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Enzymatic Studies Relating to the Mode of Action of 5-Mercapto-2′-deoxyuridine

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SUMMARY

Kinetic studies of the effects of 5-mercapto-2′-deoxyuridine (MUdR) and some of its derivatives on the thymidine kinase and thymidylate synthetase of *Escherichia coli* B are presented. It was found that MUdR and its *S*-methyl derivative are phosphorylated by thymidine kinase, while other derivatives, including the disulfide, are not. In crude extracts of *E. coli* B, thymidylate synthetase was inhibited by MUdR (but not the *S*-methyl derivative) in the presence of ATP only. MUdR 5′-monophosphate (MUdRP) was prepared by enzymatic phosphorylation of MUdR and found to be a potent inhibitor of purified *E. coli* B thymidylate synthetase ($K_i = 4.0 \times 10^{-8}$ M; $K_i/K_m = 0.0085$), competitive with the substrate, deoxyuridylate. The "anomalous" strong binding of MUdRP to this enzyme is discussed.

The results of these studies are consistent with the hypothesis that the mode of action of MUdR involves the metabolic activation of the analogue via phosphorylation by thymidine kinase and subsequent inhibition of thymidylate synthetase by the active form, MUdRP, resulting in the inhibition of DNA synthesis.

INTRODUCTION

5-Mercapto-2′-deoxyuridine (see Fig. 1), a structural analogue of thymidine, was recently synthesized by enzymatic (1) and chemical (2) methods. Preliminary results of studies involving various systems *in vitro*[2] and *in vivo*[3] [including recent clinical studies (3)] indicate that MUdR[4] is an effective antitumor agent. Its free base, 5-mercaptouracil (4, 5), and some of its derivatives were previously found to potentiate, in several experimental neoplasms, the anti-

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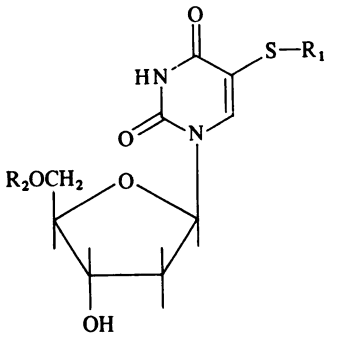
[1] Recipient of a Fellowship of the National Institute of General Medical Sciences, National Institutes of Health (GM-34,138).

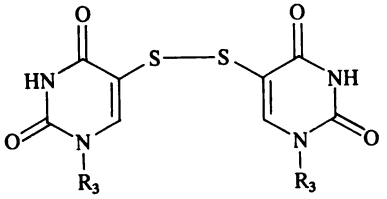
[2] In Ehrlich ascites carcinoma and Leukemia L1210 cell cultures (A. Bloch, personal communication).

[3] Against Leukemia L1210 in BDF_1 mice (J. L. Ambrus and H. L. Babbitt, personal communication).

[4] The abbreviations used are: MUdR, 5-mercapto-2′-deoxyuridine; MeMUdR, 5-methylmercaptodeoxyuridine; MUdRP, 5-mercaptodeoxyuridylate; MUdR-DS and MUdRP-DS, disulfide forms of MUdR and MUdRP; TdR, thymidine.



(a)



(b)

FIG. 1. *5-Mercaptodeoxyuridine and some of its derivatives*

a. 5-Mercapto-2′-deoxyuridine ($R_1 = R_2 = H$); 5-methylmercapto-2′-deoxyuridine ($R_1 = CH_3$, $R_2 = H$); 5-ethylmercapto-2′-deoxyuridine ($R_1 = CH_2CH_3$, $R_2 = H$); *S*-acetyl-5-mercapto-2′-deoxyuridine ($R_1 = COCH_3$, $R_2 = H$); 5-mercapto-2′-deoxyuridine 5′-phosphate ($R_1 = H$, $R_2 = PO_3H_2$).

b. 5-Mercapto-2′-deoxyuridine disulfide [R_3 = 1-(2-deoxyribosyl)]; 5-mercapto-2′-deoxyuridylate disulfide [R_3 = 1-(5-phospho-2-deoxyribosyl)].

tumor activities of 5-fluorouracil, 5-fluorodeoxyuridine, and antifolic agents (6, 7). Inhibition analysis studies in various microbiological assay systems (1) indicated that MUdR acts as an antimetabolite of the biosynthesis of the thymine moiety of DNA, and that its primary site of action appears to be the thymidylate synthetase reaction of the biosynthetic pathway. This and other observations suggested that metabolic activation of MUdR may occur, involving phosphorylation by TdR kinase. The study presented here was undertaken to demonstrate the biological activities of MUdR and its derivatives in cell-free systems and, specifically, to determine in a quantitative manner the effects of these compounds on the TdR kinase and thymidylate synthetase of *Escherichia coli* B.

