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Kinetic Isotope Effects and Stereochemical Studies on a Ribonuclease Model: Hydrolysis Reactions of Uridine 3′-Nitrophenyl Phosphate

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The reactions of a ribonuclease model substrate, the compound uridine-3′-*p*-nitrophenyl phosphate, have been examined using heavy-atom isotope effects and stereochemical analysis. The cyclization of this compound is subject to catalysis by general base (by imidazole buffer), specific base (by carbonate buffer), and by acid. All three reactions proceed by the same mechanistic sequence, via cyclization to cUMP, which is stable under basic conditions but which is rapidly hydrolyzed to a mixture of 2′- and 3′-UMP under acid conditions. The isotope effects indicate that the specific base-catalyzed reaction exhibits an earlier transition state with respect to bond cleavage to the leaving group compared to the general base-catalyzed reaction. Stereochemical analysis indicates that both of the base-catalyzed reactions proceed with the same stereochemical outcome. It is concluded that the difference in the nucleophile in the two base-catalyzed reactions results in a difference in the transition state structure but both reactions are most likely concerted, with no phosphorane intermediate. The ^{15}N isotope effects were also measured for the reaction of the substrate with ribonuclease A. The results indicate that considerably less negative charge develops on the leaving group in the transition state than for the general base-catalyzed reaction in solution.

INTRODUCTION

Ribonucleases catalyze the cleavage of the (5′–O–P bond of RNA. Because of the biological importance of this reaction its mechanistic details have been the object of numerous studies, involving both the enzyme itself and model systems (*1*). Williams and co-workers have conducted linear free energy studies on a ribonuclease model, uridine 3′-aryl phosphates (*2*). These compounds undergo the first step of the ribonuclease reaction, namely cleavage of the P–O bond, with formation of a 2′,3′ cyclic phosphate. In that work, the Brønsted parameters were measured for the general base reaction catalyzed by imidazole buffer and the specific base reaction in carbonate

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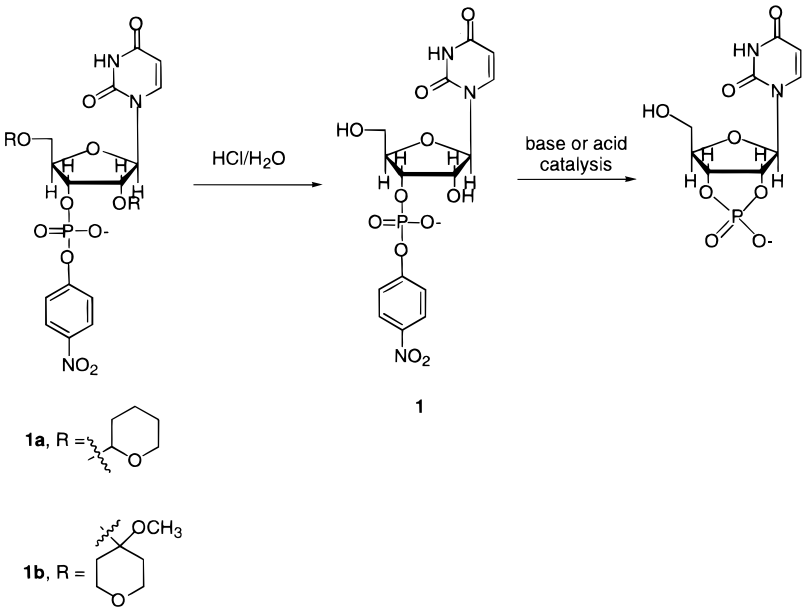
buffer. It was concluded that both the general and the specific base-catalyzed reactions were concerted, with bond cleavage to the leaving group and bond formation to the nucleophilic 2′ hydroxyl group each occurring in the transition state (*2*). In this paper, we describe the results of heavy-atom isotope effects and of stereochemical studies with uridine 3′-*p*-nitrophenyl phosphate (Scheme 1) under conditions of general base and specific base catalysis. In addition, the isotope effects were measured for the acid-catalyzed hydrolysis reaction, and the ^{15}N isotope effects were measured for the ribonuclease-A-catalyzed reaction of the substrate at pH 6.2 and 7.6.

MATERIALS AND METHODS

Pyridine was distilled from calcium hydride under nitrogen before use. DMSO was stored over KOH and 3-Å molecular sieves. Chloroform and hexane were dried in evacuated ampoules over P_2O_5 and lithium aluminum hydride, respectively, and were distilled directly into reaction vessels under vacuum. Other reagents and solvents were purchased from commercial sources and used as received unless otherwise specified. Ribonuclease A was purchased from Sigma.

The product identification NMR experiments were performed on a Bruker 500-MHz instrument, operating at 202.34 MHz for ^{31}P experiments. These ^{31}P spectra were referenced to 80% phosphoric acid in D_2O (external reference). Thin layer chromatography was performed on precoated silica gel plates (silica gel 60, F_{254}, Merck) using phosphomolybdic acid/ethanol reagent for visualization. Column chromatography was performed on silica gel 60 (40–63 mm, Merck).

