other tetrameric ring systems, *i.e.*, $S_4N_4$[32] and $P_4N_4(NMe_2)$,[33] have been used as ligands, the preferred (M–N) bonding always involves apposite nitrogen centers and clearly the presence of L is vital for OMT to be similarly bidentate.[33a]

Having established the overall donor role of HMT and OMT only one anomaly remains, namely, the inability of the former to give adduct formation with chromium(III) chloride. The possibility of exchange reactions occurring, as found in the analogous borazole–metal halides system,[34] seems remote since products obtained from the interaction of several chromium(III) chloride species and HMT were invariably nonstoichiometric with a conspicuous lack of $\nu$(SiNSi) modes in their infrared spectra. Ring degradation of cyclosilazanes by metal halides *via* cleavage of (Si–N) bonds has been demonstrated for $FeCl_3$, $CoCl_2$, and $CuCl_2$[35] with HMT > OMT in terms of susceptibility, and it is now evident that chromium(III) chloride can bring about similar skeletonal breakdown in HMT.

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# Possible Transition-State Analogs for Ribonuclease. The Complexes of Uridine with Oxovanadium(IV) Ion and Vanadium(V) Ion[1]

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**Abstract:** The hydrolysis of uridine 2′,3′-phosphate that is catalyzed by ribonuclease is competitively inhibited by uridine, 2′-deoxyuridine, and oxovanadium(IV) ion and is probably competitively inhibited by the 1:1 complexes of uridine with oxovanadium(IV) ion and vanadium(V) ion. The kinetically determined values of the dissociation constants for the binding of these inhibitors to ribonuclease at pH 7.0 and 25° are $9 \times 10^{-3}$, $45 \times 10^{-3}$, $6 \times 10^{-5}$, $1.0 \times 10^{-5}$, and $1.2 \times 10^{-5}$ $M$, respectively. Under the same conditions the value of the association constant for formation of the 1:1 complex of uridine and metal ion is 160 $M^{-1}$ in the case of oxovanadium(IV) ion and about 80 $M^{-1}$ in the case of vanadium(V) ion. These association constants were obtained from measurements of the effect of uridine upon the solubility of oxovanadium(IV) ion and of the effect of uridine upon the ultraviolet spectrum of vanadium(V) ion. The complexes probably have structures in which the *cis*-glycol function of uridine coordinates with the vanadium atom, since methyl β-D-ribofuranoside forms similar complexes and 2′-deoxyuridine does not. The strong binding of the complexes to ribonuclease may be explained by either the coordination of groups of the enzyme to vanadium or by the resemblance of structures of the complexes to the structure of the substrate portion of the transition state for ribonuclease-catalyzed hydrolysis of uridine 2′,3′-phosphate.

This paper describes the potent inhibition of the enzyme ribonuclease (RNase) by complexes that the nucleoside uridine (U) forms with oxovanadium(IV) ion (V(IV)) and with vanadium(V) ion (V(V)). The rationale for this investigation has been the hypothesis that these complexes may resemble the substrate portion of the transition state for RNase-catalyzed hydrolysis of uridine 2′,3′-phosphates and so act as transition-state analogs.[3] This hypothesis is discussed in more detail in the Discussion.

## Experimental Section

**Materials.** RNase A, lyophilized and phosphate-free, was purchased from Worthington Biochemical Corp. Stock solutions of enzyme were prepared in distilled water and were used for 3–4 weeks. The sodium salt of uridine 2′,3′-cyclic phosphate (U-2′,3′-P) was prepared from a mixture of uridine 2′- and 3′-phosphates (U-2′- and -3′-P), purchased from Sigma Chemical Corp., by the procedure of Szer and Shugar[4] with the modification that the reaction time was

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3 hr. The U-2′,3′-P was stored in an evacuated desiccator at −20°, and stock solutions in water were prepared on the day of use. The purity of each preparation of U-2′,3′-P was determined by ascending chromatography on Whatman No. 1 paper in the system saturated $(NH_4)_2SO_4$–1 *M* sodium acetate (pH 6.6)–2-propanol (80:18:2 v/v).[5] Sufficient U-2′,3′-P was spotted so that less than 0.5% U-2′- and -3′-P ($R_f$ 0.65) in the U-2′,3′-P ($R_f$ 0.5) could be detected upon illumination of the chromatogram with an ultraviolet lamp. Only preparations of U-2′,3′-P that contained less than 0.5% U-2′- and -3′-P were used in the kinetic studies. U and 2′-deoxyuridine (dU) were purchased from both Sigma Chemical Corp. and Aldrich Chemical Co.; they were used without further purification. β-Methyl riboside (methyl β-D-ribofuranoside), mp 74–76° (lit. mp 79–80°), was prepared by the method of Barker and Fletcher.[6] Stock solutions of V(IV) were made by solution of Fisher Reagent Grade $VOSO_4 \cdot 2H_2O$ in water or 0.1 *N* HCl. The V(IV) concentration was determined by titration of the stock solutions with $KMnO_4$[7] and was approximately 80% of the value expected on the basis of the weight and the empirical formula. Sodium decavanadate ($Na_6V_{10}O_{28} \cdot 18H_2O$) was a gift from Dr. Lafayette Noda. It was dissolved in $NH_4Cl$ of a molarity equivalent to the total concentration of the vanadium, and the solution was used within 10 min. By measuring the absorbance at 360 nm, we determined that less than 5% of the decavanadate hydrolyzes to less polymerized species during this period.[8]

