2′-Deoxy-2′-halonucleotides as Alternate Substrates and Mechanism-Based Inactivators of *Lactobacillus leichmannii* Ribonucleotide Reductase†

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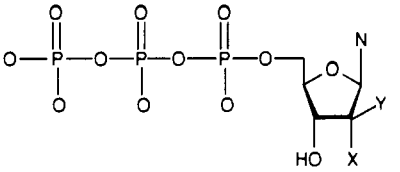
ABSTRACT: The interaction of the ribonucleoside-triphosphate reductase of *Lactobacillus leichmannii* with various 2′-halogenated ribo- and arabinonucleoside triphosphates has been investigated. All analogues examined acted as mechanism-based inactivators of the enzyme, producing base, triphosphate, and halide. In all cases, the inactive enzyme had developed the distinctive chromophore at 320 nm that is characteristic of enzyme inactivated by 2-methylene-3(2*H*)-furanone. The striking similarities between these results and those previously reported for the inactivation of this enzyme by 2′-chloro-2′-deoxyuridine triphosphate suggest a common reaction path for all 2′-halonucleotides. In the pyrimidine series, it was found that 2′-fluoro- and 2′-chloronucleotides partitioned between inactivation and formation of the normal reduction product 2′-deoxynucleotide. Normal reduction predominated with 2′-fluoronucleotides, whereas it was a minor pathway for 2′-chloro-2′-deoxyuridine triphosphate. With 2′-chloro-2′-deoxyuridine triphosphate, the relative partitioning between the two modes was pH dependent: the amount of 2′-deoxyuridine triphosphate formed increased 2.8-fold upon changing from pH 6.1 to pH 8.3. The ability of 2′-arabinohalonucleotides to inactivate ribonucleotide reductase and the variation of partitioning of the pyrimidine analogues with leaving group and reaction pH are consistent with our radical cation hypothesis and support the proposal that the difference between normal catalysis and inactivation is related to the protonation state of the reductase.

The ribonucleotide reductases catalyze the conversion of ribonucleotides to 2′-deoxyribonucleotides. These enzymes are uniquely responsible for de novo deoxynucleotide production and hence play a key role in DNA biosynthesis (Thelander & Reichard, 1979; Lammers & Follmann, 1983; Ashley & Stubbe, 1987). As such, they are prime targets for the design of new antiviral and antitumor agents. While the nucleotide specificity of the reductases has been investigated in some detail by a number of laboratories, the only alternate substrates and competitive inhibitors have possessed modified bases (Suhadolnik et al., 1968; Ludwig & Follmann, 1978). A small number of ribosyl-modified nucleotides, including 2′- and 3′-*O*-methylribonucleotides, xylonucleotides, 3′-deoxynucleotides, and arabinonucleotides, have been found to be neither substrates nor good competitive inhibitors of normal substrate reduction (Lammers & Follmann, 1983; Follmann & Hogenkamp, 1971). These results have led to the view that, although the base specificity is broad, the intact ribosyl unit is necessary for substrate recognition by ribonucleotide reductases.

In addition, a number of 2′-substituted 2′-deoxynucleotides (Table I: X = F, Cl, N_3; Y = H) have been shown to be mechanism-based inactivators of reductases from different sources (Thelander et al., 1976; Stubbe & Kozarich, 1980a,b; Ator & Stubbe, 1985; Harris et al., 1984). Inactivation of the adenosylcobalamin-dependent ribonucleoside-triphosphate reductase (RTPR)[1] from *Lactobacillus leichmannii* and the tyrosyl radical containing ribonucleoside-diphosphate reductase (RDPR) of *Escherichia coli* by 2′-chloro-2′-deoxyuridine

Table I: Substrate Analogues of RTPR



substrate 5′-triphosphate	abbreviation	N	X	Y
2′-chloro-2′-deoxyadenosine	ClATP	A	Cl	H
9-(2-chloro-2-deoxy-β-D-arabinofuranosyl)-adenine	*ara*-ClATP	A	H	Cl
2′-bromo-2′-deoxyadenosine	BrATP	A	Br	H
9-(2-bromo-2-deoxy-β-D-arabinofuranosyl)-adenine	*ara*-BrATP	A	H	Br
2′-iodo-2′-deoxyadenosine	IATP	A	I	H
2′-chloro-2′-deoxyuridine	ClUTP	U	Cl	H
2′-fluoro-2′-deoxyuridine	FUTP	U	F	H
2′-fluoro-2′-deoxycytidine	FCTP	C	F	H

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[1] Abbreviations: RTPR, ribonucleoside-triphosphate reductase; AdoCbl, (5′-deoxyadenosyl)cobalamin; ClUDP, 2′-chloro-2′-deoxyuridine 5′-diphosphate; FU, 2′-fluoro-2′-deoxyuridine; FC, 2′-fluoro-2′-deoxycytidine; dU, 2′-deoxyuridine; dC, 2′-deoxycytidine; ClA, 2′-chloro-2′-deoxyadenosine; ClU, 2′-chloro-2′-deoxyuridine; BrA, 2′-bromo-2′-deoxyadenosine; IA, 2′-iodo-2′-deoxyadenosine; *ara*-BrA, 9-(2-bromo-2-deoxy-β-D-arabinofuranosyl)adenine; *ara*-ClA, 9-(2-chloro-2-deoxy-β-D-arabinofuranosyl)adenine; PP_i, inorganic pyrophosphate; PPP_i, triphosphate; RDPR, ribonucleoside-diphosphate reductase; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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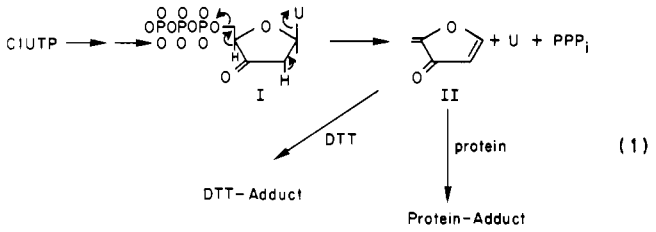
nucleotides has been studied in detail. Both reductases have been shown to produce 3′-keto-2′-deoxyuridine nucleotides as initial products. These unstable ketonucleotides subsequently undergo elimination of uracil and the phosphate moiety (PP_i or PPP_i) to produce 2-methylene-3(2*H*)-furanone, which is proposed to be responsible for enzyme inactivation (eq 1).



(1)

Such inactivation studies have provided much insight into the catalytic capabilities of these two enzymes.