[^{14}N]-*p*-Nitrophenol (*3*) and [^{15}N, phenolic-^{18}O]-*p*-nitrophenol (*4*) were prepared



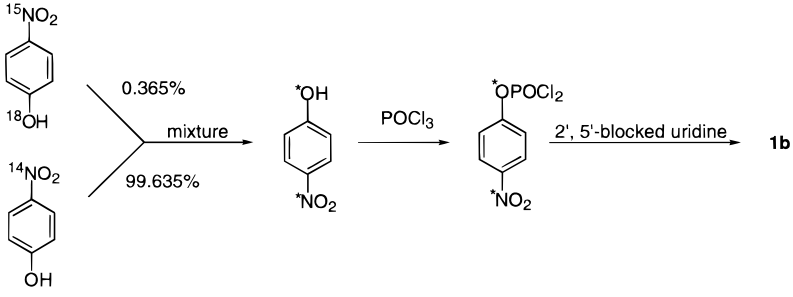
SCHEME 1

as previously described. 2′,5′-bis(*tert*-Butyldiphenylsilyl)uridine (**2**) was prepared according to Ogilvie *et al.* (*5*). [^{18}O]-Phosphorus oxychloride was prepared by hydrolysis of phosphorus pentachloride with 48% [^{18}O]-H_2O. Phosphoryl-[^{18}O]-*p*-nitrophenyl phosphorodichloridate was prepared from this material by reaction of an excess of phosphorus oxychloride with *p*-nitrophenol as described (*6*).

*Preparation of natural abundance 2′,5′-bis(tetrahydropyranyl)-uridyl-3′-p-nitrophenyl phosphate (**1a**).* This compound was prepared by the general procedure of Williams *et al.* (*2*). Uridine was benzoylated at the 3′ position using dibutyltin oxide and benzoyl chloride (*7*), followed by protection of the 2′- and 5′-hydroxy groups as their tetrahydropyranyl ethers using 5,6-dihydro-2*H*-pyran, with subsequent removal of the benzoyl group with ammonia. The resulting 2′,5′-bis(tetrahydropyranyl) uridine (900 mg) was dissolved in 4.5 mL of dry dioxane. This solution was added to a solution of *p*-nitrophenyl phosphorodichloridate (725 mg) and pyridine (450 μL) in 3 mL of dry dioxane, and the mixture was stirred under nitrogen for 6 h. Then a mixture of water (4 mL) and pyridine (1 mL) was added, and the reaction mixture was stirred for 15 min. After concentration *in vacuo*, the crude product was extracted from water with methylene chloride, purified by reverse phase chromatography eluting with methanol/water, and finally converted into the sodium salt by ion exchange. The compound was stored as the 2′,5′-bis(tetrahydropyranyl) ether and was deblocked in dilute HCl solution just before use.

Preparation of remote labeled p-nitrophenyl phosphorodichloridate (Scheme 2). Quantities of [^{14}N]-*p*-nitrophenol and [^{15}N, phenolic-^{18}O]-*p*-nitrophenol were mixed in proportion to closely duplicate the 0.365% natural abundance of ^{15}N. The isotopic ratio was confirmed by isotope ratio mass spectrometry. This mixture was then used to prepare remote labeled *p*-nitrophenyl phosphorodichloridate by the method previously described (*3*).

*Preparation of remote labeled 2′,5′-bis(4-methoxytetrahydropyran-4-yl)-uridyl-3′-p-nitrophenyl phosphate (**1b**).* Uridine was protected at the 2′- and 5′-positions as the bis (4-methoxytetrahydropyran-4-yl) ether by the same reaction sequence described above for the preparation of 2′,5′-bis(tetrahydropyranyl) uridine, except that 4-methoxy-5,6-dihydro-2*H*-pyran was used in place of 5,6-dihydro-2*H*-pyran (*8*). This compound was reacted with remote labeled *p*-nitrophenyl phosphorodichloridate and purified as described above for the natural abundance compound. The compound was



SCHEME 2

stored as the 2′,5′-bis(4-methoxytetrahydropyran-4-yl) ether and deblocked in dilute HCl solution just before use in measurements of bridge-^{18}O isotope effects. The achiral 4-methoxytetrahydropyran-4-yl ether protecting group was used to avoid differential rates in the acid-catalyzed removal of the protecting groups which could occur with the chiral tetrahydropyranyl ether.

*Preparation of 3′-O-[2′,5′-bis(tert-butyldiphenylsilyl)uridyl]-O-methyl-O-p-nitrophenyl ^{18}O-phosphate (**3**) (Fig. 1).* Protected uridine **2** (948 mg, 2 mmol) and pyridine (230 μL) were dissolved in hexane/chloroform (5:1 v/v, 5 mL) and the resulting solution was treated with a solution of *p*-nitrophenyl-[^{18}O]-phosphorodichloridate (517 mg, 2 mmol) in 1 mL of the same solvent at 0°C. A 0.5-mL aliquot of the reaction mixture was transferred into an NMR tube and the progress of the reaction was monitored by ^{31}P NMR. The formation of two diastereomers of the product giving rise to a doublet at -3 and -4 ppm was observed, along with unidentified by-products. The reaction was continued to reach the optimum yield of the product. The reaction mixture was then mixed with 230 μL of pyridine and 120 μL of methanol. TLC (hexane/acetone, 3:1) of the reaction mixture showed formation of a distinct product spot along with some unreacted protected uridine. The mixture was concentrated and purified by chromatography eluting with hexane/acetone (5:1) to give a 2:1 mixture of the diastereomers of **3**, along with some *p*-nitrophenyl dimethyl phosphate. This mixture was inseparable by TLC and was subjected to demethylation without further purification. ^{31}P NMR (C_6D_6) diastereomer a: -8.13 [^{16}O], -8.17 [^{18}O]; diastereomer b: -8.18 [^{16}O], -8.22 [^{18}O]; *p*-nitrophenyl dimethyl phosphate: -6.57 [^{16}O], -6.71 [^{18}O].