Stock solutions of V(V) were prepared by solution of Fisher purified $NH_4VO_3$ in hot water; immediately upon cooling the pH was adjusted to 7.00 with NaOH. The concentration of decavanadate in these solutions was determined from comparison of the initial absorbance at 360 nm of an aliquot in 0.2 *M* NaOH–2.5 *M* NaCl with the initial absorbance of an aliquot of the decavanadate solution in 0.2 *M* NaOH–2.5 *M* NaCl.[8] The stock solutions of V(V) contained less than 1.5% of the vanadium as decavanadate. The total concentration of V(V) in the stock solutions was determined by use of a specific colorimetric test for V(V)[9] in which the solution of V(V) is made 3 *N* in HCl and extracted with an equal volume of 1 mg/ml of *N*-benzoyl-*N*-phenylhydroxylamine in $CHCl_3$, and the absorbance of the extract at 510 nm is measured. For the standardization of this colorimetric test we used solutions of V(V) that were prepared by oxidation (see below) of solutions of V(IV) of known concentration. On the basis of this standard, the $NH_4VO_3$ was at least 98% pure by weight.

Deionized water was used to prepare all solutions. Since V(IV) is oxidized by molecular oxygen to V(V) at pH values greater than about 4,[10,11] all solutions that were used in experiments with V(IV) were either prepared with $O_2$-free water and stored under nitrogen or were deoxygenated by passing nitrogen through them. The solutions were transferred using pipets and syringes that had been flushed with nitrogen.

**Measurements of Complex Formation.** The solubility of V(IV) in the presence and absence of U, dU, and β-methyl riboside was determined in the following way. The solution of salt, buffer, and, where present, ligand was temperature equilibrated under nitrogen for 30 min in the thermostated vessel of a Radiometer pH-stat apparatus (pH meter 25SE, titrator TTTllb, titration assembly TTA31, and either Autoburette ABU12b with titrigraph SBR3a or a 0.5-ml syringe buret unit and titrigraph SBR2). An aliquot of a stock solution of $VOSO_4$ was added; and, after another 30 min, the mixture was adjusted to pH 7.00 with NaOH. In each case the concentration of V(IV) was sufficiently large (in the range from 1 to 5 m*M*) that a precipitate occurred. Between 15 and 30 min after adjustment of the pH to 7.00, the suspension was taken up into a glass syringe and passed through a HAWP 0.45-μ Millipore filter with a microfiber glass disk prefilter. In order to determine the extent of inadvertant oxidation of the V(IV), a part of the suspension was filtered directly into an equal volume of 6 *M* HCl and analyzed for V(V) by the colorimetric test described above. The total vanadium concentration in the filtrate was determined by completely oxidizing the V(IV) to V(V) and then performing the colorimetric test. In the case of V(IV) alone, the oxidation was carried out by making the filtrate $10^{-3}$ *M* in NaOH and bubbling $O_2$ through the solution for 20 min or longer; in the case of V(IV) in the presence of U and β-methyl riboside, $O_2$ was passed through the filtrate, maintained at pH 7.00, for a period of 90 min or longer; with V(IV) and dU, the filtrate was made $10^{-2}$ *N* in NaOH and treated with $O_2$ for 20 min or longer. Control experiments with V(IV) at a concentration below its solubility showed that oxidation of V(IV) to V(V) is greater than 95% complete under these conditions and that U, β-methyl riboside, dU, and V(IV) do not alter the color intensity in the colorimetric test for V(V). Another control experiment, in which V(IV) at a concentration below its solubility was passed through a Millipore filter and in which the per cent of inadvertant oxidation was measured before and after filtration, demonstrated that there was no retention of soluble V(IV) by the filter and no oxidation of V(IV) during the filtration procedure itself. Filtration of the suspensions after 15 min rather than 30 min did not result in different values for the solubility; thus, the equilibrium between soluble and insoluble V(IV), as well as that for complex formation, is established within 15 min. It is known that water liganded to V(IV) exchanges with solvent water very rapidly.[12]

In the course of the determinations of the solubility of V(IV) by itself, we discovered that V(V), formed by inadvertant oxidation of V(IV), increased the solubility of V(IV). In 0.225 *M* NaCl–$2.5 \times 10^{-5}$ *M* sodium phosphate at 25.0°, soluble V(IV) was found to equal 0.38 m*M* + 2.2 [V(V)]. The values of the total concentration of V(IV) in solution that are given in Figure 1 have been corrected for the concentration of V(IV) in solution due to V(V) by subtracting 2.2[V(V)] from the actual values. This correction was less than 10% of the total concentration of V(IV) in solution, except for one experiment in which it was 20%.

The accurate titrations to pH 7.00 of 0.10 and 0.20 m*M* $VOSO_4$–0.225 *M* NaCl alone and in the presence of U, dU, and β-methyl riboside were carried out at 24.7° under nitrogen in the vessel of the Radiometer pH-stat apparatus on a 5-ml scale with addition of 7.75 m*M* NaOH from the syringe of the Radiometer apparatus. At the end of each titration, the reaction mixture was analyzed for V(V) by the colorimetric test; less than 3% oxidation occurred. Titration of mixtures without V(IV) were performed in order to obtain blank values. In addition, our stock solution of 10 m*M* $VOSO_4$, prepared by solution of $VOSO_4 \cdot 2H_2O$ in water, contained about 2.5 m*N* strong acid, since its pH was 2.70 rather than the value of 3.7 that is expected from reported titration data.[13] The ratio of the equivalents of acidic impurity (probably $H_2SO_4$) to V(IV) was accurately determined to be 0.34 by passing a solution of 0.1 *M* $VOSO_4$ through an ion exchange column of Bio-Rad AG50WX8, $H^+$ form, and titrating the effluent.