We now report the first alternate substrates for the ribonucleoside-triphosphate reductase (RTPR) from *L. leichmannii* having modified ribosyl moieties, 2′-fluoro-2′-deoxyuridine 5′-triphosphate (FUTP), 2′-fluoro-2′-deoxycytidine 5′-triphosphate (FCTP), and 2′-chloro-2′-deoxyuridine 5′-triphosphate (ClUTP). In addition, a number of 2′-arabinohalo-2′-deoxynucleoside triphosphates are shown to be mechanism-based inactivators of RTPR. Inactivation appears to proceed by a mechanism similar to that previously elucidated for the inactivation of RTPR by ClUTP. The implications of these findings to the proposed radical cation mechanism for ribonucleotide reduction are discussed (Ashley et al., 1986).

Materials and Methods

General. Ribonucleoside-triphosphate reductase was isolated from *Lactobacillus leichmannii* (ATCC 7830) according to the procedure of Ashley et al. (1986) and had a specific activity of 1.2–1.5 μmol min^{-1} mg^{-1}. The enzyme was judged to be nearly homogeneous by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). Protein concentrations were determined by UV/vis spectrophotometry, with E_{280} (1%) = 1.33 (Blakley, 1978). Prereduced RTPR was prepared as described by Ashley et al. (1986). All reactions involving AdoCbl were carried out in the dark at 37 °C.

Standard activity assays were carried out in 500 μL of a mixture containing 10 mM ATP, 30 mM DTT, 12 μM AdoCbl, 1 M sodium acetate, and 50 mM potassium phosphate, pH 7.8, at 37 °C in the dark. The dATP formed was quantitated by the diphenylamine procedure (Blakley, 1978).

Carrier-free $H_3{}^{32}PO_4$ was purchased from New England Nuclear. $POCl_3$, triethyl phosphate, and carbonyldiimidazole were purchased from Aldrich Chemical Co. Sep-Pak C_{18} cartridges were from Waters Associates. *E. coli* alkaline phosphatase (30 μmol min^{-1} mg^{-1}), nucleoside diphosphate kinase (1084 μmol min^{-1} mg^{-1}), and ATP from Sigma Chemical Co. Polynucleotide phosphorylase was from P-L Biochemicals. Deoxyuridine hydroxylase (0.33 μmol min^{-1} mg^{-1}) was isolated by the procedure of Stubbe (1985), and 2-(ethylthiomethyl)-3(2*H*)-furanone was prepared according to Harris et al. (1984). HPLC analyses were performed on an Altex HPLC system equipped with a 4 × 300 mm Alltech C_{18} reversed-phase column at a flow rate of 1.7 mL/min. NMR spectra were obtained on a Bruker 270 MHz spectrometer. Release of fluoride and bromide was measured with Orion ion-selective electrodes 96-09 and 90019/943500, respectively. Standard curves were prepared from Orion fluoride and bromide standard solutions immediately before and after the experiments. The adenosine analogues were prepared by S. D. Hawrelak and M. J. Robins (Robins, 1982; Robins et al., 1983), while 2′-fluoro-2′-deoxyuridine was prepared by R. L. Tolman by a modification of a published procedure (Codington et al., 1964) and purified on Merck AG silica gel 7734 with $CH_2Cl_2/CH_3OH/H_2O$ (90:10:1) as eluant. All nucleosides were phosphorylated to the monophosphates with $POCl_3$ in triethyl phosphate according to Yoshikawa et al. (1967) and then to the triphosphates with carbonyldiimidazole and tributylammonium pyrophosphate according to Hoard and Ott (1965).

Reaction mixtures containing enzymes were deproteinized before injection onto the HPLC by heating for 1 min in a boiling water bath followed by centrifugation to pellet the denatured proteins. Anion-exchange chromatography was performed on DEAE-Sephadex A-25 resin, elution being with linear gradients of 0–0.8 M triethylammonium bicarbonate.

Characterization of ATP Analogues. Conversion of the AMP analogues to their corresponding triphosphates according to Hoard and Ott (1965) generally produced a mixture of the desired ATP analogue (70–90%) and the 3′-(methoxycarbonyl)-ATP analogue (10–30%). This latter compound, identified by NMR spectroscopy, arises during destruction of excess carbonyldiimidazole with CH_3OH. We have recently established that this contaminant can be avoided by replacing the CH_3OH with H_2O in the original procedure. All ATP analogues were isolated from the reaction mixture by anion-exchange chromatography. BrATP and ClATP were further purified by ion-pairing reversed-phase HPLC (50 mM potassium phosphate, pH 4.8, 5 mM tetrabutylammonium bromide, and 30% CH_3OH). The retention times were as follows: BrATP, 6 min, 3′-(methyoxycarbonyl)-BrATP, 18 min; ClATP, 7.5 min, 3′-(methoxycarbonyl)-ClATP, 24 min. The other analogues were not purified in this manner due to the small amount of material available. To ensure the identity of these analogues, 13 nmol of *ara*-ClATP, *ara*-BrATP, and IATP were each treated with 1.6 units of alkaline phosphatase in 100 μL of 0.2 M Tris, pH 8.5, and 5 mM $MgCl_2$, at 37 °C for 2 h. In each case, a single nucleoside that cochromatographed with authentic nucleoside was observed by reversed-phase HPLC. Retention times in 5 mM potassium phosphate, pH 6.7, and 15% CH_3OH were 17.4 min for *ara*-ClA and 9.4 min for IA and in 5 mM potassium phosphate, pH 6.7, and 20% CH_3OH 8 min for *ara*-BrA.

Reaction of FUTP and FCTP with RTPR: Elimination of F^- and Uracil and Production of dUTP or dCTP. All measurements were made in 10-mL polystyrene beakers thermostated at 37 °C. A standard curve for the fluoride electrode covering the range of 0.005–1.0 mM F^- was prepared before and after each experiment by repeated additions of the standard fluoride solution to a 1-mL sample containing 50 mM potassium phosphate, pH 7.8, 1 M sodium acetate, and 3 mM DTT. The incubation mixture contained 50 mM potassium phosphate, pH 7.8, 1 M sodium acetate, 0.5 mM FUTP, 3 mM DTT, 1 mM EDTA, 13 nmol of RTPR, and 0.11 mM AdoCbl in a total volume of 1.0 mL. A control omitting RTPR was run in parallel. Reaction was initiated by addition of AdoCbl, and release of F^- was monitored continuously. After 20 min, the reaction was diluted with 50 mL of H_2O and chromatographed on DEAE-Sephadex A-25 (2 × 6.4 cm), with collection of 4.0-mL fractions. Fraction 1–22 were pooled and evaporated to dryness in vacuo, and the residue was dissolved in 0.6 mL of H_2O. A 0.2-mL aliquot of this material was analyzed by HPLC. Fractions containing uracil (retention times = 3.6 min) were pooled, evaporated to dryness, and redissolved in a small volume of H_2O. The uracil concentration

was determined by UV spectrophotometer with ϵ_{260} = 8200 M^{-1} cm^{-1}. Addition of NaOH to the sample resulted in a spectral shift to λ_{max} = 284 nm with a decrease in ϵ, characteristic of uracil. The amount of uracil recovered was 36 nmol.