*Preparation of 3′-O-[2′,5′-bis(tert-butyldiphenylsilyl)uridyl] O-p-nitrophenyl phosphate (**4**).* The triesters **3a,b** (141 mg, 0.205 mmol) and tetra-*n*-butylammonium iodide (150 mg, two-fold excess) were dissolved in dry acetonitrile (0.5 mL), and the solution was stored at room temperature. After 12 h TLC could detect no remaining substrate. The mixture was chromatographed on silica gel, eluting first with chloroform/methanol (20:1) to elute excess tetrabutylammonium iodide and some *p*-nitrophenol and then with chloroform/methanol (7:1) to give pure product (178 mg). ^{31}P NMR (CD_3OD) δ -7.70, -7.73 ppm.

*Desilylation of **4** to diester **5** and subsequent cyclization to cyclic 2′,3′-uridine phosphate **6*** (Fig. 1). Cleavage of the TBDMS groups in **4** with tetra-*n*-butylammonium fluoride in THF or with HCl in aqueous methanol were found to give unsatisfactory results due to the rapid formation of 2′,3′-cyclic UMP following deprotection of the 2′-hydroxyl group. The tetra-*n*-butylammonium salt of diester **4** (120 mg) from the previous step was converted to the potassium salt using the potassium form of Dowex 50-X8 cation exchange resin. This salt was dissolved in methanol/water (5:3) and the solution was treated with Dowex 50-X8 resin in the proton form. ^{31}P and ^{1}H NMR were used to monitor the progress of deprotection of the 5′-hydroxyl group, and the reaction was judged complete within approximately 0.5 h as revealed by an upfield shift of the ^{31}P chemical shift by 0.25 ppm. Deprotection of the 2′-hydroxyl was slower and required several hours and resulted in a further upfield shift of 0.18 ppm. In parallel with deprotection, cyclization and hydrolysis of 2′,3′-cyclic UMP proceeded which manifested itself by the appearance of two new signals at 2.9 and 2.7 ppm, which were ascribed to 2′- and 3′-UMP. After 3 h the concentration of

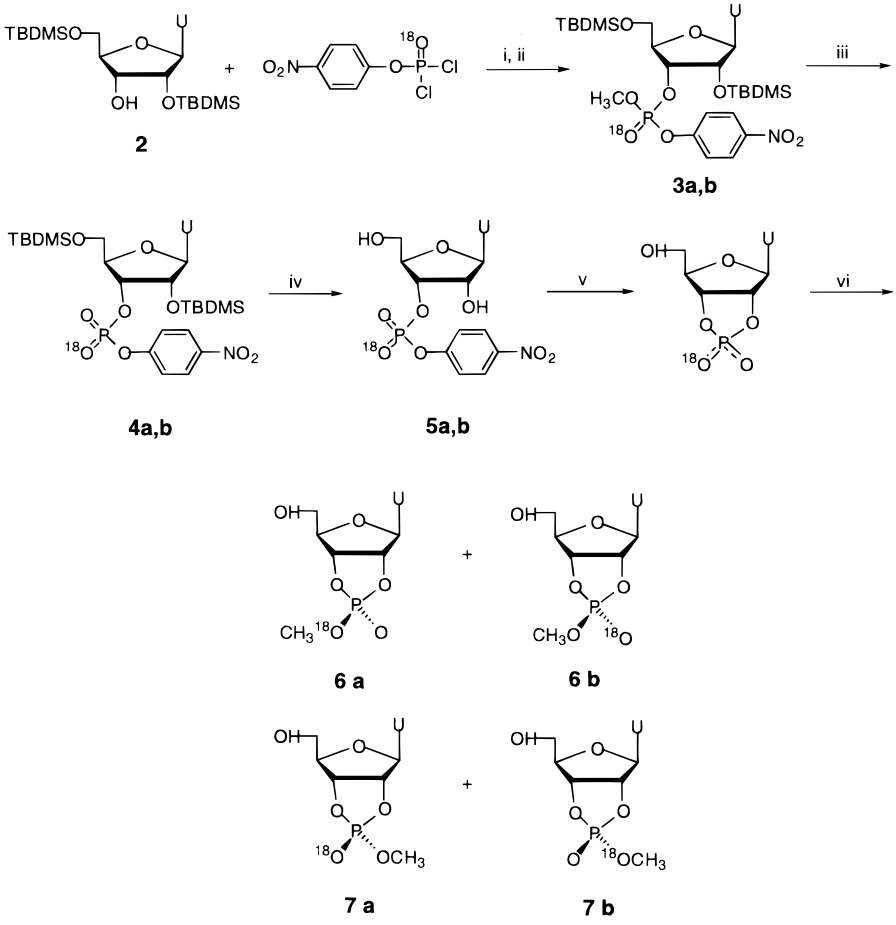


FIG. 1. Outline of the sequence used to produce the diastereomeric mixture of **5**, its subsequent cyclization to cUMP with imidazole or carbonate buffer, and subsequent methylation for ^{31}P NMR analysis. i: Pyridine; ii: methanol, pyridine; iii: $Bu_4N^+I^-$; iv: H^+-Dowex 50-X8; v: K_2CO_3 (pH 9.6) or imidazole (pH 6.7) buffers; vi: CH_3I, dibenzo-18-crown-6.