MATERIALS AND METHODS

Materials

Tris (enzyme grade, three times recrystallized), ammonium sulfate, and streptomycin sulfate were purchased from Nutritional Biochemicals Corporation, and tetrahydrofolic acid, from General Biochemicals. Dithiothreitol, TdR, ATP, and 2-mercaptoethanol were obtained from Calbiochem. 2-^{14}C-TdR (specific activity, 30 mCi/mmole and 59.2 mCi/mmole) and 6-^{14}C-orotic acid (specific activity, 6.2 mCi/mmole) were purchased from New England Nuclear Corporation; 8-^{14}C-ATP (specific activity, 0.83 mCi/mmole), from Schwarz BioResearch; and γ-^{32}P-ATP (specific activity, 3.2 Ci/mmole) from Amersham/Searle. All other chemicals were Fisher Scientific Company reagent grade products. Bovine serum albumin and frozen *E. coli* B cells (Kornberg type, late log phase) were obtained from Armour Pharmaceutical Company, Chicago, and Grain Processing Corporation, Muscatine, Iowa, respectively. Solutions were made with deionized, distilled water.

Preparation of TdR Kinase

TdR kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) was partially purified from *E. coli* B extracts by the method of Okazaki and Kornberg (8); fraction V was used throughout this study. Preparations were routinely obtained with specific activities of 0.4–0.9 unit/mg of protein (1 unit is defined as the amount catalyzing the phosphorylation of 1 μmole of TdR per minute at 37°). Protein was determined by the modified Folin procedure of Lowry *et al.* (9), using bovine serum albumin as standard.

Assay of TdR Kinase

Method A (for potential substrate activity). This assay is based on the method of Okazaki and Kornberg (8), which follows the conversion of 8-^{14}C-ATP to 8-^{14}C-ADP in the presence of a phosphate acceptor by measuring the radioactivity of ATP and ADP after their electrophoretic separation.

The incubation mixture contained, unless otherwise stated, the following constituents: 7.5 μmoles of Tris-HCl buffer, pH 7.4; 120 mμmoles of 8-^{14}C-ATP (specific activity, 0.83 mCi/mmole); 120 mμmoles of $MgCl_2$; TdR or other nucleosides in varying amounts; 30 μg of bovine serum albumin; enzyme (2.1 μg of protein); and 5% ethanol in a total volume of 0.1 ml. When MUdR (α or β) was assayed, 100 mμmoles of dithiothreitol were included in order to keep MUdR in its reduced state (10, 11). After incubation at 37° for 30 min, the reaction was terminated by cooling to 0°, followed by the addition of 5 μl of 0.05 M disodium EDTA containing about 0.1 M carrier ATP and ADP. Aliquots (in parallel) were spotted on Whatman No. 3MM paper strips and subjected to electrophoresis at a potential gradient of 20 V/cm, using a 0.05 M sodium EDTA–H_3BO_3 electrolyte, pH 5.1 [relative mobilities: 1.00 (ATP), 0.78 (ADP), 0.57 (thymidylate), 0.35 (AMP), and −0.05 (TdR)]. Radioactivity of ATP and ADP was counted on a Nuclear-Chicago 4π scanner. The extent of conversion was expressed as nanomoles of ADP formed during 30 min under the conditions of the assay. Values for ADP were obtained as percentages of the total counts (ATP + ADP) after subtracting the corresponding value of a "blank" (3–4% of total counts) from which the phosphate acceptor was omitted.

Method B (for kinetics of phosphorylation of effective substrates). This assay measures directly the transfer of the γ-phosphate of γ-^{32}P-ATP to the various nucleoside acceptors. The incubation mixture contained the following: 7.5 μmoles of Tris-HCl buffer, pH 7.6; 120 mμmoles of γ-^{32}P-ATP (specific activity, 0.2–0.4 mCi/mmole); 200 mμmoles of $MgCl_2$; 500 mμmoles of dithiothreitol; TdR, MUdR, or MeMUdR in varying amounts; 30 μg of bovine serum albumin; and enzyme (2 μg of protein) in a total volume of 0.1 ml. After 30 min of incubation at 37°, the reaction was terminated by cooling below 0°, followed by the addition of 10 μl of a solution of EDTA (0.1 M) and dithiothreitol (1.0 M). The radioactive materials were separated by horizontal chromatography (12) on Whatman No. 4 paper, using a 1-butanol–acetone–acetic acid–5% ammonium acetate (7:5:3:5 by volume) solvent mixture containing 0.02 M dithiothreitol. The distribution of radioactivity on the paper strips was counted as described above for method A, and the extent of phosphorylation of the nucleosides was calculated as a percentage of the counts under the monophosphate spot relative to the total counts on each strip and expressed as nanomoles per 30 min.

