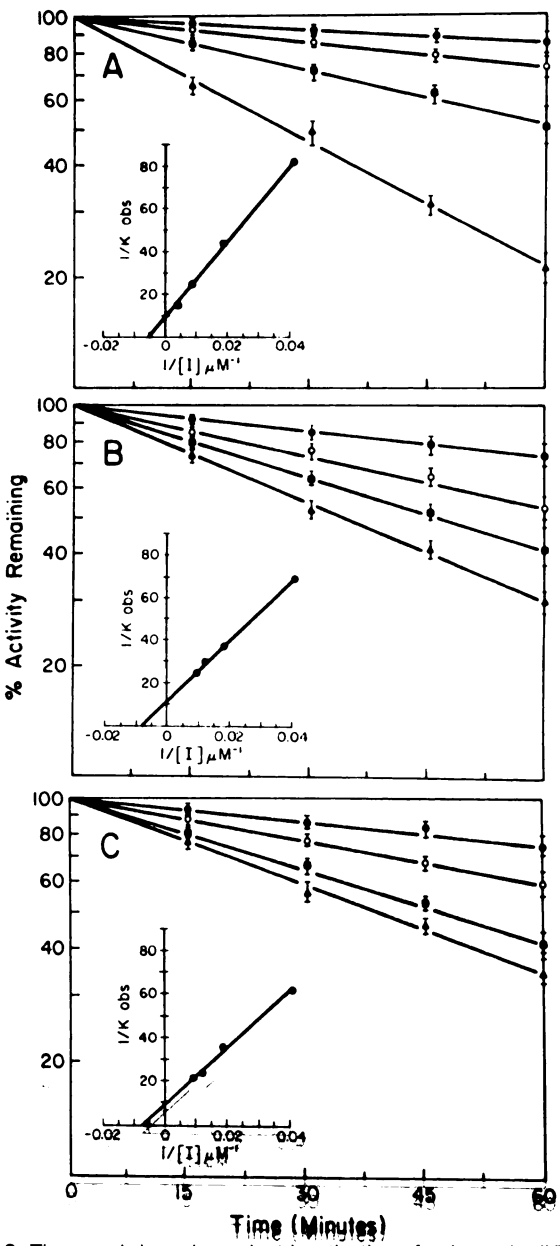

Fig. 2. Time- and dose-dependent inactivation of eukaryotic dUTPases by HgdUTP. Inhibition studies were performed as described in Materials and Methods. Aliquots of the reaction mixture were removed at 15-min intervals and examined for residual dUTPase activity by further incubation at 37°. *Inset*, K_i determination. A, KB dUTPase, 0.045 unit/assay. ●, 25 μM; ○, 50 μM; ■, 100 μM; ▲, 200 μM. B, HSV-1-induced dUTPase, 0.032 unit/assay. ●, 25 μM; ○, 50 μM; ■, 75 μM; ▲, 100 μM. C, HSV-2-induced dUTPase, 0.049 unit/assay. ●, 25 μM; ○, 50 μM; ■, 75 μM; ▲, 100 μM.

the specificity of the compound so that it only acts as an inhibitor of the HSV-induced dUTPases.

Our results regarding the inhibitory action of HgdUTP for the eukaryotic dUTPases are in contrast to those of Ingraham and Goulian (17), who reported that HgdUTP did not act as a substrate, as demonstrated by the lack of hydrolysis of HgdUTP, nor did it prevent the hydrolysis of dUTP by a partially purified dUTPase from human lymphoid cells. Although these differences could be due to differences in the dUTPases, they are more probably due to differences in the procedures used to examine HgdUTP inhibition of the dUTPase. In fact, we believe their data support this study. Our