diester **5** reached a maximum and began to decline. At this point the mixture was split into two equal portions. The first was added to imidazole–HCl buffer (20 mL, 0.2 M, pH 6.7), and the second was added to potassium carbonate buffer (20 mL, 0.2 M, pH 9.6). The pH of the solutions was checked immediately and found to be within 0.1 unit of the pH of the original buffer. ^{31}P NMR spectra recorded shortly after mixing showed formation of the cyclic UMP at 19.2 ppm. The sample in carbonate buffer was passed through a Dowex 50-X8 column in the Et_3NH^+ form, the eluate concentrated under vacuum, and the residue was chromatographed on DEAE–Sephadex eluting with triethylammonium bicarbonate buffer. The pure cUMP obtained was freed of the residual buffer salt by absorbption on charcoal, washes with excess water, and elution with ethanol/aqueous ammonia (100:3). The eluate

was concentrated, passed through a Dowex 50-X8 column in the potassium form, and concentrated to give cUMP as its potassium salt. ^{31}P NMR δ 19.10 [^{16}O], 19.07 [^{18}O]. The sample treated with imidazole buffer was worked up in a similar manner except that it was treated sequentially with charcoal, DEAE–Sephadex chromatography, and cation exchange with K^+-Dowex.

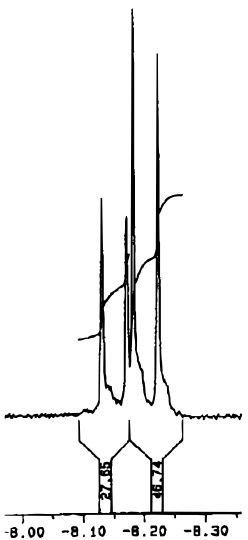
Configurational analysis of [^{16}O, ^{18}O] cUMP. An aqueous mixture of the foregoing potassium salts of [^{16}O, ^{18}O] cUMP (half of the total quantity) was transferred to a 5-mm NMR tube and freeze-dried. The solid residue was made anhydrous by repeated dispersion in warm, dry dioxane and freeze-drying. The anhydrous solid was dissolved in dry DMSO (0.4 mL), dibenzo-18-crown-6 (15 mg) was added, and the homogeneous sample was treated with methyl iodide (100 μL) at room temperature. The progress of the reaction to form **6** and **7** was followed by ^{31}P NMR. The reaction was complete within 3 h, as evidenced by the appearance of two groups of signals at 19.5 and 18.8 ppm. In addition to the two groups of signals arising from exo- and endomethyl esters of [^{16}O, ^{18}O] cUMP, the formation of a minor quantity of upfield (carbonate sample) and down field (imidazole sample) was observed (Fig. 2). These signals displayed a similar intensity pattern as the major peak clusters and are probably the product of further alkylations occurring on the uridine moiety. **7**: ^{31}P NMR (DMSO) δ 19.476 (^{16}O–P=^{16}O), 19.459 (^{18}O–P=^{16}O), 19.434 (^{16}O–P=^{18}O), 18.796 (^{16}O–P=^{16}O), 18.778 (^{18}O–P=^{16}O), 18.753 (^{16}O–P=^{18}O).

General procedures for isotope effect determinations. Removal of the protecting groups on the 2′- and 5′-hydroxyl groups was accomplished by dissolving in 30 mL of dilute HCl (pH 1.5) for 45 min at room temperature in the case of 2′,5′-tetrahydropyranyl groups and by identical conditions for 30 min for labeled 2′,5′-(4-methoxytetrahydropyran-4-yl) groups. NMR experiments monitoring the removal of the tetrahydropyranyl groups versus hydrolysis showed that under these conditions deprotection was complete while hydrolysis of the deprotected diester was undetectable by ^{13}P NMR. For ^{15}N isotope effects, the natural abundance 2′,5′-bis(tetrahydropyranyl)uridine-3′-p-nitrophenyl phosphate (**1a**) was used, and for the ^{18}O isotope effect experiments, remote labeled 2′,5′-bis(4-methoxytetrahydropyran-4-yl) uridine-3′-p-nitrophenyl phosphate (**1b**) was used. It was noted that the four diastereomers of **1a** resulting from the chiral tetrahydropyranyl groups exhibited slightly different rates of deprotection. There was a possibility that if the diastereomers differed slightly in their isotopic composition, then in the isotopic mixture used for the bridge ^{18}O isotope effects differential deprotection rates might result in slightly different isotopic mixtures from experiment to experiment. For this reason the achiral protecting groups used in **1b** were used for the remote label experiments.