Method C (inhibitory activity test). This assay measures the effects of various nucleoside analogues on the conversion of ^{14}C-TdR to ^{14}C-thymidylate in the presence of an internal radioactivity standard (^{14}C-orotate). The enzyme (0.05 μg of protein) was incubated for 30 min at 37° in a total volume of 0.1 ml of the reaction mixture, which contained the following: Tris-HCl, 7.5 μmoles, pH 7.5; ATP, 600 mμmoles; $MgCl_2$, 600 mμmoles; ^{14}C-TdR (specific activity, 30 mCi/mmole), 4–32 mμmoles; ^{14}C-orotic acid (specific activity, 6.2 mCi/mmole), 2.2–18 mμmoles; bovine serum albumin, 30 μg; ethanol, 5% (v/v); inhibitors; and dithiothreitol, 100 mμmoles, when indicated. The reaction was terminated by immersing the tubes in a boiling water bath for 2.5 min. Carrier thymidylate and orotate were added and, after centrifugation, aliquots of the supernatant solution were applied in parallel on Whatman No. 3MM paper strips and subjected to electrophoresis for 4 hr at a potential gradient of 19 V/cm, using sodium citrate electrolyte, 0.05 M, pH 4.5 [relative mobilities: 1.00 (orotate), 0.78 (thymidylate), and −0.05 (TdR)]. Portions of the paper strips bearing the orotate and thymidylate spots (detected under ultraviolet light) were counted for radioactivity in a manner similar to method A. The ratio of the radioactivities of the internal standard (^{14}C-orotic acid) and the substrate (^{14}C-TdR) in the radioactive stock solution was separately determined. The radioactivity of the product was expressed as a percentage of ^{14}C-TdR phosphorylated, which could be calculated directly from the ratio of the radioactivities of the ^{14}C-thymidylate spot and the ^{14}C-orotate spot on each individual paper strip, and then converted to nanomoles.

Preparation of Thymidylate Synthetase

The effects of inhibitors on thymidylate synthetase were examined both in crude and in partially purified preparations of the enzyme from *E. coli* B cells. The crude preparation was obtained as the 105,000 × *g* supernatant fraction of sonicated cells. A 100-fold purification was achieved by a procedure adopted from Wahba and Friedkin (13) and Friedkin *et al.* (14), involving streptomycin sulfate treatment, DEAE-cellulose column chromatography, and $(NH_4)_2SO_4$ fractionation (45–85% saturation), yielding a specific activity of 1.9 units/mg of protein.

Assay of Thymidylate Synthetase

For the study of nucleosides in the crude system, the spectrophotometric method of Wahba and Friedkin (15) was employed, using a Gilford model 2000 recording spectrophotometer thermostated at 30°. The incubation mixture contained 35 μmoles of Tris-HCl buffer, pH 7.4; 100 μmoles of 2-mercaptoethanol; 0.7 μmole of sodium EDTA; 0.13 μmole of *dl*,L-tetrahydrofolate, 12 μmoles of formaldehyde; 20 μmoles of $MgCl_2$; and 0.2 ml of crude *E. coli* B extract in a total of 1.0 ml, including inhibitors in varying amounts. The mixture was incubated for 12 min at 30° in the presence and absence of 5×10^{-3} M ATP; then the reaction was started by the addition of deoxyuridylate (2.5×10^{-5} M or 2.5×10^{-4} M). The rates were calculated from the initial linear portions of the recorded curves and were corrected by subtracting the rate of a "blank reaction" from which deoxyuridylate was omitted. Inhibitions were expressed as percentages calculated from the rates of the reaction in the presence and absence of the inhibitors.

For the study of MUdRP in the purified thymidylate synthetase system, essentially the same assay procedure as above was employed, except that the reaction mixture contained deoxyuridylate in varying amounts (20, 30, 50, and 200 mμmoles). MUdRP in varying amounts (0.1, 0.25, and 0.75 mμmole), and 22.5 μg of purified enzyme preparation. The reaction mixture was incubated for 10 min prior to the addition of deoxyuridylate and MUdRP, unless otherwise indicated; the latter was introduced in its disulfide form, which, however, was reduced instantaneously to the thiol by the large excess of mercaptoethanol present in the medium (see below). The rates were expressed as micromoles of thymidylate formed per hour per milligram of protein at 30°. The data represent average values of 5–10 separate determinations.