Fractions from DEAE-Sephadex containing nucleoside triphosphates (84–100) were pooled, evaporated to dryness, and redissolved in 2 mL of H_2O. Recovered nucleotides were quantitated with ϵ_{260} = 10 000 M^{-1} cm^{-1}, giving 500 and 520 nmol in the experiment and control, respectively. A 0.1-μmol aliquot was treated with 1.6 units of alkaline phosphatase in 100 μL of 80 mM Tris-HCl, pH 8.5, and 2 mM $MgCl_2$. The resulting nucleosides were separated by HPLC (2% CH_3OH/H_2O), yielding 260 nmol of dU (retention time = 12 min) from the experiment and 310 nmol of FU (retention time = 16.5 min) from the control.

In a similar experiment, 0.7 mM FCTP replaced 0.5 mM FUTP. Cytosine was quantitated by UV spectrophotometry after isolation by HPLC (retention time = 3.0 min) with ϵ_{267} = 6100 M^{-1} cm^{-1}. Upon addition of NaOH, the spectrum shifted to λ_{max} = 282 nm with a slight increase in ϵ, which is characteristic of cytosine. The amount of cytosine recovered was 100 nmol. Nucleotide triphosphates (560 nmol from the experiment and 590 nmol from the control) were recovered and treated with alkaline phosphatase as above. HPLC separation of the nucleosides (25 mM potassium phosphate, pH 6.6) gave 180 nmol of dC and 93 nmol of FC from the experiment, while 297 nmol of FC was recovered from the control.

Formation of dUTP during RTPR-Catalyzed Turnover of ClUTP. The incubation mixture contained 50 mM potassium phosphate, pH 7.8, 1 M sodium acetate, 3 mM DTT, 1 mM EDTA, 2 mM [5′-^{3}H]ClUTP (3.1 × 10^6 cpm/μmol), 21.2 nmol of RTPR, and 1.6 mM AdoCbl in a total volume of 1.2 mL. A control omitting AdoCbl was run in parallel. The samples were kept at 37 °C in the dark, and additional DTT was added after 15 and 45 min to bring the total DTT concentration to 5.25 mM. After 60 min, unlabeled dUTP (20 nmol) and ClUTP (200 nmol) were added as carriers. The samples were diluted to 5 mL with cold H_2O and chromatographed on DEAE-Sephadex A-25 (1 × 6 cm). The nucleoside triphosphate fractions were pooled and evaporated to dryness. The residue was treated with 1.6 units of alkaline phosphatase in 100 μL of 75 mM Tris-HCl, pH 8.5, and 6 mM $MgCl_2$, for 1 h at 37 °C. The resulting nucleosides were analyzed by HPLC (2% CH_3OH/H_2O for 22 min, followed by a linear gradient to 20% CH_3OH over 5 min). dU and ClU migrated with retention times of 12 and 30 min, respectively. Fractions of 1.7 mL were collected and analyzed for radioactivity by scintillation counting. The dU was further analyzed by conversion to uridine as described below.

Effect of Reaction pH on Conversion of ClUTP to dUTP. Buffers used were as follows: sodium acetate, pH 5.5; sodium citrate, pH 6.1, Tris-HCl, pH 7.4 and pH 8.3. Each reaction mixture contained 3 nmol of RTPR, 260 nmol of [5′-^{3}H]-ClUTP (1.4 × 10^7 cpm/μmol), 260 nmol of AdoCbl, 30 mM DTT, and 0.3 M buffer in a total volume of 260 μL. Reactions were initiated by addition of AdoCbl and were kept for 1 h at 37 °C. Unlabeled dUTP (100 nmol) was added, and the mixture was filtered through a Sep-Pak C_{18} cartridge with H_2O to remove cofactor and cofactor byproducts. The filtrate was lyophilized, and the residue was treated with 1.6 units of alkaline phosphatase in 450 μL of 0.5 M Tris-HCl, pH 8.5, 10 mM $MgCl_2$, and 10 mM $ZnCl_2$. After 3 h at 37 °C, the mixture was neutralized with 40 μL of 6 N HCl, deproteinized, and analyzed by HPLC (H_2O). Eluted dU (retention time = 12 min) was analyzed by scintillation counting. As dUTP produced during the reaction would have a specific activity of 1.4 × 10^7 cpm/μmol, addition of 100 nmol of unlabeled dUTP as carrier before analysis would give dUTP having an observed specific activity of 1.4 × 10^5 for every 1 nmol of dUTP produced from ClUTP. Thus, production of [5′-^{3}H]-dUTP during turnover was calculated from the measured specific activity of the isolated dU with a conversion factor 1.4 × 10^5 cpm/μmol for each 1 nmol of [5′-^{3}H]dUTP produced.

Conversion of Deoxyuridine to Uridine Catalyzed by Deoxyuridine Hydroxylase. The reaction mixture contained in a total volume of 50 μL 1 mM α-ketoglutarate, 3 mM ascorbate, 1.5 mM $Fe(NH_4)_2(SO_4)_2$, 10 300 units of catalase, 0.30 mM dU, 25 mM sodium phosphate, pH 7.5, and 0.16 unit of deoxyuridine hydroxylase. A control using authentic dU was run in parallel. After 30 min at 25 °C, the mixture was deproteinized, and the supernatant was analyzed by HPLC (4% CH_3OH/H_2O). Uridine (retention time = 6 min) and dU (retention time = 8.5 min) were quantitated from peak areas. Identical amounts of turnover were observed in the experiment and control.

Time-Dependent Inactivations. Inactivation mixtures contained 50 mM potassium phosphate, pH 7.8, 1 M sodium acetate, 0.2–1.0 mM substrate analogue, 0.013–0.26 mM AdoCbl, 3 mM DTT, 1 mM EDTA, and 3.7 nmol of RTPR in a total volume of 140 μL and were kept at 37 °C. Aliquots of 20 μL were removed at intervals and added to 500 μL of the standard activity assay mixture. Time-dependent inactivations in the absence of DTT were carried out by the same procedure using prereduced RTPR (Ashley et al., 1986).

RTPR Inactivation by ara-ClATP: Formation of A_{320}. RTPR was inactivated by *ara*-ClATP as described above in a volume of 340 μL. A control omitting AdoCbl was run in parallel. After 45 min at 37 °C, the protein was isolated by gel filtration on Sephadex G-50 (1.2 × 29 cm) with 10 mM potassium phosphate, pH 7.3. The UV/vis spectrum was recorded from 400 to 230 nm.