The reaction mixtures used to determine the equilibrium constants for polymerization of V(V) and for formation of the complexes of V(V) were prepared from stock solutions of the individual components that had been adjusted to pH 7.00, were themselves adjusted to pH 7.00, and were temperature equilibrated for 10 min. The absorbance values were then measured against blanks lacking V(V) on a Ziess PMQII or a Gilford 240 spectrophotometer. The absorbance values were stable for 15 hr. The formation of complexes of V(V) monoanion have been shown to occur in milliseconds.[14]

**Kinetic Measurements.** The initial rates of RNase-catalyzed hydrolysis of U-2′,3′-P were obtained by measurement of the consumption of 1.0 m*N* sodium hydroxide in the Radiometer pH-stat apparatus.[15] To the titration cell, thermostated at 25.0° and flushed with argon or nitrogen, was added 0.20–1.80 ml of 25 m*M* U-2′,3′-P in 0.25 *M* NaCl, enough 0.25 *M* NaCl to bring the volume to 1.8 ml, 5 μl of 10 m*M* potassium phosphate (pH 7.0), and 0.20 ml of water or inhibitor solution. This solution was allowed to temperature equilibrate for 5 min, while at the same time it was titrated to pH 6.98 with a few μl of 0.1 *N* NaOH. At this point the pH-stat was activated in order to bring the solution to the end point of 7.00 with the 1 m*N* NaOH in the instrument. The reaction was initiated by the addition of a small aliquot, usually 10 μl, of 1 mg/ml of RNase. The concentrations of enzyme and substrate were such that less than 0.5% of the substrate was hydrolyzed during

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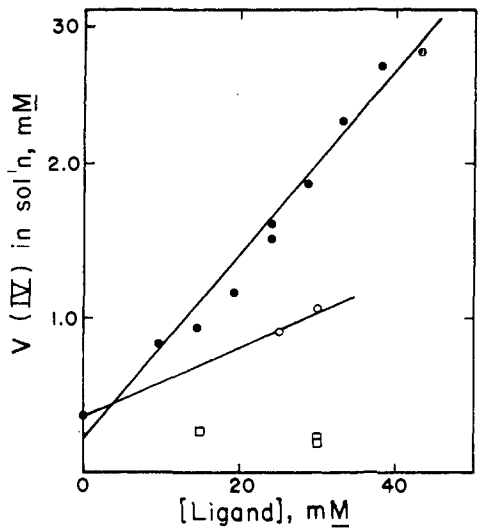


Figure 1. The solubility of V(IV) in 0.225 $M$ NaCl–2.5 $\times$ $10^{-5}$ $M$ potassium phosphate (pH 7.00) at 25.0° as a function of the concentration of U (●), β-methyl riboside (○), and dU (□). The concentrations of V(IV) shown in the figure have been corrected for the concentration in solution due to complexation with V(V) by the method given in the Experimental Section. The line through the points with U is the one given by a least-squares analysis.

the initial several minutes of reaction. It is necessary to determine the initial velocities before significant produce formation, since U-3′-P is a potent inhibitor of the reaction (see Table I). We calculate on the basis of the values of the Michaelis constant for the substrate and the dissociation constant for U-3′-P (see eq 4 and Table I) that with 10 m$M$ U-2′,3′-P, after 1.0% reaction the rate is only 90% of its initial value because of product inhibition. During the first few minutes the uptake of base was found to be linear with time. In the absence of RNase, there was no consumption of base, except when V(IV) alone was the inhibitor. With this inhibitor there was a background rate of less than 5% of the rate in the presence of the enzyme, which was corrected by subtraction of this background rate. At the beginning and end of each series of kinetic runs, the rate of hydrolysis of 18 or 10 m$M$ U-2′,3′-P in the absence of inhibitor was measured. These two values were the same within 3% and were used to normalize the data for each series to exactly the same concentration of active enzyme. Our value of the turnover number for the enzyme, calculated from the maximal velocity (see Figure 3) and the concentration of the enzyme based upon its weight, is 3.6 $sec^{-1}$, which is in fair agreement with the value of 4.16 $sec^{-1}$ reported by del Rosario and Hammes.[16]

The final concentration of V(IV) in each reaction mixture was less than its solubility so that no precipitation occurred. However, the insolubility of V(IV) did cause us to prepare stock solutions of the different inhibitors at different pH values. The stock solutions of U plus V(IV) were sufficiently concentrated in U that no precipitation of V(IV) occurred at pH 7.00. These stock solutions were prepared by mixing aliquots of solutions of $VOSO_4$ and U, adjusting the pH to 7.00 with NaOH under nitrogen, and transferring the mixture with a syringe to a nitrogen-flushed flask sealed with a rubber septum. Aliquots of the mixture were withdrawn with a syringe for use in the rate assays. The stock solutions of $VOSO_4$ alone and of $VOSO_4$ plus dU were maintained at about pH 3, rather than at pH 7.00, in order to avoid precipitation of V(IV). In our preliminary report of this research,[16a] we described the inhibition of RNase by V(IV) that was added to the reaction mixture partly as precipitate and partly as a solute due to the fact that the stock solution was adjusted to pH 7.00. Our subsequent work has revealed that the inhibition by V(IV) added in this way is substantially less than the inhibition caused by addition of V(IV) entirely in solution at pH 3. Using the Millipore filtration technique, we have discovered that when a suspension of V(IV) at pH 7.00 is diluted at 25° in 0.225 $M$ NaCl–2.5 $\times$ $10^{-5}$ $M$ potassium phosphate (pH 7.00), to a concentration at which all the V(IV) should be soluble (less than 0.3 m$M$), the precipitated V(IV) does not dissolve to a significant extent after 30 min of stirring. Evidently, the precipitated form of V(IV) is not effective as an inhibitor of RNase. Generally at the completion of a kinetic run with V(IV) present, the reaction mixture was acidified with an equal volume of 6 $N$ HCl, and the concentration of V(V) formed through inadvertant oxidation was determined by the colorimetric test. Usually less than 15% of the V(IV) was oxidized. The results of the few runs with higher percentages were discarded.