Enzymatic Preparation of 5-Mercapto-2′-deoxyuridine 5′-Phosphate

Enzymatic phosphorylation of MUdR was carried out at 37° for 12 hr under an atmosphere of N_2 in a solution containing 15 μmoles of MUdR (3.9 mg), 15 μmoles of NaOH, 15 μmoles of dithiothreitol, 12 μmoles of ATP, 12 μmoles of $MgCl_2$, 300 μmoles of Tris-HCl (pH 7.4), 36 μmoles of creatine phosphate, 0.3 mg of bovine serum albumin, 0.2 mg of creatine kinase (specific activity, 20 units/mg), and 0.6 mg of partially purified *E. coli* B TdR kinase (fraction IV; specific activity, 0.15 unit/mg; see above) in a total volume of 1.5 ml.

Following incubation, the proteins were precipitated by heat denaturation and separated by centrifugation. After air oxidation (10, 11) of the 5-mercaptopyrimidines to the disulfide form (1 day), the solution was applied to Whatman No. 3MM paper and subjected to electrophoresis at a potential gradient of 20 V/cm in 0.05 M sodium citrate buffer, pH 4.5 [relative mobilities: 2.55 (ATP), 2.01 (MUdRP-DS), 1.00 (AMP), and −0.13 (MUdR-DS)]. The MUdRP-DS bands were eluted with distilled water, concentrated under vacuum, and further purified by sequential paper chromatography in two solvent systems (16): 1-butanol–NH_4OH–H_2O, 86:4:10 (by volume), and 1-butanol–formic acid–H_2O, 77:10:13 (by volume). MUdRP-DS was eluted with H_2O, evaporated to dryness under vacuum, and taken up in distilled H_2O. The ultraviolet spectra of this solution showed the same λ_{max} at different pH values as those previously reported (10) for MUdR-DS and also showed the characteristic change to the spectra of the reduced

mercapto form on the addition of dithiothreitol (10).

Since the observed λ_{max} of the 5-mercaptopyrimidine derivatives in the 330–335 mμ region was found to be a property of the thiolate ion (12, 13), this absorption band was used for the determination of the pK_a value of the sulfhydryl group of MUdRP as previously described in the case of MUdR (10). The spectrophotometric titration curve shown in Fig. 2 gives a pK_a of 5.34 (± 0.02).

The concentration of the stock solution of MUdRP used in the assays was estimated on the basis of the absorbance in the presence of 10^{-3} M dithiothreitol at pH 7.5 (ϵ_{333} = 5×10^3 $M^{-1}cm^{-1}$).

RESULTS

Inhibition of Thymidylate Synthetase in Extracts of E. coli B Cells

The crude cell free system used in this study is of interest because it simulates the conditions (relative enzyme concentrations, etc.) present in the intact cell and permits the study of the mode of action of nucleoside analogues directly in the form in which these antimetabolites are applied to the living organisms. The TdR kinase present in the crude preparation may convert the nucleoside analogues to the corresponding 5′-phosphates, provided that ATP is made available. [A similar type of analysis was employed by Hartmann and Heidelberger (17), using Ehrlich ascites carcinoma cell extracts.] The results are summarized in Table 1. It is shown that, in the presence of ATP, MUdR at 10^{-6} M concentration caused 76% inhibition of thymidylate synthetase in the case of a 25-fold excess of the substrate (deoxyuridylate). The inhibition was reversed proportionally by a 10-fold increase (250-fold excess) of the concentration of deoxyuridylate, indicating a competitive relationship between substrate and inhibitor. Complete inhibition of thymidylate synthetase was achieved at MUdR concentrations corresponding to one-fourth of that of the substrate. MeMUdR was at least three orders of magnitude less inhibitory than MUdR. In the absence of ATP, none of the compounds exhibited significant inhibitory activity at the concentrations employed.