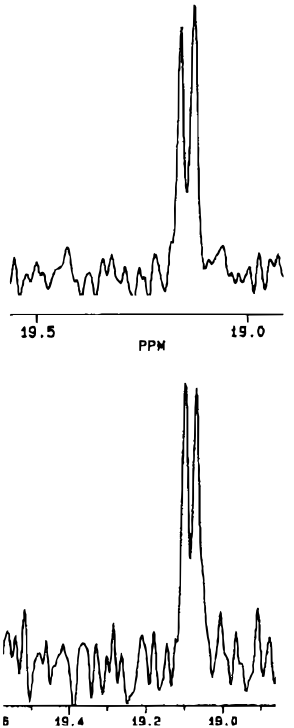
Deblocking of **1a** and **1b** was monitored by adding 10-μL samples to a cuvette containing 0.4 N NaOH and reading the absorbance at 400 nm. The deblocked substrate undergoes nearly instantaneous release of *p*-nitrophenolate under these conditions. After deblocking was complete, the solution was divided into three equal portions treated under the specific reaction conditions described below.

The isotope effects were calculated using the isotopic ratios of the nitrogen atom in the nitro group in the product nitrophenol. This ratio was measured for the product isolated after partial reaction and that in the unreacted substrate following its complete hydrolysis. The isotope ratio in the product and that in the residual substrate, compared

Mix of diastereomers of the triester 3



cUMP products from imidazole (top) and carbonate (bottom) reactions



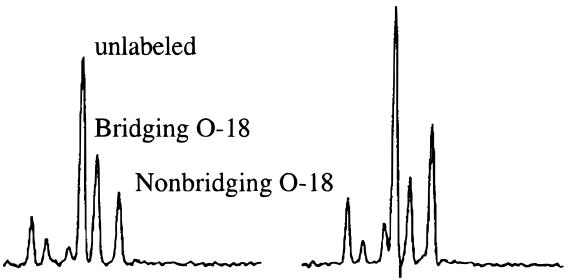
cUMP products after methylation

Imidazole buffer products

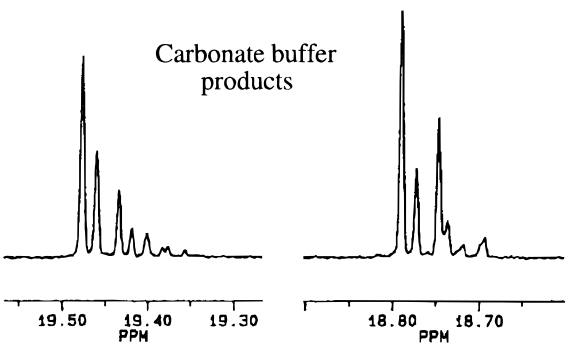
unlabeled

Bridging O-18

Nonbridging O-18



Carbonate buffer products



with the isotopic ratio in the starting material, were used for calculations of the isotope effects as previously described (*4*). The nitrogen in nitrophenol was converted to molecular nitrogen for isotopic analysis as previously described (*4*).

Determination of the isotope effect in the specific base reaction (carbonate buffer). A total of 220 mg of either substrate **la** or substrate **1b** was deprotected by treatment with HCl as described. The resulting uridine-3′-*p*-nitrophenyl phosphate solution was divided into three 10-mL portions and to each was added 35 mL of ice-cold water followed by 5 mL of cold 0.5 M carbonate buffer, pH 9.7. Under these conditions the substrate has a half-life for hydrolysis of approximately 90 s. At times ranging from 60 to 120 s, reactions were stopped by rapid titration with 5 N sulfuric acid to give a pH of about 4. The reaction mixtures were extracted with diethyl ether (3×50 mL) to isolate the *p*-nitrophenol product. After ether was removed by rotary evaporation, the nitrophenol was taken up in a known volume of water and assayed by adding an aliquot to 0.1 N NaOH and measuring the absorbance at 400 nm. The aqueous layer from the original reaction mixture, containing the unreacted substrate, was titrated to pH 10 with 5 N NaOH and left stand at room temperature for 1 h. The *p*-nitrophenol liberated from the residual substrate in this manner was assayed as above. The aqueous layers were then acidified and extracted with ether to isolate *p*-nitrophenol which was purified by vacuum sublimation at 90°C prior to isotopic analysis.

Determination of the isotope effect in the general base reaction (imidazole buffer). Substrate **1a** or **1b** was deprotected as described above for the specific base reaction; then to each 10-mL aliquot of the substrate 40 mL of 0.625 M imidazole buffer (pH 6.75) was added giving a final buffer concentration of 0.5 M. Under these conditions at 22°C the half-life for hydrolysis was approximately 15 min. Reactions were stopped at times ranging from 10 to 25 min by titration to pH 3 with 5 N sulfuric acid and were then extracted with ether (3×50 mL). The ether extracts were concentrated by rotary evaporation, dissolved in 50 mL of water, and assayed for *p*-nitrophenol as described above. The solution was titrated to pH 11 and washed with methylene chloride (50 mL) and then with ether (2×40 mL). After acidification to pH $\sim$ 3, *p*-nitrophenol was extracted with ether (3×50 mL). These ether layers were dried over magnesium sulfate and concentrated by rotary evaporation. This back extraction procedure yielded *p*-nitrophenol free from contamination by imidazole.