Release of Adenine from ATP Analogues. A typical reaction contained 50 mM potassium phosphate, pH 7.8, 1 M sodium acetate, 0.26 mM substrate analogue, 3.7 nmol of RTPR, and 0.26 mM AdoCbl in a total volume of 140 μL. A control omitting RTPR was run in parallel. Reaction was initiated by addition of AdoCbl, and 20-μL aliquots were periodically removed to assay for RTPR activity by addition to 500 μL of the ATP assay mix described above. At the end of the reaction, the mixture was deproteinized, and the supernatant was analyzed by HPLC (10% CH_3OH/H_2O). Adenine and 5′-deoxyadenosine migrated with retention times of 7.5 and 15 min, respectively.

Preparation of [β-^{32}P]-ara-BrATP. The reaction mixture contained 0.1 M Tris-HCl, pH 8.7, 5 mM $MgCl_2$, 0.3 mM EDTA, 3.8 mM *ara*-BrADP, 2.5 mM KH_2PO_4, 1.5 mCi of $H_3{}^{32}PO_4$, and 7.5 units of polynucleotide phosphorylase in a total volume of 0.5 mL. After 12 h at ambient temperature, 20 μL of 1 M Tris–acetate, pH 7.2, 2.5 μmol of UTP, and 20 units of nucleoside diphosphate kinase were added. After 20 min, the sample was diluted with cold H_2O and chromatographed on DEAE-Sephadex A-25 (1.2 × 12 cm). Nucleotide fractions were pooled and evaporated to dryness in vacuo. The residue was dissolved in H_2O and purified by ion-pairing reversed-phase HPLC in 50 mM potassium phosphate, pH 6.0, 5 mM $Bu_4N^+Br^-$, and 30% CH_3OH. UTP and *ara*-BrATP had retention times of 4 and 7.5 min, respectively. Ion-pairing reagents were removed by a second chromatography on

Table II: Inactivation of RTPR by 2′-Deoxy-2′-halonucleoside Triphosphates

substrate	concn (mM)	$t_{1/2}$ (min) + 3 mM DTT fast phase	$t_{1/2}$ (min) + 3 mM DTT slow phase	concn (mM)	$t_{1/2}$ (min), no DTT fast phase	$t_{1/2}$ (min), no DTT slow phase
ClATP	0.5	1.5 ± 0.5[a]	13 ± 3	0.26	1 ± 0.5	
ara-ClATP	0.5	4.5 ± 0.5[a]	19 ± 2	0.26	1[a]	5
BrATP	0.5	3.8 ± 0.5[a]	19 ± 7	0.26	1.3[a]	10
ara-BrATP	0.5	4.0 ± 0.5[a]	31 ± 1	0.26	3.5 ± 0.5	
IATP	0.5	4.0 ± 0.5[a]	26 ± 6	0.26	16	
ClUTP	2	9 ± 2		2	<1.0	
FUTP	0.5	24, 40			ND[b]	
FCTP	0.5	44			ND	

[a] Biphasic kinetics. [b] ND, not determined.

DEAE-Sephadex as before. Nucleoside triphosphate containing fractions were pooled and evaporated to dryness to give 0.79 μmol of [β-^{32}P]-*ara*-BrATP, specific activity = 4.0 × 10^7 cpm/μmol. Phosphate analysis (Ames & Dubin, 1960) demonstrated 3.0 mol of phosphate/mol of nucleoside. A sample of the product was also treated with alkaline phosphatase (100 μL of 0.1 M Tris-HCl, pH 8.5, 2 mM $MgCl_2$); the resulting nucleoside comigrated with authentic *ara*-BrA by HPLC.

Release of [β-^{32}P]PPP$_I$, Bromide, and Adenine in the Reaction of RTPR with [β-^{32}P]-ara-BrATP. Reactions were run in 30-mL glass beakers thermostated at 25 °C. The reaction mixture contained 100 mM Tris–acetate, pH 7.2, 1 M sodium acetate, 250 nmol of [β-^{32}P]-*ara*-BrATP (4.0 × 10^7 cpm/μmol), 50 nmol of RTPR, and 100 nmol of AdoCbl in a total volume of 1.0 mL. A control omitting AdoCbl was run in parallel. RTPR activity was measured in 2-μL aliquots removed at the beginning and end of the reaction. The reaction was initiated be addition of AdoCbl, and release of Br^- was monitored continuously until no further changes were noted. A Br^- standard curve was subsequently generated by addition of known quantities of NaBr to the control mixture, and quantitation of Br^- released in the experiment was determined from this standard curve. After 10 min at 37 °C, the reaction mixture was divided into two portions: (1) release of adenine was determined on 320 μL by HPLC as described above, and (2) release of [β-^{32}P]PPP$_I$ was determined by mixing 500 μL of the sample with 9.4 μmol of unlabeled PPP$_i$ carrier and 0.25 g of acid-washed charcoal in 10 mL of 75 mM ammonium acetate, pH 5.0. After 10 min, the charcoal was removed by centrifugation, and the supernatant was filtered through glass wool. The amount of PPP$_i$ was determined by scintillation counting, and the overall recovery of PPP$_i$ was measured by phosphate assay (Ames & Dubin, 1960). A control omitting RTPR was run in parallel.

Inactivation of RTPR by 2-(Ethylthiomethyl)-3(2H)-furanone. The stock solution of 2-(ethylthiomethyl)-3-(3*H*)-furanone in ethanol was quantitated by UV spectrophotometry, with ϵ_{260} = 4000 M^{-1} cm^{-1}. The reaction mixture contained RTPR (0.1 mg), 20 μM AdoCbl, and 1.0 mM inactivator in 300 μL of 50 mM potassium phosphate, pH 7.3. A control substituting ethanol in place of the ethanolic inactivator solution was run in parallel. Aliquots of 50 μL were periodically removed and added to 500 μL of the standard assay mixture.

Results

Time-Dependent Inactivation of RTPR by 2′-Halonucleotides. Studies designed to examine the effects of a variety of ribo and arabino halogenated nucleotide substrate analogues (Table I) on RTPR reveal that all of these compounds act as mechanism-based inactivators in a fashion similar to the previously described ClUTP (eq 1; Harris et al., 1984; Stubbe et al., 1983b). The $t_{1/2}$ values for the inactivations are listed in Table II. While DTT was included in early experiments, later investigations with ClUTP (Harris et al., 1984) showed that a reductant was not essential for inactivation and in fact typically slowed the inactivation process; therefore, inactivation experiments were repeated in the absence of DTT with prereduced RTPR (Ashley et al., 1986). In the presence of DTT, the 2′-halo-ATP analogues displayed biphasic kinetics with a rapid initial phase resulting in 20–30% activity loss followed by a slower second phase. These results contrast with those from studies using ClUTP, FUTP, or FCTP, where pseudo-first-order inactivation kinetics are observed.