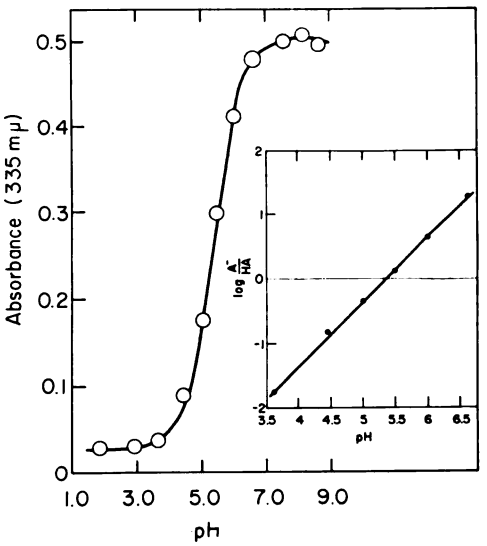


FIG. 2. *Spectrophotometric titration curve of 5-mercaptodeoxyuridylate*

The absorbance values at 335 mμ of 10^{-4} M MUdRP in 0.1 M EDTA solutions (containing 10^{-3} M dithiothreitol) were determined at 25° in a Beckman DU spectrophotometer and are plotted against the corresponding pH values (Radiometer M26); below pH 3, dilute HCl solutions were employed. The inset shows dependence on pH of the logarithm of the ratio of the anionic (A^-) and the undissociated (HA) species of MUdRP; the points were obtained from the experimental data, and the solid line represents a theoretical line corresponding to the ionization of an acid (HA) with a pK_a of 5.34 ($K_a = [H^+][A^-]/[HA]$).

Substrate Properties of MUdR and Its Derivatives in the TdR Kinase Reaction

In order to determine whether MUdR and a series of its derivatives may serve as phosphate acceptors in the TdR kinase system, the method of Okazaki and Kornberg (8) was employed to follow the conversion of ^{14}C-ATP to ^{14}C-ADP (method A). This method permits convenient screening of a variety of nucleoside analogues for their potential substrate activity by a uniform procedure. The results given in Table 2 show that MUdR and MeMUdR promote the dephosphorylation of ATP by TdR kinase of *E. coli* B and thus appear to serve as

TABLE 1

Inhibition of thymidylate synthetase in crude extracts of E. coli B

The effects of various concentrations of MUdR and MeMUdR on the methylation of deoxyuridylate in crude extracts of *E. coli* B were measured in the presence and absence of ATP at the indicated substrate concentrations by following the absorbance change at 340 mμ. For further details of the assay conditions, see MATERIALS AND METHODS.

Inhibitor	UdRP[a]	ATP, 5×10^{-3} M	Inhibition of reaction at various inhibitor concentrations[b]				
			10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
	M		%	%	%	%	%
MUdR	2.5×10^{-5}	−		2	0	0	0
	2.5×10^{-5}	+		100	97	76	15
	2.5×10^{-4}	−		0	0	0	
	2.5×10^{-4}	+		100	67	22	
MeMUdR	2.5×10^{-5}	−	0	0	0		
	2.5×10^{-5}	+	31	11	0		
	2.5×10^{-4}	−	0	0	0		
	2.5×10^{-4}	+	0	0	0		

[a] UdRP = deoxyuridylate.

[b] The inhibitions were calculated from the reaction rates obtained in the presence and absence of the inhibitors, corrected by the corresponding "blank" reactions measured in the absence of deoxyuridylate.

TABLE 2

Utilization of 5-mercapto-2'-deoxyuridine and congeners as substrates of E. coli B thymidine kinase

The various analogues (NdR) were tested at the indicated concentrations as phosphate acceptors in the purified TdR kinase system, by assaying the formation of 8-^{14}C-ADP in the reaction NdR + 8-^{14}C-ATP → NdR 5'-phosphate + 8-^{14}C-ADP. For detailed conditions, see MATERIALS AND METHODS (method A).

NdR added	ADP formed (37°)					
	MUdR	α-MUdR[a]	MeMUdR	α-MeMUdR[a]	AcMUdR[b]	MUdR-DS
mμmoles	*mμmoles/30 min*					
10	1.4	0.2	2.5	0.0	0.0	0.0
50	3.9	0.0	7.5	0.0	0.0	0.1
500	15.1	0.0	16.3	0.0	0.0	0.0

[a] The α-anomer of the corresponding nucleoside.

[b] *S*-Acetyl-5-mercaptodeoxyuridine.

phosphate acceptors. None of the other compounds tested, i.e., *S*-acetyl-5-mercaptodeoxyuridine, MUdR-DS, and the α-anomers of MUdR and MeMUdR, showed any activity as phosphate acceptors at concentrations up to 5×10^{-3} M.

The two compounds which showed activity in the above assay system were then compared with the natural substrate by studying the kinetics of the direct transfer of the γ-phosphate group of γ-^{32}P-ATP to the respective nucleosides (method B). The results represented in the Lineweaver-Burk plots in Fig. 3 indicate that MUdR and MeMUdR are indeed utilized as substrates by TdR kinase, although they are bound less strongly to the enzyme than the natural substrate. The numerical values of the apparent Michaelis constants, given in the legend of Fig. 3, are valid only for the suboptimal ATP concentration (18) used, owing to the nature of this assay.