Stock solutions of V(V) plus U were prepared in two ways, by mixing of the individual components and adjustment of the pH to 7.00 and by bubbling oxygen through a solution of 1 m$M$ V(IV) plus 0.1 $M$ U at pH 7.00 for 15 min. The colorimetric test for V(V) showed that oxidation was completed in the 15-min period. These stock solutions gave identical inhibition (see Table II). Some of the measurements of rates in the presence of V(V) plus U were made with the same precautions taken to exclude oxygen as were taken with V(IV). Since colorimetric analysis for V(V) at the end of these rate measurements gave the expected concentration, no reduction of V(V) occurs under these conditions. A control experiment in which rates of the enzymatic reaction in the presence of U plus decavanadate were measured showed that the inhibition found with V(V) plus U is not due to the small fraction of decavanadate in the solutions of $NH_4VO_3$.

## Results

**Complex Formation.** We have investigated the formation of complexes of U, dU, and β-methylribose with V(IV) and V(V). In the case of V(IV), our initial observations were that titration of 10 m$M$ $VOSO_4$ by itself or in the presence of 0.10 $M$ dU from its pH of 2.7 to pH 7.0 resulted in the gray precipitate of V(IV) hydroxide,[10,17] whereas no precipitation occurred upon titration of 10 m$M$ $VOSO_4$ in the presence of 0.10 $M$ U or 0.10 $M$ β-methyl riboside from pH 2.7 to 7.0. These findings suggested that the *cis*-glycol group is the liganding functional group. In order to determine the stoichiometry and equilibrium constants for complexation, we measured the solubility of V(IV) at pH 7.00 as functions of the concentrations of U, β-methyl riboside, and dU (Figure 1). The dependence of the solubility of V(IV) upon the concentration of U is approximately a first-power dependence, and we have analyzed the data upon the assumption that there is a 1:1 complex formed between the two (eq 1 and 2, where [C], [V], and

$$K_c = [C]/[V][N] \quad (1)$$

$$[V]_t = [V](1 + K_c[N]) \quad (2)$$

[N] refer to the concentrations of the complex, uncomplexed vanadium, and free nucleoside, respectively, and $[V]_t$ is the total concentration of vanadium in solution). The value of $K_c$ obtained in this way is 160 $M^{-1}$. Similar analysis of the limited data for β-methyl riboside gives an equilibrium constant of 60 $M^{-1}$ for complexation. dU appears to decrease slightly in the solubility of V(IV). Since V(IV) is known to dimerize in solution at slightly acid pH values,[13,18] it is possible that V(IV) in dilute solution at pH 7.00 exists predominantly in a polymeric form and that the complex(es) with U and β-methyl riboside are ones in which there is a single U and several V(IV). Our solubility data do distinguish between this possibility and that of a 1:1 complex.

In order to determine the average charge on V(IV) and its complexes at pH 7.00, we titrated solutions of 0.1 and 0.2 m$M$ $VOSO_4$ in 0.225 $M$ NaCl that contained no organic ligands, 30 m$M$ dU, 30 m$M$ U, and 30 m$M$ β-methyl riboside. At these concentrations of V(IV),

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there is no precipitation of V(IV) hydroxides at pH 7.0 (Figure 1). After correction for the uptake of NaOH due to acid in the stock $VOSO_4$ and due to the other components of the mixture (see the Experimental Section), the equivalents of NaOH consumed per mole of $VOSO_4$ were 2.15 in the absence of ligands, 2.15 in the presence of dU, 2.88 in the presence of U, and 2.53 in the presence of β-methyl riboside. With the above values of the equilibrium constants for complex formation, we calculate that the equivalents of NaOH consumed per mole of complexed V(IV) are 3.01 with U and 2.75 with β-methyl riboside. Thus, the average charge per V(IV) complexed with U is −1.0. Presumably, two of the three protons released upon formation of the complex at pH 7.0 originate from the *cis*-glycol function, and the third arises from the ionization of a liganded water molecule (see the Discussion and Figure 4).

In order to investigate the formation of complexes with V(V), it was first necessary to determine the species of V(V) that exist in solution under our conditions. V(V) is known to occur as a trimer, and may possibly occur as a tetramer, at pH 7.00.[19] Following the procedure of Schiller and Thilo,[20] we determined the apparent molar absorbance at 322 nm of V(V) in 0.225 *M* NaCl–2.5 × $10^{-5}$ *M* sodium phosphate at pH 7.00 and 25.0° as a function of the total concentration of V(V). The value decreased from a value of 925 $M^{-1}$ $cm^{-1}$, obtained at 20 m*M* and greater V(V), to an asymptotically approached value of 470 $M^{-1}$ $cm^{-1}$ (490 $M^{-1}$ $cm^{-1}$ at 0.10 m*M* and 475 $M^{-1}$ $cm^{-1}$ at 0.05 m*M*). The dependence of the apparent molar absorbance upon the total concentration of V(V) accurately fit the equation for a monomer–trimer equilibrium.[20] The value of the dissociation constant is 1.2 × $10^{-6}$ $M^2$; Schiller and Thilo[21] report a value of 2.8 × $10^{-6}$ $M^2$ at pH 7.2 in 0.005 *M* Tris·HCl buffer at an unspecified temperature.