The residual substrate in the aqueous layer of the reaction mixture was titrated to

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FIG. 2. At the left are the ^{31}P NMR signals of the 2:1 mixture of the diastereomers of 3′-*O*-[2′,5′-bis(tert-butyldiphenylsilyl)uridyl]-*O*-methyl-*O*-*p*-nitrophenyl phosphate (**3**). To the right of this are the ^{31}P NMR signals of the cUMP produced in the imidazole-buffered (top) and carbonate-buffered (bottom) reactions of **5**. Prior to methylation the signals for the diastereomers cannot be distinguished; the two signals seen are those for the unlabeled and the ^{18}O-labeled cUMP. At the right are the spectra of the cUMP products after methylation, corresponding to the four compounds **6a,b** and **7a,b** in Fig. 3. Each diastereomer gives a set of three signals consisting of, in order from left to right, a peak for the unlabeled compound, for the bridge ^{18}O-labeled, and for the nonbridge ^{18}O-labeled compounds. These are labeled in the first of the four spectra in the set. The smaller set of signals upfield (in the imidazole spectra) and downfield (in the carbonate spectra) are ascribed to products from overmethylation of the cUMP.

pH 7, left to stand for 3 h, and then assayed for *p*-nitrophenol. The *p*-nitrophenol thus liberated was then isolated by the same method.

Determination of the isotope effect in the acid hydrolysis reaction. After deprotection, the substrate solution was made 1 N in HCl by addition of 5 N HCl. Under these conditions the half-life for hydrolysis was about 12 min. Reactions were effectively stopped by addition of cold 1 N Hepes buffer, pH 8, until the reaction mixtures had a pH of about 4. The reaction mixtures were extracted with ether (3×40 mL). After evaporation of ether, the *p*-nitrophenol was dissolved in water and assayed. The residual substrate solution was titrated to pH 10 by addition of 5 N NaOH, allowed to stand for >10 half-lives, and then assayed for *p*-nitrophenol. The *p*-nitrophenol thus liberated from residual substrate was isolated after acidification by ether extraction and purified by sublimation before isotopic analysis.

Determination of the isotope effect in the ribonuclease A reaction. After deprotection, 5 mL of the substrate solution was diluted with 75 mL of distilled water and added to 20 mL of a 500 mM solution of either Bis–Tris buffer at pH 6.2 or of Tris at pH 7.6. The final buffer concentration was 100 mM, and substrate concentration was 0.5 mM. A 100-μL aliquot of a 0.05 mg/mL enzyme solution was added to initiate the reaction at pH 6.2; 1 mL of a 0.5 mg/mL enzyme solution was used for the reactions at pH 7.6. The amounts of enzyme were chosen in order to keep the enzyme-catalyzed reaction at least 10 times faster than the competing solution reaction. The progress of the reaction was monitored by following the absorbance at 330 nm of a 50-μL aliquot added to 950 μL of distilled water. When the fraction of reacted substrate was in the 40 to 60% range, reactions were stopped by acidification with acid to pH 3, followed by extraction of the *p*-nitrophenol product with ether (3×50 mL). The aqueous layer containing the residual substrate was titrated back to pH 6 and 500 μL of the RNase solution was added. This solution was allowed to stand for >10 half-lives and then assayed for *p*-nitrophenol. The ether layers containing the product *p*-nitrophenol after partial reaction were evaporated to dryness and the residue was dissolved in 50 mL of water and assayed for *p*-nitrophenol. The fraction of reaction reached when the enzymatic reaction was stopped was determined from the assay of the initial nitrophenol product and that released from hydrolysis of the residual substrate. Both samples of *p*-nitrophenol were isolated by acidification of the aqueous solutions followed by ether extraction and then purified by sublimation before isotopic analysis.

Product determination NMR experiments. 2′,5′-bis(Tetrahydropyranyl) uridine 3′-*p*-nitrophenyl phosphate (7.5 mg) was dissolved in 600 μL of D_2O and 25 μL of 1 N HCl was added. ^{31}P NMR was used to monitor the deblocking of the tetrahydropyranyl groups. When this was complete (approximately 30 min), reactions were initiated by addition of 0.5 M carbonate buffer (500 μL, pH 9.7), imidazole buffer (0.6 N, pH 6.75, 500 μL), or HCl (5.0 N, 100 μL). Formation of products was followed by ^{31}P NMR.

RESULTS

The course of the reaction. The courses of the general base, specific base, and acid hydrolysis reactions were each followed by proton-decoupled ^{31}P NMR. The 2′,5′-bis(tetrahydropyranyl) uridine 3′-*p*-nitrophenyl phosphate exhibited a group of

four signals between −4.277 and −4.442 ppm corresponding to four diastereomers due to the presence the chiral tetrahydropyranyl groups. As the deblocking reaction progressed, these signals were gradually replaced by a single resonance at −3.84 ppm.

After addition of imidazole buffer, the resonance of the deblocked substrate was gradually replaced by a single resonance at 22.14 ppm which did not undergo further reaction over 45 min. Acidification with 100 μL of 5 N HCl resulted in the immediate replacement of this resonance by a pair of nearly equal resonances at 1.08 and 1.13 ppm. Addition of authentic uridine 3′-phosphate resulted in an enhancement of the more upfield of these two signals.

When carbonate buffer was used to initiate the reaction, the resonance of the deblocked substrate was gradually replaced by a single resonance at 22.23 ppm. This signal was unchanged over 2 h, whereupon acidification with 100 μL of 5 N HCl resulted in the immediate replacement this signal by a pair of nearly equal resonances at 1.19 and 1.24 ppm. Addition of authentic uridine 3′-phosphate resulted in an enhancement of the more upfield of these two signals.