MUdR and Its Derivatives as Inhibitors of TdR Kinase

The inhibitory effects of MUdR and its derivatives on the phosphorylation of TdR by purified *E. coli* B TdR kinase, determined by method C, are represented graphically in Fig. 4. The results show that MUdR is a competitive inhibitor of thymidine kinase, with an apparent K_i of 1.6 (± 0.7) × 10^{-3} M (K_i/K_m = 80). Similarly, the *S*-alkyl derivatives MeMUdR and 5-ethylmercaptodeoxyuridine showed competitive inhibition of this enzyme, with apparent K_i values of 3.2 (± 0.9) × 10^{-3} M (K_i/K_m = 160) and 3.5 (± 0.8) × 10^{-3} M (K_i/K_m = 175), respectively. The α-anomers of MUdR and MeMUdR showed no inhibitory activity.

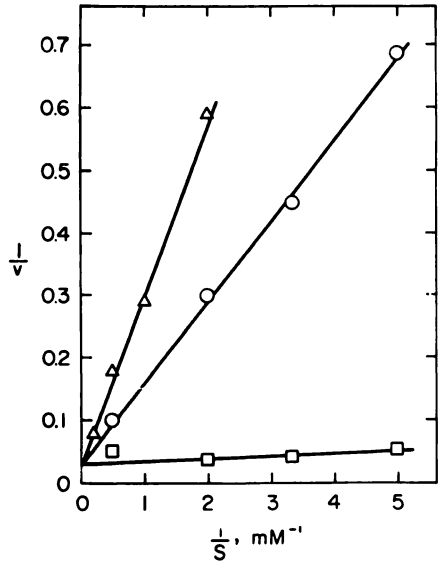


FIG. 3. *Phosphorylation of thymidine, 5-mercaptodeoxyuridine, and 5-methylmercaptodeoxyuridine by E. coli B thymidine kinase*

The reciprocal plots represent the concentration dependence of the phosphate transfer catalyzed by purified *E. coli* B TdR kinase from γ-^{32}P-ATP to various phosphate acceptors. *S* = TdR (□), MUdR (△), MeMUdR (○); *v* = nanomoles of ^{32}P transferred in 30 min at 37°. For details of assay conditions, see MATERIALS AND METHODS (method B). The apparent K_m values calculated from the slopes are 1.6 × 10^{-4} M, 9.4 × 10^{-3} M, and 4.5 × 10^{-3} M for TdR, MUdR, and MeMUdR, respectively.

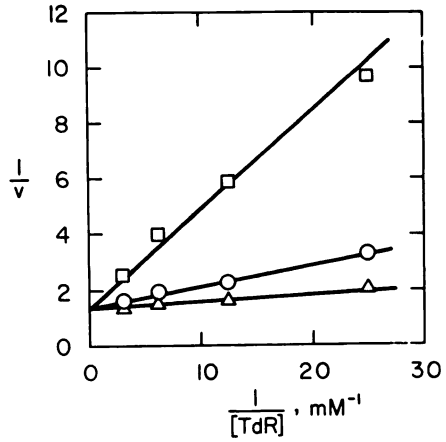


FIG. 4. *Reciprocal plots of inhibition of E. coli B thymidine kinase by 5-mercaptodeoxyuridine*

The phosphorylation of 2-^{14}C-TdR by purified *E. coli* B TdR kinase was measured in the absence of MUdR (△) and in the presence of 4 × 10^{-3} M (○) or 10^{-2} M (□) MUdR; *v* = nanomoles of thymidylate formed in 30 min at 37°. Other conditions were the same as described in MATERIALS AND METHODS (method B).

Linear double-reciprocal plots could not be obtained in the cases of *S*-acetyl-5-mercaptodeoxyuridine and MUdR-DS. These compounds irreversibly inactivated thymidine kinase; the former, by acetylation of the essential sulfhydryl group(s) of the enzyme (19), and the latter, presumably by a thiol-disulfide exchange reaction. Detailed studies of the effects of these derivatives will be reported.

Inhibition of Thymidylate Synthetase by MUdRP

The kinetics of the inhibition of purified *E. coli* B thymidylate synthetase by the 5′-monophosphate of MUdR prepared enzymatically (see MATERIALS AND METHODS) is represented graphically in Fig. 5. It is shown that MUdRP is a potent inhibitor of thymidylate synthetase, and that the inhibition is strictly competitive with respect to the substrate, deoxyuridylate. Essentially the same results were obtained whether the inhibitor was present in the reaction mixture during a 10-min preliminary incubation period or added together with the substrate. An apparent K_i value of 4.0 (± 0.2) × 10^{-8} M was obtained for MUdRP from the inter-