The complexation of V(V) by U was followed spectrophotometrically with 0.15 m*M* V(V), at which concentration about 95% of the V(V) is the monomer (Figure 2). The data in Figure 2 were analyzed on the basis of the assumptions that the trimer does not complex with U and that only a 1:1 complex forms between U and monomeric V(V). In this case the concentration of uncomplexed, monomeric V(V) is given by

$$[V]^3 + \left(\frac{\epsilon_V - \epsilon_C}{\epsilon_{V3} - 3\epsilon_C}\right) K_t[V] - \frac{K_t}{(\epsilon_{V3} - 3\epsilon_C)} \frac{\Delta A}{l} = 0 \quad (3)$$

where $\epsilon_V$, $\epsilon_C$, and $\epsilon_{V3}$ are the molar absorbances of uncomplexed, monomeric V(V), of the 1:1 complex, and of trimeric V(V), respectively. $K_t$ is the dissociation constant of the trimer; and $\Delta A$ is the difference between the absorbance at intermediate and at saturating concentrations of U, divided by the path length of the cuvette, $l$. The value of $\epsilon_C$ was calculated from the limiting value of the absorbance at high concentrations of U (Figure 2), according to the expression, $A = \epsilon_C l \cdot [V(V)]_{total}$. The values of the equilibrium constant for formation of the 1:1 complex were then calculated according to eq 1. The values obtained from the data at 5.0, 7.5, 10, 15, and 25 m*M* U are 58, 70, 77, 82, and 96 $M^{-1}$, respectively. The 1.6-fold increase in $K_c$ with the fivefold increase in the concentration of U suggests that a 2:1 complex may also form; however, there is not sufficient information to calculate values of equilibrium constants for the formation of 1:1 and 2:1 complexes. Since β-methyl riboside causes a decrease in the absorbance of V(V) that is identical with that caused by U, whereas dU has no effect upon the absorbance of V(V) (Figure 2), the structure of the 1:1 complex with U is very likely one in which the oxygen atoms of the *cis*-glycol group are ligands.

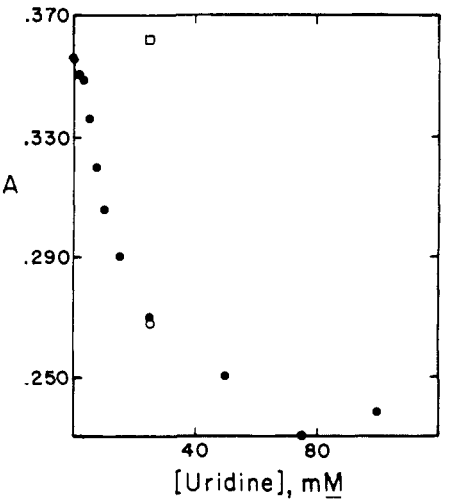


Figure 2. The absorbance of 0.15 m*M* V(V) at 322 nm in 0.225 *M* NaCl–2.5 × $10^{-5}$ *M* sodium phosphate, at pH 7.0 and 25.0°, as a function of the concentration of U. The open circle and the square give the results of single measurements with β-methyl riboside and dU, respectively. Path length, 5.00 cm.

Other measurements, made at a higher total concentration of V(V) (1.0 m*M* rather than 0.15 m*M*), are qualitatively in agreement with those presented in Figure 2. Thus, at pH 7.0, the 322-nm absorbance of V(V) alone and in the presence of 30 m*M* dU was 0.65, whereas the absorbance in the presence of 30 m*M* U and of 30 m*M* β-methyl riboside fell to 0.33 (1-cm light path).

**Kinetics.** The effects of U, dU, V(IV), V(V), and mixtures of U with V(IV), U with V(V), dU with V(IV), and dU with V(V) upon the initial rates of RNase-catalyzed hydrolysis of U-2′,3′-P were determined. With the exception of V(V), which did not inhibit at 0.10 m*M*, the individual species and the mixtures inhibited RNase (Figure 3). The reciprocal plots of the initial rates against substrate concentration in the presence of the inhibitors are linear and, within experimental error, intersect the ordinate at the same point that the plot of the initial rates in the absence of inhibitors does (Figure 3 and similar data not presented). These findings show that the inhibition by both the individual inhibitors and the mixtures is competitive with the substrate and thus suggest that the inhibitors bind at the active site of RNase.[21]

The dissociation constants of the enzyme–inhibitor complexes ($K_i$'s) were calculated from the kinetic data. In the cases of inhibition by the single species, the expression for the initial velocity is