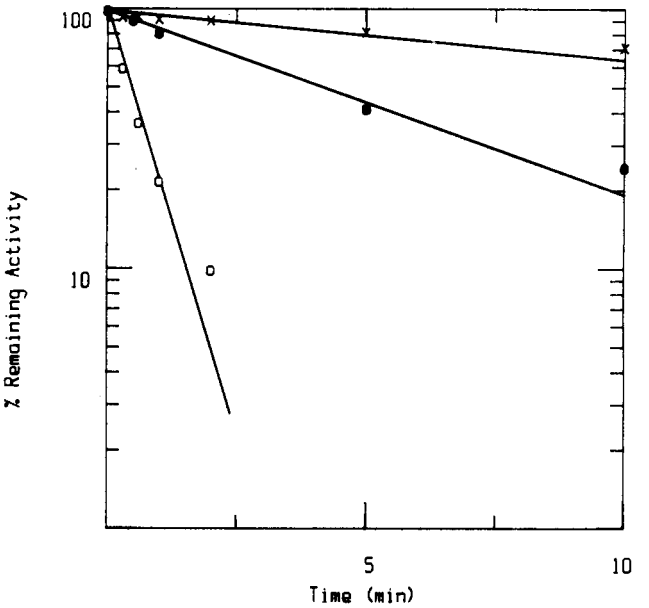


FIGURE 1: Time-dependent inactivation of ribonucleotide reductase by 2′-halogenated ATP analogues. Prereduced enzyme was treated with inactivator in the absence of DTT as described under Materials and Methods: (○) ClATP; (●) *ara*-BrATP; (×) IATP.

With ClATP, IATP, and *ara*-BrATP, use of prereduced RTPR eliminated the biphasic behavior (Figure 1). *Ara*-ClATP and *ara*-BrATP continued to show complex kinetics in the absence of DTT (Figure 2), however. The reason for this behavior is not clear at present.

Inactivation of RTPR Is Accompanied by an Increase in Protein Absorbance at 320 nm. RTPR was inactivated by all of the analogues in this study, and in each case, the UV/vis spectrum of the RTPR showed an increase in the absorbance near 320 nm. A representative example using *ara*-ClATP is shown in Figure 3, and results for other analogues are given in Table III. Recent model studies have indicated that this new absorbance results from further reaction of enzyme-bound dihydrofuranone [generated by alkylation of protein residues by 2-methylene-3(2*H*)-furanone] with protein amino groups to form β-amino enones (G. W. Ashley, unpublished results).

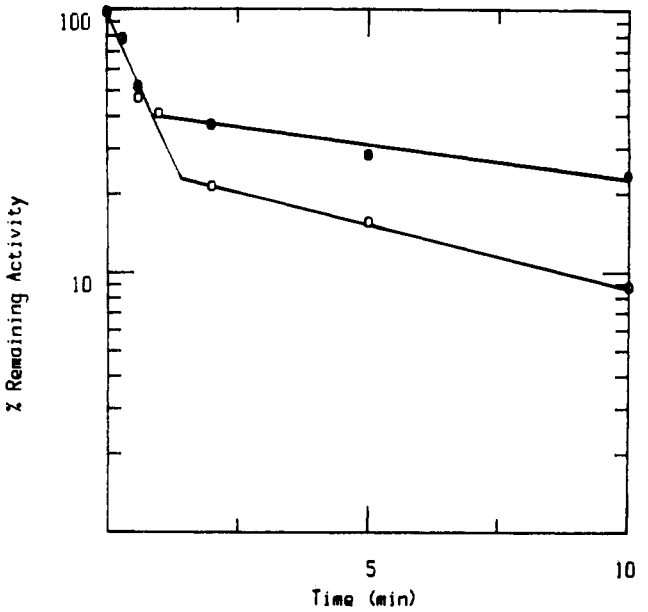


FIGURE 2: Time-dependent inactivation of ribonucleotide reductase by 2′-halogenated ATP analogues showing biphasic kinetics. Prereduced enzyme was treated with inactivator in the absence of DTT as described under Materials and Methods: (○) *ara*-ClATP; (●) BrATP.

Table III: Formation of A_{320} on RTPR following Inactivation[a]

substrate	concn (mM)	time (min)	% activity remaining	A_{280}/A_{320}
ClATP	0.5	45	26	4.5
ara-ClATP	0.5	45	38	4.8
BrATP	0.5	45	49	5.9
ara-BrATP	0.5	45	20	4.1
IATP	0.13	60	35	6.3
ClUTP	2.0	45	20	3.7
FCTP	0.7	60	51	5.4
FUTP	0.5	60	68	11.3

[a] Reactions were performed with prereduced RTPR in the absence of DTT except for FCTP and FUTP, which were performed in the presence of 3 mM DTT. At the end of the indicated reaction time, the protein was isolated by gel filtration chromatography, and its optical spectrum was recorded.

Thus, it appears that all the 2′-halonucleotides examined in this study are capable of forming 2-methylene-3(2*H*)-furanone.

Release of Adenine from 2′-Halo-ATP Analogues. If the inactivation of RTPR by the 2′-halo-ATP analogues proceeds via formation of 3′-keto-dATP and subsequently 2-methylene-3(2*H*)-furanone in a mechanism similar to that proposed for ClUTP (eq 1), then inactivation of RTPR should be accompanied by formation of adenine. This hypothesis was tested by analyzing the inactivation mixtures containing ClATP, *ara*-ClATP, BrATP, *ara*-BrATP, and IATP, by HPLC. In all cases, 2–6 equiv of adenine was observed per equivalent of RTPR inactivated (Table IV). While the estimated error in adenine quantitation by this method is ±50%, controls omitting RTPR established the enzyme dependence of adenine formation.

Release of Adenine, Bromide, and PPP_i from ara-BrATP. In analogy with studies on ClUTP, release of PPP_i should also accompany the inactivation of RTPR by *ara*-BrATP. Treatment of 50 nmol of RTPR with 250 nmol of [β-^{32}P]-*ara*-BrATP led to complete inactivation of RTPR and concomitant formation of 150 nmol of Br^- and 145 nmol of ^{32}P-labeled material not absorbable on charcoal. Attempts to demonstrate the identity of this material by cochromatography with authentic PPP_i were thwarted by the presence of a contaminating inorganic tripolyphosphatase activity in the RTPR preparation. Thus, anion exchange of the charcoal-treated material demonstrated only that all ^{32}P label was present as inorganic phosphate. Assay of the RTPR preparation used in this experiment showed inorganic tripolyphosphatase activity capable of converting 7 nmol of PPP_i to inorganic phosphate per minute per milligram of protein, sufficient to destroy 8 times the quantity of PPP_i formed in the experiment. As only 0.5 nmol of ^{32}P label was not absorbed by charcoal in a control reaction omitting AdoCbl, the ^{32}P label found as inorganic phosphate in the experiment cannot have arisen from phosphatase activity on [β-^{32}P]-*ara*-BrATP; thus, given the demonstrated tripolyphosphatase contaminant, it is reasonable to postulate PPP_i formation in this reaction. The amount of adenine formed in this experiment was determined after isolation by HPLC to be approximately 113 nmol. These results are consistent with the mechanism of *ara*-BrATP inactivation being analogous to that proposed for ClUTP. Control experiments indicated that no PPP_i, Br^-, or adenine is formed in the absence of either RTPR or AdoCbl.