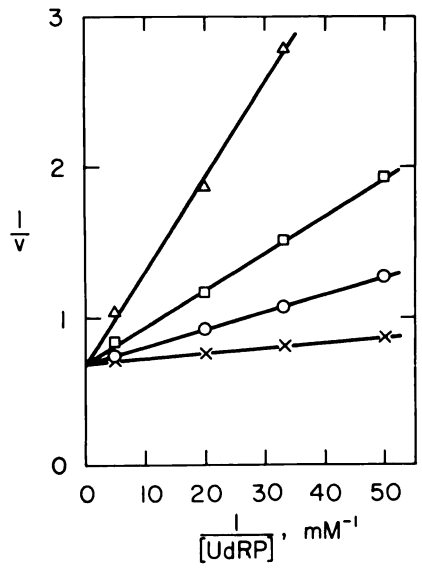


FIG. 5. *Reciprocal plots of inhibition of E. coli B thymidylate synthetase by 5-mercaptodeoxyuridylate*

Purified *E. coli* B thymidylate synthetase was assayed spectrophotometrically in the presence and absence of MUdRP by following the change in optical density at 340 mμ, corresponding to the conversion of methylenetetrahydrofolate to dihydrofolate (15). MUdRP levels were: ×, none; ○, 10^{-7} M; □, 2.5×10^{-7} M; △, 7.5×10^{-7} M; UdRP = deoxyuridylate; v = micromoles of thymidylate formed per hour per milligram of protein at 30°. Other conditions of the assay were the same as described in MATERIALS AND METHODS.

cepts of the Lineweaver-Burk plots in Fig. 5 ($K_i/K_m = 0.0085$).[5] In contrast, the nonphosphorylated nucleoside, MUdR, produced only 16% inhibition even at the highest applicable concentration (2×10^{-4} M, in the presence of a substrate concentration of 2×10^{-5} M), thus showing approximately three orders of magnitude lower activity than the corresponding 5′-phosphate.

DISCUSSION

Since the inhibition of TdR kinase by MUdR is of a relatively low magnitude, it is unlikely that this effect bears a causal relationship to the cytotoxicity of the analogue. Moreover, the concentrations of MUdR required for half-maximal growth inhibition were found to be 2×10^{-8} M and 6×10^{-8} M in *E. coli*[6] and *Lactobacillus leichmannii* (1), respectively, i.e., much lower than the K_i value of MUdR for TdR kinase. In contrast, the K_i value of MUdRP for thymidylate synthetase is virtually identical with the concentrations of MUdR required for inhibition of these bacterial systems. Therefore, the mode of action of MUdR in the cell appears to involve metabolic activation by TdR kinase and inhibition of thymidylate synthetase by the phosphorylated analogue, MUdRP, as illustrated by the scheme shown in Fig. 6.

It is of particular interest that MUdR-DS is not phosphorylated by TdR kinase. It has been demonstrated previously that MUdR undergoes very rapid, trace metal-catalyzed autoxidation in neutral (or alkaline) solutions (11) to MUdR-DS, which, however, shows the same growth-inhibitory activity as MUdR in the microbiological test systems (1). It is clear that reduction of the disulfide form prior to phosphorylation is a prerequisite for the inhibitory activity at the thymidylate synthetase site. Since alkyl thiols (including glutathione[7]) readily reduce MUdR-DS to MUdR (10), an oxidation-reduction cycle may operate in the cell, mediated by glutathione reductase and dependent on the availability of NADPH. Evidence has been obtained concerning the existence of a similar mechanism for the intracellular reduction of the disulfide of 2-mercaptopyridine (21).

The very strong binding of MUdRP to thymidylate synthetase appears to be anomalous in comparison with other known deoxyuridylate analogues. In decreasing order of binding ability, the following relationship has been observed (17, 22–27) with various substituents: $F \gg Cl > Br > I > H > CH_3$ (van der Waals' radii, 1.35, 1.80, 1.95, 2.15, 1.20, and 2.0 A, respectively; approximate pK_a values of the corresponding nucleosides, 7.8, 8.1, 8.1, 8.25, 9.35, and 9.8). Thus, an inverse relationship is apparent between the strength of binding and

[5] MUdRP has been prepared recently by chemical synthesis (20). The analytical sample yielded a K_i value of 3.4×10^{-8} M.

[6] A. Bloch, personal communication.

[7] T. I. Kalman and T. J. Bardos, unpublished observations.