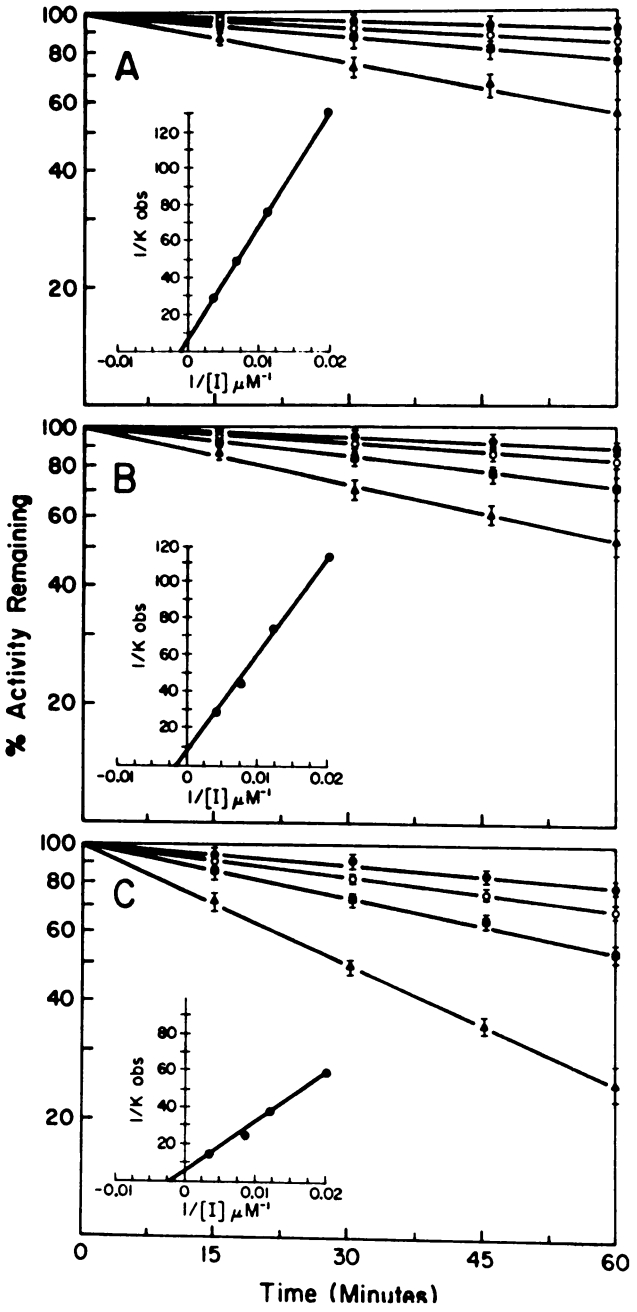

Fig. 3. Time- and dose-dependent inactivation of eukaryotic dUTPases by HgdCTP. Inhibition studies were performed as described in Materials and Methods. Aliquots of the reaction mixture were removed at 15-min intervals and examined for residual dUTPase activity by further incubation at 37°. Concentrations of HgdCTP were: 50 μM (●), 80 μM (○), 120 μM (■), and 240 μM (▲). *Inset*, K_i determination. Units per assay were: A, KB dUTPase, 0.038; B, HSV-1-induced dUTPase, 0.044; C, HSV-2-induced dUTPase, 0.039.

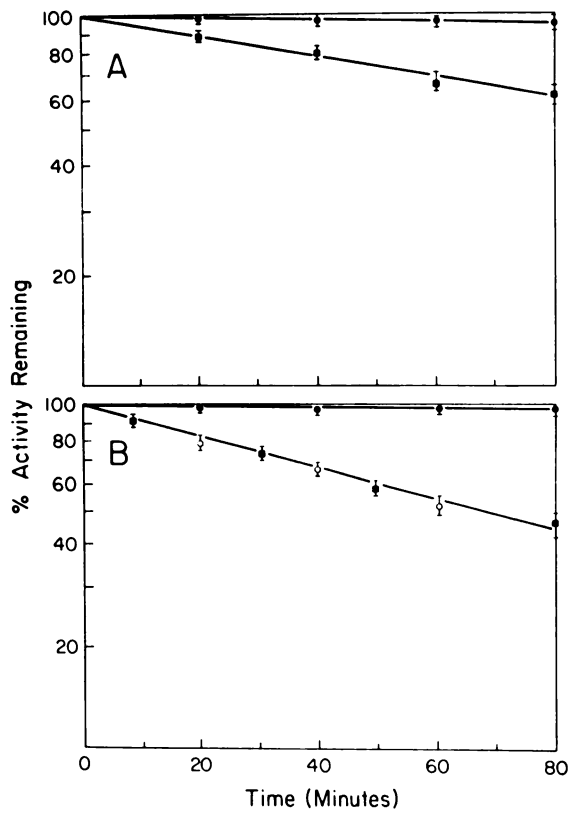

Fig. 4. Antagonism of HgdUTP and HgdCTP inhibition by dUTP and dCTP. Inhibition studies were performed as described in Materials and Methods in the presence and absence of either dUTP (80 μM) or dCTP (1 mM). The concentration of HgdUTP was 100 μM and the concentration of HgdCTP was 120 μM. Aliquots of the reaction mixture were removed at 15-min intervals and residual dUTPase activity was determined by further incubation at 37°. KB-induced dUTPase was employed at 0.045 unit/assay. A, HgdUTP: without dUTP, ■; with dUTP, ●. B. HgdCTP: without dUTP or dCTP, ■; with dCTP, ○; with dUTP, ●.

TABLE 2

Effect of 2-mercaptoethanol on the inhibition of various dUTPases by HgdUTP

The standard reaction mixture as described in Materials and Methods was employed in all assays. The amount of purified enzyme used per assay was 0.03 unit. The reaction mixtures were incubated at 37° for 60 min. The values represent the mean ± standard deviation for at least four experiments.