It was observed that the Br^- electrode was extremely sensitive to low concentrations of thiols. Addition of 10 μM DTT to a standard curve determination led to vast changes in millivolt readings (differences of up to 20 mV) over the standard curve. Although the DTT used to prereduce the RTPR was removed by Penefsky column (Penefsky, 1977) before beginning the experiment, addition of RTPR to the reaction mixture led to a drop of 15 mV, even in the absence of *ara*-BrATP. Whether this is due to incomplete removal of

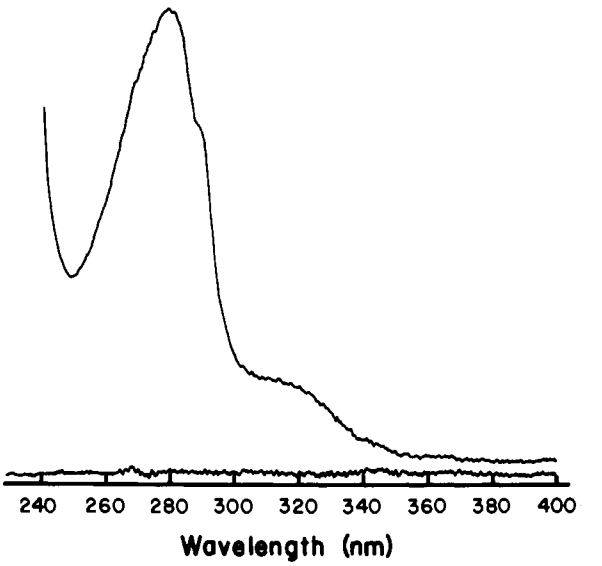


FIGURE 3: Absorbance spectrum of ribonucleotide reductase after inactivation by *ara*-ClATP. Inactivation was performed as described under Materials and Methods, and the protein was isolated by gel filtration. Inactivation results in formation of a new protein-bound chromophore absorbing maximally near 320 nm.

Table IV: Release of Adenine from 2′-Halogenated ATP analogues[a]

substrate	concn (mM)	% activity remaining	equiv of adenine	
			obsd	corrected[b]
ClATP	0.26	0	3.2	3.2
ara-ClATP	0.26	6	5.1	5.4
BrATP	0.26	ND	1.4	ND
ara-BrATP	0.25	0	2.3	2.3
IATP[c]	0.52	45	1.7	3.1

[a] Protocol is described under Materials and Methods. [b] Calculated amount of adenine for 100% inactivation of RTPR, calculated as (observed adenine)/(100 − % activity remaining). [c] Reaction time of 40 min.

Table V: Products Isolated from Reaction of RTPR with FUTP, FCTP, and ClUTP[a]

substrate	F^- (nmol)	dNTP (nmol)	base (nmol)	substrate remaining (nmol)[b]
FUTP (500 nmol)	400	260	36	0
control – RTPR	0	0	0	310
FCTP (700 nmol)	510	185	100	94
control – RTPR	0	0	0	297
ClUTP (2400 nmol)		10.8	ND	0
control – RTPR		0		2220

[a] Reactions performed in the presence of 3 mM DTT as described under Materials and Methods. [b] Indicated yields are not corrected for percent recoveries from HPLC chromatography.

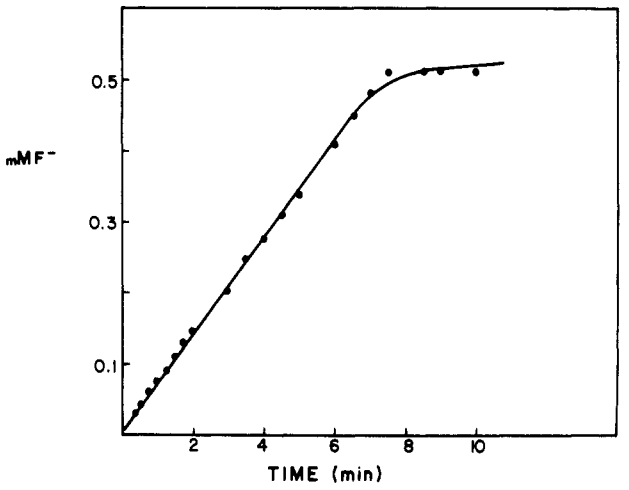


FIGURE 4: Time course for F^- release during the reaction of ribonucleotide reductase with FCTP. The reaction was performed as described under Materials and Methods, with F^- being continuously monitored with a fluoride electrode.

DTT or due to protein thiols (RTPR was present at 50 μM and contains approximately 18 mol of cysteine/mol of enzyme) is not known. To correct for this effect, a standard curve was prepared by addition of NaBr to a control mixture containing all reaction components except AdoCbl.

Interaction of FUTP and FCTP with RTPR. As shown in Table V, the product distribution from reaction of 2′-fluoronucleotides with RTPR is strikingly different from that observed with other 2′-halonucleotides: incubation of 13 nmol of RTPR with 500 nmol of FUTP results in formation of 400 nmol of F^- and subsequent isolation of 260 nmol of dUTP and 36 nmol of uracil. The dUTP was identified by removal of phosphates by alkaline phosphatase treatment and isolation of dU by HPLC. The dU was then converted to uridine with deoxyuridine hydroxylase (Stubbe, 1985), confirming the assignment. Thus, RTPR catalyzes the formation of approximately 1 mol of dUTP for every 1.5 mol of F^- produced. Similar results are observed with FCTP (Table V). In this case, dC was identified solely by comigration on HPLC with authentic dC. RTPR catalyzes formation of dC in 1 out of every 2.8 turnovers of FCTP.

Although both FUTP and FCTP inactivate RTPR in the presence of DTT (Table II) and cause an increase in the protein absorbance at 320 nm (Table III), the rates of enzyme inactivation (0.03 min^{-1} for 0.5 mM FUTP and 0.016 min^{-1} for 0.7 mM FCTP) are 200–300 times slower than rates of F^- production as observed by the F^- electrode (6.2 min^{-1} for FUTP and 5.3 min^{-1} for FCTP). Figure 4 shows a typical time course for F^- formation. No F^- is observed in the absence of either RTPR or AdoCbl. The large differences in inactivation and F^- release rates suggest a different mode of enzymic processing for these analogues.