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \quad (4)$$

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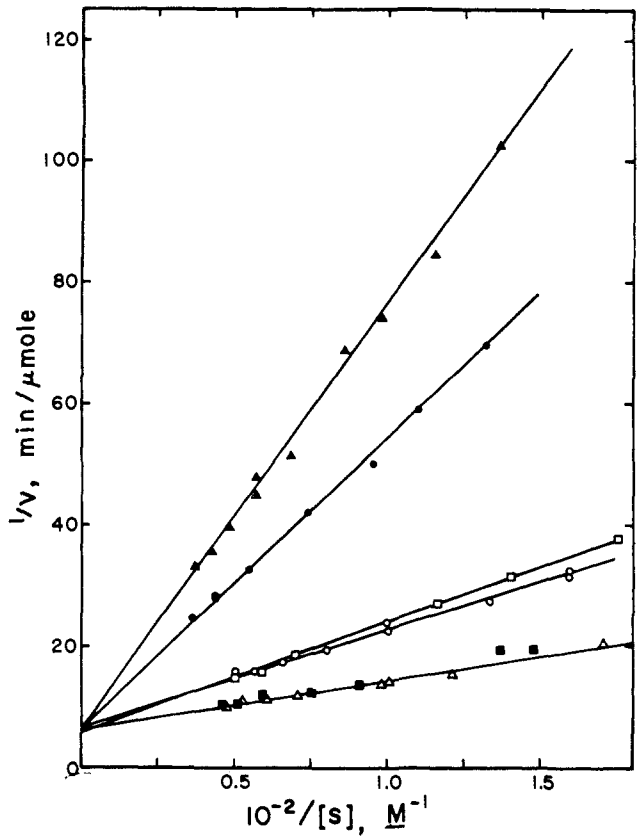


Figure 3. Reciprocal plots of initial velocity *vs.* substrate concentration for RNase-catalyzed hydrolysis of U-2′,3′-P, at 25.0° and pH 7.00. The velocities are the ones for a 2-ml volume. The inhibitors present were: none (△); 10 m*M* U (□); 0.10 m*M* V(IV) (○); 10 m*M* U–0.10 m*M* V(IV) (▲); 0.10 m*M* V(V) (■); 10 m*M* U–0.10 m*M* V(V), prepared by oxidation with $O_2$ of stock 100 m*M* U–1 m*M* V(IV), at pH 7.0 (●). All velocities have been normalized to the same concentration of enzyme.

where $K_m$ is the Michaelis constant and $V_{max}$ is the maximal velocity.[21] The values of $K_i$ were obtained from the slopes of the reciprocal plots by use of the value of $K_m/V_{max}$ that was determined in the absence of inhibitor (Table I).

In the case of inhibition by the mixtures of nucleosides and vanadium ions (V), the following set of equilibria must be considered, where the symbol E represents free enzyme and the other symbols have the same meaning as in eq 1. In addition to the ternary complex of RNase with the complex of nucleoside and V(EC), there may form an enzymatically inactive ternary complex of RNase, nucleoside, and V in which the nucleoside and V are not directly complexed with each other (ENV). With these equilibria, the dependence of the initial velocity upon concentrations is

$$\text{E} + \text{N} \rightleftarrows \text{EN} \qquad K_{iN} = [\text{E}][\text{N}]/[\text{EN}]$$

$$\text{E} + \text{V} \rightleftarrows \text{EV} \qquad K_{iV} = [\text{E}][\text{V}]/[\text{EV}]$$

$$\text{E} + \text{C} \rightleftarrows \text{EC} \qquad K_{iC} = [\text{E}][\text{C}]/[\text{EC}]$$

$$\text{EN} + \text{V} \rightleftarrows \text{ENV} \qquad K_{iNV} = [\text{EN}][\text{V}]/[\text{ENV}]$$

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[N]}{K_{iN}} + \frac{[V]}{K_{iV}} + \frac{[C]}{K_{iC}} + \frac{[N][V]}{K_{iN}K_{iNV}}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \quad (5)$$

**Table I.** The Inhibition of RNase by Nucleosides and Ions of Vanadium, at pH 7.00 and 25°

| Inhibitor | Concn, m*M* | $K_i$,[a] m*M* |
|---|---|---|
| U-2′,3′-P | | 10.6[b] |
| U | 10 | 8.0[c] |
| | 40 | 9.1 |
| | 75 | 9.7 |
| dU | 25 | 45[d] |
| U-3′-P | | 0.44[e] |
| V(IV) | 0.10 | 0.065 |
| | 0.20 | 0.060 |
| | 0.30 | 0.065 |
| V(V) | 0.10 | >0.3[f] |

[a] Calculated according to eq 4 in the text. [b] $K_m$ value for the substrate. A value of 7.3 m*M* under similar conditions but with Tris-acetate buffer was reported in ref 16. [c] D. R. Pollard and J. Nagyvary [*Biochemistry*, **12**, 1065 (1973)] report a value of 2.3 m*M* in 0.05 *M* Tris-HCl–0.05 *M* sodium acetate–0.1 *M* NaCl, at pH 7.0 and 25°. We have no explanation for the discrepancy. [d] In our preliminary communication (ref 1a) we reported a value of 10 m*M*. Redeterminations of $K_i$ with new samples of dU from both the Aldrich and Sigma Companies gave the value reported in this table. [e] From D. G. Anderson, G. G. Hammes, and F. G. Walz, Jr., *Biochemistry*, **7**, 1637 (1968). [f] A lower limit based upon the absence of detectable inhibition.