In the acid hydrolysis reaction the single resonance of the deblocked substrate was gradually replaced by a pair of nearly equal resonances at 1.08 and 1.13 ppm. Addition of authentic uridine 3′-phosphate resulted in an enhancement of the more upfield peak.

Isotope effects. The isotope effects and their standard errors are tabulated in Table 1 for the solution and enzymatic reactions. At least four experiments were conducted for each set of conditions. Each experiment yields two independent determinations of the isotope effect, one calculated from the isotope ratios in the product and that of the starting material and the other from the ratios of the residual substrate and starting material. These isotope effects agreed in all cases within the standard errors and were averaged together to give the mean values reported in Table 1. In the experiments with the remote-labeled compound for determination of $^{18}k_{\text{bridge}}$, the observed isotope effect is that due to both ^{15}N and ^{18}O substitution. This observed effect was corrected for the ^{15}N contribution and for incomplete isotopic incorporation, as previously described (*9*), to give the values reported in Table 1.

Stereochemistry. The substrate for the reaction **5** was generated *in situ* from the potassium salt of **4** prepared as a 2:1 diastereomeric mixture. Acid-catalyzed deprotection was accomplished using Dowex 50-X8 in the proton form. A small amount of cyclization to cUMP and subsequent hydrolysis was observed to occur during deprotection. When the concentration of the deprotected diastereomeric mixture of isomers of substrate **5** had reached a maximum, the solution was divided into two

TABLE 1

Isotope Effects for Reactions of Uridine 3′-*p*-Nitrophenyl Phosphate

Reaction	^{15}k	$^{18}k_{\text{bridge}}$
1, Imidazole buffer	1.0009 ± 0.0002	1.0067 ± 0.0009
1, Carbonate buffer	1.0001 ± 0.0002	1.0059 ± 0.0004
1, Acid catalysis	1.0010 ± 0.0002	1.0063 ± 0.0011
1, RNase, pH 6.2	1.0002 ± 0.0001	
1, RNase, pH 7.6	1.0005 ± 0.0001	

portions. One of these was treated with imidazole buffer and the other with carbonate buffer. The cUMP product obtained under both general base and specific base conditions was subsequently methylated, allowing the diastereomeric products to be analyzed by ^{32}P NMR. Prior to methylation of the cyclic UMP, the signals for the isotopic diastereomers cannot be distinguished. Following methylation, the exo- and endomethyl esters (**6** and **7**) (Fig. 2) of cUMP are resolved into separate clusters of signals. Each cluster consists of signals, from left to right, for the unlabeled compound, with bridging ^{18}O and with nonbridging ^{18}O. Unmethylated cUMP with exo- ^{18}O will produce, after methylation, a mixture of exomethyl ester with bridging ^{18}O (**6a**) and endomethyl ester with nonbridging ^{18}O (**7a**). Similarly, cUMP with endo- ^{18}O after methylation will produce a mixture of endomethyl ester with bridging ^{18}O (**7b**) and exomethyl ester with nonbridging ^{18}O (**6b**). The results show essentially identical stereochemical outcomes for the general base and the specific base-catalyzed reactions. The approximately 2:1 ratio of diastereomers present in the starting material is essentially unchanged in the products.

DISCUSSION

In the ^{31}P NMR experiments following the products of the general base (imidazole) and specific base (carbonate) reactions, the signal of the deblocked substrate was gradually replaced by a more downfield resonance having a chemical shift typical of 5-membered ring cyclic phosphates. The slight difference in the chemical shift of this signal between these two sets of conditions was attributed to the difference in pH of the reactions. This initial product is stable under the conditions of both of the base-catalyzed reactions, but upon acidification it undergoes ring opening to form a mixture of 3′- and 2′-uridine phosphates. The relative intensities of these two signals in the ^{31}P spectra were similar in the two reactions, with the more downfield peak being slightly larger. The intermediate cyclic phosphate was not observed in the acid catalyzed reaction, but its intermediacy is inferred from the fact that the same two final products are formed, in the same ratio. This result rules out the alternative reaction of direct acid hydrolysis of the uridine 3′-nitrophenyl phosphate, since this process would form uridine 3′-phosphate as the single product. Although isomerization of nucleotide 3′- and 2′-phosphates under acidic conditions is known to occur, this reaction is too slow under the reaction conditions employed to have occurred in the time period of these experiments. These results indicate that, under all three conditions, the reaction proceeds via formation of the 2′,3′-cyclic phosphate, as shown in Scheme 1.

Interpretation of the isotope effects. Changes in bonding to the *p*-nitrophenyl leaving group in the transition state are measured by the isotope effects $^{18}k_{\text{bridge}}$ and ^{15}k. The ^{15}k isotope effect monitors how much negative charge had developed on the leaving group in the transition state. The primary isotope effect $^{18}k_{\text{bridge}}$ gives information about the extent of P–O bond cleavage. As a primary isotope effect, $^{18}k_{\text{bridge}}$ has contributions from factors other than zero point energy and may not be linearly related to bond cleavage. In particular, resonance contributions can cause this isotope effect to exhibit a nonlinear response to transition state structure. In an early transition state, there is little charge delocalization into the aromatic ring and the nitro group, and thus the phenolic oxygen atom remains singly bonded to the phenyl ring. In a later

transition state there will be more charge delocalization and thus partial double bond character between the phenolic oxygen atom and the ring. This stiffening of the C–O bond can compensate partially for the loss of P–O bond order and reduce the isotope effect. The $^{18}k_{bridge}$ isotope effect can, however, distinguish between the considerable bond cleavage typical of reactions of phosphate monoesters, where this bond is mostly broken in the transition state (where $^{18}k_{bridge}$ is in the range 1.02 to 1.03) and more associative transition states where the degree of bond cleavage is much smaller, as in phosphodiester reactions (where $^{18}k_{bridge}$ is <1.01).