Additive		Enzyme source		
2-Mercaptoethanol	HgdUTP	KB	HSV-1	HSV-2
mM		*% activity remaining*		
0.2		100 ± 2	100 ± 3	100 ± 2
0.2	0.2	11 ± 3	0 ± 1	0 ± 1
0.4	0.2	73 ± 3	30 ± 2	14 ± 2
0.6	0.2	85 ± 4	46 ± 3	23 ± 4
0.8	0.2	90 ± 3	63 ± 2	57 ± 3
1.0	0.2	93 ± 5	77 ± 3	71 ± 4
2.0	0.2	97 ± 3	86 ± 3	79 ± 4

results demonstrated that HgdUTP acts as an irreversible active site-directed inhibitor of the dUTPases and, thus, no reaction products of HgdUTP hydrolysis would be expected. Furthermore, at the concentrations of dUTP and HgdUTP that Ingraham and Goulian (17) employed, HgdUTP would not prevent the hydrolysis of dUTP since dUTP is a more effective substrate for the enzyme.

In conclusion, the results of this study demonstrate that various dUTPases can be distinguished based upon differences in the affinities for various mercury (II) compounds and that mercurithio-derivatives of dUTP can be used to elucidate differences in the steric binding properties of the eukaryotic dUTPases. We have been able to demonstrate the presence of an Epstein-Barr virus-induced dUTPase based in part upon its sensitivity to the 5-mercurithioguanosine derivative of dUTP (18).

These results also suggest that it may be possible to develop a class of antiviral agents that may act as suicide inhibitors (19, 20) of the HSV-induced dUTPases. However, further stud-

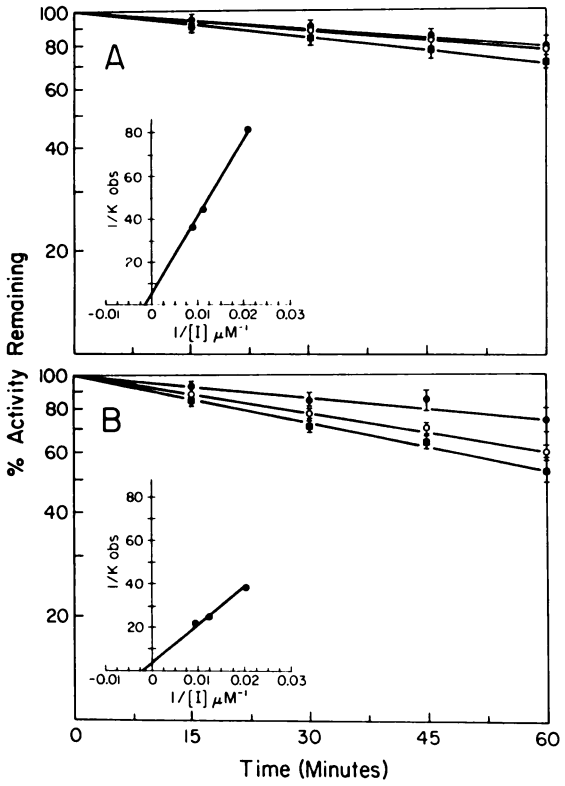

Fig. 5. Time- and dose-dependent inactivation of HSV-induced dUTPases by MEdUTP. HgdUTP was mixed with a 3-fold molar excess of 2-mercaptoethanol and incubated at 37° for 15 min. The resulting solution was employed for inhibition studies which were conducted as described in Materials and Methods. Aliquots of the reaction mixture were removed at 15-min intervals and residual dUTPase activity was determined by further incubation at 37° for 60 min. The concentrations of MEdUTP were: 50 μM (●), 75 μM (○), and 100 μM (■). *Inset*, K_i determination. A, HSV-1-induced dUTPase, 0.043 unit/assay; B, HSV-2-induced dUTPase, 0.039 unit/assay.

TABLE 3

Effect of various mercaptans on the activity of HgdUTP

The concentration of HgdUTP was 0.2 mM and the final concentration of the mercaptans was 2 mM. The reaction mixture without enzyme was incubated at 37° for 15 min prior to the addition of the enzyme to allow for the formation of the mercurithio-dUTP derivative. The amount of purified enzyme used per assay was 0.03 unit. The reaction mixtures were incubated for 60 min following the addition of the enzyme. The values represent the mean ± standard deviation for at least three experiments.

Additive	Enzyme source		
	KB	HSV-1	HSV-2
	% activity remaining		
None	100 ± 4	100 ± 3	100 ± 5
HgdUTP	15 ± 3	2 ± 1	0 ± 1
HgdUTP + glutathione	100 ± 3	83 ± 5	78 ± 3
HgdUTP + dithiothreitol	98 ± 3	84 ± 5	76 ± 3
HgdUTP + 2-mercaptoethanol	97 ± 4	86 ± 3	81 ± 4
HgdUTP + L-cysteine	95 ± 4	81 ± 3	83 ± 3
HgdUTP + α-thioglycerol	92 ± 4	85 ± 2	88 ± 3
HgdUTP + 6-mercaptoguanosine	97 ± 6	41 ± 3	37 ± 4
HgdUTP + 2-thiouracil	28 ± 5	5 ± 3	3 ± 2

ies are necessary to isolate the Hg-dUTPase adduct and to elucidate the mechanism by which the mercurithio-derivatives of dUTP inhibit the virus-induced dUTPases.

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