Since our results on complexation of uridine with V have shown that [C] approximately equals $K_c$[V][N], eq 5 becomes

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[N]}{K_{iN}} + \frac{[V]}{K_{iV}} + \frac{[N][V]}{K_{iC}}K_c + \frac{[N][V]}{K_{iN}K_{iNV}}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \quad (6)$$

Using the values of $K_m$, $K_{iN}$, and $K_{iV}$ that were obtained in separate kinetic experiments and the values of the concentration of uncomplexed V that are obtained from the equilibrium constants for the formation of C, we have calculated values of the composite term, [N]·[V]($K_c/K_{ic}$ + $1/K_{iN}K_{iNV}$), from the slopes of the reciprocal plots (Table II). With the mixtures of U and V, this term is a significant fraction of the total term, 1 + [N]/$K_{iN}$ + [V]/$K_{iV}$ + [N][V]($K_c/K_{iC}$ + $1/K_{iN}K_{iNV}$ (Table II). Thus, a significant fraction of the inhibition that is observed with these mixtures is due to formation of a ternary complex of RNase, V, and nucleoside. Without direct information about the structure(s) of this ternary complex, it is not possible to decide with certainty whether the ternary complex is predominantly EC, in which case $K_c/K_{iC}$ is dominant in the term, $K_c/K_{iC}$ + $1/K_{iN}K_{iNV}$, or predominantly ENV, in which case $1/K_{iN}K_{iNV}$ is dominant. However, since the mixtures of dU and V, which do not form complexes with each other, show little or no detectable inhibition due to ternary complexes (Table II), it seems likely that U is complexed with V in the ternary complexes with RNase. On this basis, we have calculated the minimal values for $K_{iC}$ for inhibition of RNase by the complexes of U with V(IV) and V(V) (Table II). The values obtained for the complex of U and V(IV) from measurements at different concentrations of U and of V(IV) are approximately the same, with the exception of the value given by data at 5 m*M* U. We have no explanation for the deviation of this value.

A more detailed way of considering the data in Table II that leads to the same interpretation is the following.

The overall equilibrium constant for dissociation of

**Table II.** Inhibition of RNase by Mixtures of Nucleosides and Vanadium Ions, at pH 7.00 and 25.0°

| Inhibitors | Concn, m$M$[a] | | $1 + [N]/K_{iN} + [V]/K_{iV} + [N][V](K_c/K_{iC} + 1/K_{iN}K_{iNV})$[b] | $[N][V](K_c/K_{iC} + 1/K_{iN}K_{iNV})$[b] | $K_{iC}$, m$M$[c] |
|---|---|---|---|---|---|
| U and V(IV) | 5.0 and | 0.10 | 4.5 | 2.1 | 0.021 |
| | 10 | 0.05 | 6.4 | 4.0 | 0.008 |
| | 10 | 0.10 | 8.6 | 5.9 | 0.011 |
| | 10 | 0.20 | 16.8 | 13.5 | 0.009 |
| | 20 | 0.10 | 10.2 | 6.6 | 0.012 |
| | 30 | 0.10 | 14.1 | 10.8 | 0.008 |
| dU and V(IV) | 23 | 0.04 | 2.0 | −0.2 | |
| U and V(V) | 10 | 0.10[d] | 5.8 | 3.68 | 0.011 |
| | 10 | 0.10 | 5.5 | 3.43 | 0.012 |
| dU and V(V) | 23 | 0.10 | 1.6 | +0.1 | |

[a] These are the total concentrations of V. [b] See eq 6 in the text. [c] For dissociation of the ternary complex of RNase and C to form RNase and C, calculated on the assumption that the entire term in the adjacent column is equal to $[N][V]K_c/K_{iC}$. [d] Prepared by oxidation of 1 m$M$ V(IV)–100 m$M$ U with molecular oxygen at pH 7.00.

the ternary complexes of N, V, and RNase, whatever their structures, to free N, V, and RNase is given by the reciprocal of $(K_c/K_{iC} + 1/K_{iN}K_{iNV})$, the values of which can be calculated from the values given in Table II for the term $[N][V](K_c/K_{iC} + 1/K_{iN}K_{iNV})$. These values are $6 \times 10^{-8}$ $M^2$, $1.5 \times 10^{-7}$ $M^2$, greater than $2 \times 10^{-6}$ $M^2$, and greater than $6 \times 10^{-6}$ $M^2$ for the pairs U–V(IV), U–V(V), dU–V(IV), and dU–V(V), respectively. If the binding of N did not effect in any way the binding of V and *vice versa*, the values of the dissociation constants for the ternary complexes would simply be equal to the products of the individual dissociation constants of the binary complexes of RNase with these ligands. The values of these products are $5 \times 10^{-7}$ $M^2$, greater than $3 \times 10^{-6}$ $M^2$, $3 \times 10^{-6}$ $M^2$, and greater than $1.3 \times 10^{-5}$ $M^2$ for the above pairs given in the same order (Table I). Thus, stabilities of the ternary complexes of RNase with U plus V(IV) and with U plus V(V) are 10 and more than 20 times the respective values predicted for the case of no mutual enhancement of binding. On the other hand, the maximum stability of the ternary complex of RNase with dU and V(IV) is no greater than that predicted on the basis of no interaction between the two ligands. The case of the complex with dU and V(V) is indeterminate, although the minimal values for the equilibrium constants indicate an increased stabilization of no more than twofold for the ternary complex. These considerations indicate that the binding of V strengthens the binding of U but not dU. The most likely explanation is that the U is complexed with the V in the ternary complexes with RNase.