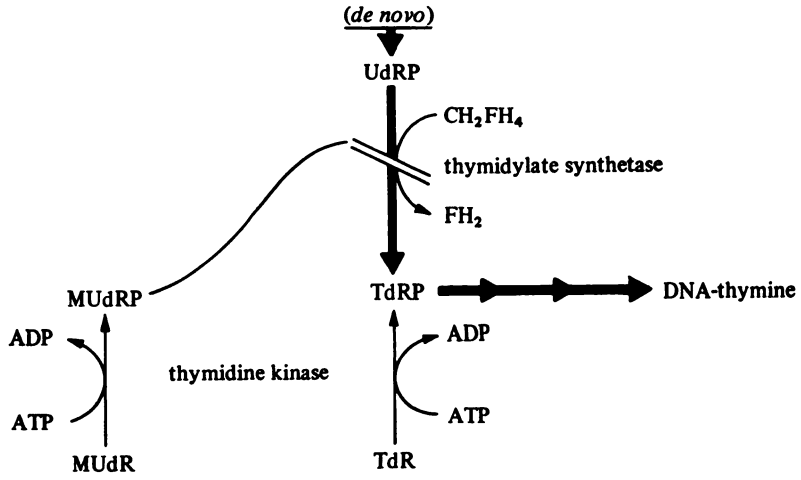


FIG. 6. *Hypothetical mode of action of 5-mercaptodeoxyuridine*

UdRP, deoxyuridylate; TdRP, thymidylate; CH_2FH_4, 5,10-methylenetetrahydrofolate; FH_2, dihydrofolate.

the size of the 5-substituent, as well as the pK_a of the N—H proton at position 3 of the ring. It has been suggested (28) that the strong binding of 5-fluorodeoxyuridylate to the enzyme may be due to the acidic proton at N-3 of the pyrimidine ring. By analogy, MUdRP should have very little or no inhibitory activity, since the pK_a for the ionization of the N-3 hydrogen of the 5-mercaptopyrimidines is 10.5–10.6 (10), and the ionic radius of S^- is 2.19 A (the van der Waals' radius of S is 1.85 A); despite this fact, MUdRP is a close second to 5-fluorodeoxyuridylate in its strength of binding to thymidylate synthetase. However, since the sulfhydryl group of MUdRP is essentially ionized at physiological pH (pK_a 5.34, Fig. 2), it appears that the "anomalous" high activity of this analogue may be due to a significant contribution of the negative charge on the S^- anion to the binding. This conclusion is supported by the observation that the *S*-methyl analogue, MeMUdR,[8] did not show significant inhibition of thymidylate synthetase in the cell extract and demonstrated very low cytotoxicity[9] in the same microbiological systems in which MUdR showed high activity. The similar substrate activities of MeMUdR and MUdR in the TdR kinase system rule out the possibility that the inactivity of MeMUdR may be due to a lack of phosphorylation.

The exceptional behavior of MUdRP can be explained if we assume that there is an appropriately positioned cationic binding site on the enzyme that can interact either with the thiolate anion of MUdRP or with the ionized species of 5-fluorodeoxyuridylate, as illustrated in Fig. 7. This would satisfactorily account for the strong, competitively reversible binding of both substrate analogues. It is important to note, however, that there is a significant difference between

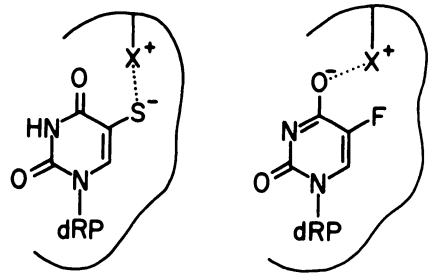


FIG. 7. *Postulated mode of ionic binding of MUdRP and 5-fluorodeoxyuridylate to thymidylate synthetase*

dRP, deoxyribose 5-phosphate; X^+, cationic binding site of the enzyme.

[8] The pK_a (NH-3) of MeMUdR is 9.0 (T. I. Kalman, unpublished observations).

[9] T. I. Kalman, K. Baranski and T. J. Bardos, unpublished observations.

the modes of interaction of MUdRP and 5-fluorodeoxyuridylate with thymidylate synthetase. It has been reported by several investigators that preliminary incubation of thymidylate synthetase with 5-fluorodeoxyuridylate markedly decreases the K_i value of the inhibitor and results in a change to noncompetitive or mixed kinetics (23–25, 27, 29). In contrast, prior incubation of the enzyme with MUdRP did not affect the strictly competitive kinetics of the inhibition produced by the latter compound. This difference in the behavior of the two pyrimidine nucleotide analogues may be due to the opposite polarization of the 5,6-double bond by the respective 5-substituents. The fluoro substituent activates the 6-position of the pyrimidine ring (30) to attack by a nucleophilic group on the enzyme (31), possibly leading to a pseudo-irreversible enzyme-inhibitor complex. In contrast, the negative charge of the ionized thiol group of MUdRP increases the electron density in the pyrimidine ring, making the latter unreactive toward nucleophilic attack.

Further studies with these and related analogues may be helpful in the elucidation of the nature of the active site of thymidylate synthetase.

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