Table VI: Effect of Reaction pH on ClUTP Partitioning[a]

reaction pH	nmol of ClUTP converted	dU sp act.	nmol of dUTP formed	total turnovers per reduction
5.5	260	2.0×10^5	1.4	190
6.1	251	1.8×10^5	1.3	200
7.4	235	2.9×10^5	2.1	112
8.3	224	4.4×10^5	3.1	72

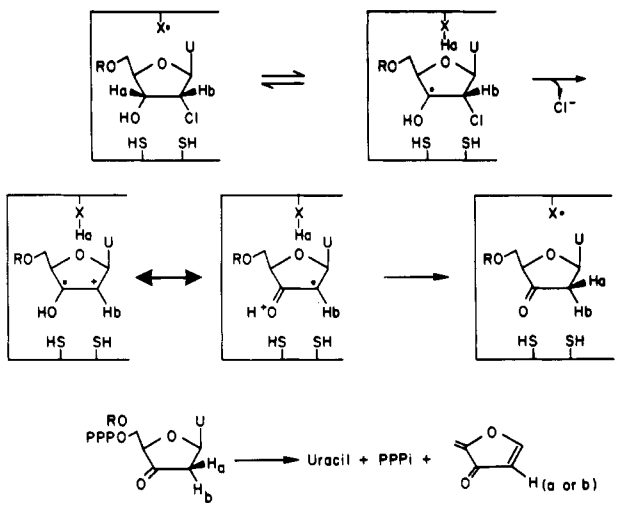
[a] RTPR (3 nmol) was treated with 260 nmol of [5′-^{3}H]ClUTP and 260 nmol of AdoCbl as described under Materials and Methods. After addition of 100 nmol of unlabeled dUTP and treatment with alkaline phosphatase, the specific activity of the dU isolated from HPLC was determined and used to calculate the nanomoles of [5′-^{3}H]dUTP formed from catalytic reduction of substrate. Total nanomoles of ClUTP converted was determined from the difference of the starting nanomoles of ClUTP and recovered nanomoles of ClU.

The large amount of normal reduction products formed in these reactions necessitates addition of DTT to the reaction mixture in order to maintain RTPR in the reduced form. The 2-methylene-3(2*H*)-furanone formed may thus react with DTT rather than the protein. To investigate the possibility that the slow inactivation observed was due to the reaction of RTPR with DTT-trapped 2-methylene-3(2*H*)-furanone rather than the free furanone, RTPR was incubated with 1 mM 2-(ethylthiomethyl)-3(2*H*)-furanone, a synthetically prepared analogue of the DTT adduct. The ethanethiol adduct of 2-methylene-3(2*H*)-furanone was chosen rather than the DTT adduct because of its greater stability and ease of purification. RTPR is inactivated in the presence of 1 mM 2-(ethylthiomethyl)-3(2*H*)-furanone at a rate of 0.04 min^{-1}. Furthermore, an increase in the protein absorbance at 320 nm followed inactivation. Given the lower concentrations of DTT-trapped species in the above experiments, it is feasible that inactivation in this case is due to such a furanone–thiol adduct.

Formation of dUTP during ClUTP Turnover. Production of dUTP during conversion of FUTP prompted investigation of the formation of dUTP in the reaction of ClUTP with RTPR. With [5′-^{3}H]ClUTP, dUTP was detected in low concentrations (Table V) by conversion to dU with alkaline phosphatase and analysis on HPLC. Firm identification of this material as dU was provided by subsequent conversion to uridine with deoxyuridine hydroxylase. The ratio of total turnovers to dUTP formed varies with pH (Table VI): higher pH values leading to greater formation of dUTP. The amount of dUTP formed also varies with the concentration of exogenous reductant. With 30 mM DTT at pH 7.4, dUTP is formed in 1 out of every 120 turnovers of ClUTP, while with 3 mM DTT dUTP is formed in 1 out of 220 turnovers.

Lack of Destruction of AdoCbl during Incubations with Analogues Other Than ClUTP. Incubation of RTPR and ClUTP has been previously shown to result in irreversible cleavage of 0.6–0.8 mol of AdoCbl to cob(II)alamin and 5′-deoxyadenosine for each mole of ClUTP consumed (Stubbe et al., 1983b). As the cob(II)alamin/5′-deoxyadenosine combination inhibits RTPR, the breakdown of ClUTP was found to be limited by the concentration of AdoCbl in the reaction mixture. Incubation mixtures of the analogues described in this study were examined for cofactor destruction. The analogues were incubated with 3 mM DTT and RTPR in the presence of a 2–100-fold excess of AdoCbl. The initial concentration of AdoCbl had no effect on either the rate or the extent of reaction. In addition, no 5′-deoxyadenosine was detected in HPLC analyses of the reaction mixtures. Consequently, no AdoCbl destruction was detected with any 2′-

Scheme I



halonucleotides other than ClUTP.

Discussion

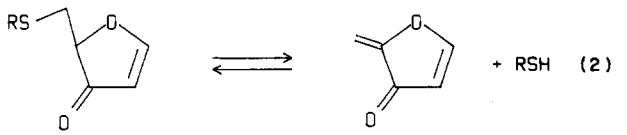
In 1976, Thelander et al. observed that the ribonucleoside-diphosphate reductase (RDPR) of *E. coli* was inactivated by ClUDP and that inactivation was accompanied by production of uracil and Cl^- and an increase in the protein absorbance near 320 nm. Extensive investigations in our laboratory on both RDPR and RTPR from *L. leichmannii* have begun to unravel the mechanism of this complex transformation (Ashley & Stubbe, 1987). Present experimental data are in accord with the mechanism shown in Scheme I. We have demonstrated that both RDPR and RTPR catalyze cleavage of the 3′ carbon–hydrogen bond of the nucleotide. By analogy to the chemical precedent of Fenton's reagent (Walling & Johnson, 1975; Gilbert et al., 1972), we proposed that Cl^- is lost from the initial substrate 3′-radical to form a resonance-stabilized radical cation, a process differing from the normal catalytic sequence in that protonation of the leaving group is not required. Replacement of the hydrogen atom originally abstracted from the 3′-carbon on the 2′-carbon now generates 3′-keto-2′-deoxyuridine nucleotide. We have demonstrated this hydrogen atom shift from 3′ to 2′ with both the RDPR and RTPR reactions (Ator & Stubbe, 1985; G. W. Ashley and G. Harris, unpublished results). Release of the ketonucleotide into solution is followed by a double β-elimination of uracil and phosphate moiety to generate 2-methylene-3(2*H*)-furanone. This species is thought to be responsible for enzyme inactivation (Stubbe & Kozarich, 1980a,b; Stubbe et al., 1983a; Harris et al., 1984; Ator & Stubbe, 1985).