## Discussion

There is kinetic evidence that the $K_m$ value for U-2′,3′-P that is obtained from steady-state kinetic data is a true dissociation constant.[22] Therefore, the association constant for the binding of the V(IV)–U complex to RNase is 1000 times larger than that for binding of the substrate and 40 times larger than that for binding of the product U-3′-P (Tables I and II). Similarly, the V(V)–U complex binds to RNase 1000 times more tightly than U-2′,3′-P and 40 times more tightly than U-3′-P. In addition, V(IV) itself is an effective inhibitor of RNase; its association constant is 160 times larger than the value of the association constant for U-2′,3′-P. Without direct information on the structures of the complexes of RNase with these inhibitors, we cannot state with any certainty what the explanation(s) for the unusually strong binding of these inhibitors to RNase is (are). However, there are two possible explanations that we think are worth describing at this point.

First, one or more groups at the active site of the enzyme may coordinate to the vanadium. For example, the imidazole group of either His-12 or His-119, or possibly both imidazole groups, which are part of the active site,[23] may function as ligands. Cu(II) and Zn(II) are inhibitors of RNase-catalyzed reactions.[24,25] At pH 7.0 and 37°, the value of the dissociation constant of the enzyme–Cu(II) complex that is obtained from inhibition of the initial velocities of the hydrolysis of the benzyl ester of cytidine 2′-phosphate is $5 \times 10^{-6}$ $M$.[24] Crystallographic[26] and other evidence[27,28] has shown that an imidazole group and nearby peptide groups are the ligands for the Cu(II) that binds at the active site.

The second possible explanation is that the inhibitor possesses, or can adopt without a large increase in energy, a structure that is similar to the substrate portion of the transition state for RNase-catalyzed hydrolysis of phosphate esters. In this case the inhibitor would be a transition-state analog. The theoretical reason and experimental support for the proposition that any such analog should bind very much more tightly than the corresponding substrate have been described elsewhere.[3]

Considerable evidence indicates that the transition state for RNase-catalyzed hydrolysis of U-2′,3′-P probably resembles a trigonal bipyramid of pentaoxophosphorus in which the entering and leaving groups are axial (Figure 4).[23,29,30] The favored geometry of

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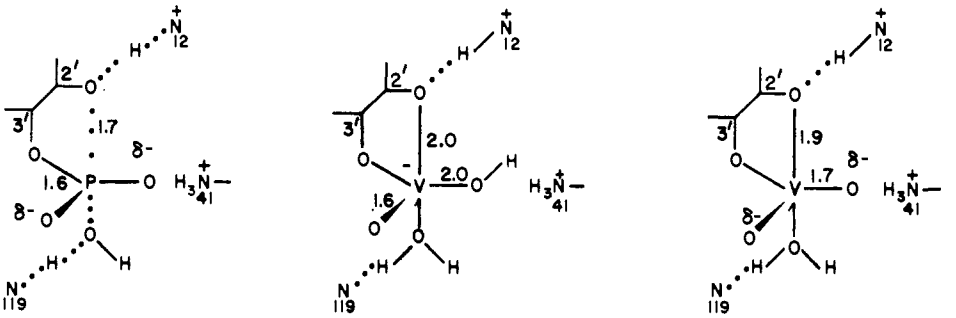

Figure 4. Hypothetical partial structures for the transition state of RNase-catalyzed hydrolysis of U-2′, 3′-P (left), for the analogous complex of RNase with the complex of U and V(IV) (center), and for the analogous complex of RNase with the complex of U and V(V) (right). N-12 and N-119 refer to nitrogen atoms of the imidazole groups of His-12 and -119; $H_3N^+$-41 refers to the amino group of Lys-41. The numbers on the bonds are the approximate bond lengths in Å of similar bonds in stable compounds (ref 19, 31, and F. Rameriz, *Accounts Chem. Res.*, **1**, 168 (1968)).

crystalline complexes of oxovanadium(IV) ion appears to be a square-pyramidal one in which the oxygen atom bonded only to vanadium occupies the axial position.[31] However, some crystalline complexes possess a trigonal-bipyramidal structure, which may occur as the result of unfavorable nonbonded interactions in the square-pyramidal geometry.[32] In aqueous solution the structures of the V(IV) complexes are probably the same, with the exception that water coordinates weakly with the vacant sixth position of the square pyramid to give a distorted octahedral structure.[12,33] These facts suggest that energy required for the transformation of the U–V(IV) complex from its normal solution structure to a trigonal bipyramid may be small enough, relative to the release in energy upon binding of the trigonal bipyramid to RNase, so that the ternary complex with RNase may be a trigonal bipyramid, as shown in Figure 4. The analogy between this structure and the transition state is imprecise, since the V–O bond lengths, with the exception of one, are about 0.3 Å greater than the P–O bond lengths (Figure 4). Also, although the net charge on both the substrate and the complex of U with V(IV) is minus one at pH 7.00, the number of protons in the transition state is smaller than the number in the analogous complex by one (Figure 4).

It also seems possible that the complex of U and V(V) might easily adopt, or even exist as, a trigonal-bipyramidal structure, as shown in Figure 4. The vanadium atoms in crystalline, hydrated metavanadates ($VO_3^- \cdot H_2O$) are five-coordinate with oxygen atoms, and the geometry is approximately trigonal bipyramidal.[19] In solution at pH 7, the predominant species of monomeric V(V) is a monoanionic species,[19] and thus bears the same charge per vanadium atom as does crystalline hydrated metavanadate. The number of oxygen atoms in the primary coordination sphere of the monoanionic species in solution has not yet been clearly determined. It may either be four, five, or six. At very alkaline pH V(V) exists as the tetrahedral $VO_4^{3-}$,[19] whereas the geometry of complex between V(V) and ethylenediaminetetraacetic acid is octahedral.[34]

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