The difference between normal turnover and abnormal turnover to produce 3′-keto-2′-deoxynucleotides is thought to be related to the leaving-group ability of the 2′-substituent. In the reaction of Fenton's reagent with glycols and halohydrins, protonation is essential when the leaving group is OH, but with Cl^- as leaving group it is not required. By analogy, with RTPR and UTP an enzymic group would be necessary for general acid catalysis to assist in elimination of the 2′-OH group during formation of a radical cation intermediate, while with RTPR and ClUTP no acid catalysis would be required. In the abnormal turnover observed with 2′-halogenated substrate analogues, the enzyme may be left in an improper protonation state for subsequent catalytic reduction, and the normally unfavorable side reaction of ketone formation may prevail. This hypothesis has led to a number of predictions that have been tested in this paper: (1) enzyme catalysis of halide release from the 3′-radical species should not be necessary for inactivation; (2) partitioning between normal and abnormal turnover may be observed with leaving groups of the appropriate pK_a; (3) partitioning between normal and abnormal turnover may be dependent on the protonation state of enzyme groups.

Enzyme catalysis of halide release was investigated with a stereochemical argument. After abstraction of the 3′-hydrogen atom from the substrate, RTPR should normally be set up to catalyze loss of 2′-ribo substituents and would not be expected to catalyze loss of 2′-arabino substituents located on the opposite face of the ribosyl ring. Uncatalyzed heterolysis of the 2′-substituent should, on the other hand, be relatively independent of the stereochemistry of the substituent. As demonstrated in Table II, *ara*-ClATP and *ara*-BrATP inactivate comparably to their ribo isomers. The products of the inactivation reactions, adenine and PPP_i, are the same, and inactivation by each of the four compounds results in an increase in the protein absorbance near 320 nm, indicative of inactivation via 2-methylene-3(2*H*)-furanone. Interestingly, *ara*-ATP binds to RTPR and causes reversible cofactor homolysis (Chen et al., 1974; Tamao & Blakley, 1973), yet it does not cause inactivation, suggesting the absence of an appropriate acid catalyst on the top face of the ribosyl ring. These results support the mechanism shown in Scheme I and indicate that the step involving heterolysis of the carbon–halogen bond in 2′-chloro- and 2′-bromonucleotides is not enzyme catalyzed.

The observation of partitioning of FUTP, FCTP, and ClUTP between normal production of 2′-deoxynucleotide and abnormal formation of 3′-keto-2′-deoxynucleotide is also in accord with the predictions of Scheme I. With 2′-OH (pK_a = 16) as the leaving group, only normal reduction is observed. With 2′-F (pK_a = 3.2), normal reduction predominates over abnormal ketone formation. With 2′-Cl (pK_a = −7), the situation reverses and abnormal ketone formation predominates over normal reduction. These results can be accommodated by partitioning of a common 3′-radical intermediate to produce the same radical cation (Scheme I) with the subsequent reaction pathway dependent upon the protonation state of the protein. Thus, as the leaving group ability increases and the requirement for general acid assistance decreases, the abnormal reaction to produce 3′-keto-2′-deoxynucleotide becomes more important.

Interestingly, the rates of release of F^- from FUTP and FCTP are 200–300 times faster than the observed rates of enzyme inactivation. Due to the large number of reductions relative to abnormal ketone formations characteristic of these analogues, a reductant such as DTT must necessarily be included in the reaction mixture to recycle the protein dithiol. We have previously demonstrated the efficacy of thiols such as DTT at trapping 2-methylene-3(2*H*)-furanone (Harris et al. 1984). As β-elimination of thiol from the trapped furanone may occur, DTT-trapped furanone may act as a slow source of 2-methylene-3(2*H*)-furanone (eq 2).



In support of this, treatment RTPR with chemically synthesized 2-(ethylthiomethyl)-3-furanone results in inactivation at a rate comparable to that observed with FUTP and FCTP in the presence of DTT. Interestingly, the rate of inactivation by 2-(ethylthiomethyl)-3(2*H*)-furanone ($t_{1/2}$ = 17 min) is also quite similar to that observed for the slow phase of the inac-

tivation by other 2′-halonucleotides in the presence of DTT (Table II). We postulate that the slow inactivation by 2′-fluoronucleotides and the slow second phase of inactivation by other 2′-halonucleotides in the presence of DTT are due to initial trapping of the 2-methylene-3(2*H*)-furanone by DTT, followed by slow, reversible regeneration of the inactivating species (eq 2). The more rapid inactivation of RTPR seen with ClUTP in the presence of DTT is likely related to the relative concentrations of DTT adduct formed: most FUTP goes to dUTP, while most ClUTP goes to DTT adduct.

The effect of reaction pH on formation of dUTP during ClUTP turnover was investigated. In accord with our proposed mechanism, the amount of normal reduction increased from 1 in 200 at pH 6.1 to 1 in 72 at pH 8.3. This increase, although not dramatic, clearly reveals a trend that is consistent with our proposed mechanism. Deprotonation of the crucial functional groups (of present unknown structure and pK_a) on the enzyme apparently promotes the normal reduction process.

Finally, original studies with ClUTP and RTPR demonstrated that a large portion of the AdoCbl cofactor was destroyed during turnover (Stubbe et al., 1983b). These results differed from the observed retention of free radical during the reaction of RDPR with ClUDP. Investigation of other 2′-halonucleotides with RTPR have shown that loss of AdoCbl is specific to ClUTP: no other analogue tested results in irreversible conversion of AdoCbl into cob(II)alamin and 5′-deoxyadenosine. Given the other similarities noted between ClUTP and these analogues, it appears unlikely that destruction of cofactor is intimately involved in the inactivation mechanism.

In summary, a variety of 2′-halonucleotides have been evaluated as substrates/inactivators of the ribonucleoside-triphosphate reductase from *L. leichmannii*. Several novel observations have resulted: (1) inactivation occurs independent of the stereochemistry of the 2′-halogen; (2) inactivation produces similar products in all cases, suggesting similar reaction mechanisms; (3) FUTP, FCTP, and ClUTP partition between normal 2′-deoxynucleotide and abnormal 3′-keto-2′-deoxynucleotide formation; (4) partitioning between normal and abnormal turnover varies predictably with 2′-substituent leaving group ability and reaction pH. These aspects of the inactivation of RTPR by 2′-halonucleotides are in accord with the proposed radical cation mechanism.

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