The oxygen (primary) and the nitrogen (secondary) equilibrium isotope effects on the deprotonation of *p*-nitrophenol are $^{18}K_{eq} = 1.0153 \pm 0.0002$ (*10*) and $^{15}K_{eq} = 1.0023 \pm 0.0001$ (*11*), respectively. However, deprotonation involves breaking a bond to a hydrogen atom, not to a phosphoryl group; a phosphoryl group is more electron withdrawing, as evidenced by the β_{eq} value of $+0.73$ for an aryl group in a phosphodiester (*12*). This value is very close to those for aryl groups in acetate esters (*13*). The positive value reflects electron donation from the bridge oxygen atom to the phosphoryl group in the ground state, with resultant effects on this bond order. Primary $^{18}k_{bridge}$ isotope effects in phosphoryl and acyl transfer reactions are as large as 1.03 (*4,10,14*), and this is a better indicator of the maximum for this isotope effect than the equilibrium isotope effect for deprotonation. Secondary ^{15}k isotope effects of 1.003 in reactions of the dianion of *p*-nitrophenyl phosphate, where bond cleavage is nearly complete, are also larger than the 1.0023 equilibrium ^{15}k isotope effect for deprotonation.

Isotope effects for the alkaline hydrolysis and for the acid hydrolysis reactions of two other diesters where *p*-nitrophenol is the leaving group, *p-tert*-butylphenyl *p*-nitrophenyl phosphate (**8**) and 3,3-dimethylbutyl *p*-nitrophenyl phosphate (**9**) have previously been measured (*15*) and are reported in Table 2 for comparison with the values measured in the present work. The prior studies with **8** and **9** were conducted at higher temperatures, so an estimation of these isotope effects at 25°C was made using the equation ln(KIE at temp x) = (y °K/x °K) ln (KIE at temp y), which assumes the isotope effect becomes unity at infinite temperature. The isotope effects increase by about 20% from this correction.

The three solution reactions studied here differ in some of their mechanistic details. In the general base reaction of **1**, the nucleophile is the 2′-hydroxyl group with the proton partially abstracted in the transition state. Brønsted analysis indicates that the proton is about 2/3 abstracted by the general base in the transition state (*2*). In the

TABLE 2

Isotope Effects for Reactions of Other Diesters of *p*-Nitrophenyl Phosphate (Data from Ref. (*15*))

Reaction	^{15}k	$^{18}k_{bridge}$
8, alkaline hydrolysis	1.0012 ± 0.0001	1.0052 ± 0.0008
8, acid hydrolysis	1.0008 ± 0.0001	1.0071 ± 0.0005
9, alkaline hydrolysis	1.0019 ± 0.0002	1.0072 ± 0.0005
9, acid hydrolysis	1.0011 ± 0.0002	1.0048 ± 0.0004

Note. Corrected for temperature as described in the text.

specific base reaction in carbonate buffer the nucleophile is the fully ionized alkoxide. This more powerful nucleophile manifests itself in the faster rate of this reaction compared to the general base reaction. The acid-catalyzed hydrolyses of diesters proceed through the nucleophilic attack of water on the protonated, neutral phosphate ester (*16*). In comparing the isotope effects under the three conditions it is apparent that bond cleavage and charge development are, within experimental error, indistinguishable in the acid hydrolysis reaction and the general base (imidazole) reaction.

For the acid-catalyzed reaction, the data do not rule out a mechanism with more extensive bond cleavage to the leaving group than in the basic reactions, but in which protonation of the leaving group occurs to lower the isotope effects. However, it is unlikely that acid catalysis would involve protonation of the leaving group in the transition state, for the following reasons. The acid hydrolysis of phosphate diesters proceeds via the neutral, phosphoryl protonated species (alkyl nitrophenyl diesters have a pK_a of about -0.5 (*3*)). The bridging oxygen of the neutral diester will have an extremely low pK_a in the region of -9 or lower, by analogy with carboxyl esters. In the transition state, the pK_a of the bridging oxygen will be somewhere between this value and $+7.1$, the pK_a of nitrophenolate. Unless the extent of bond cleavage in the transition state is considerably greater than 50%, the pKa of the bridging oxygen atom will still be well below the pH of the reaction. In addition, the finding of a significant ^{15}k isotope effect argues against protonation.

Both $^{18}k_{\text{bridge}}$ and ^{15}k for the acid reaction and the general base-catalyzed reaction of the uridine substrate are similar to those previously measured for the acid hydrolysis of phosphodiesters **8** and **9**. Thus, the data indicate that the intramolecular nature of the reaction and the ring strain introduced in the transition state have no measurable effect on leaving group bond cleavage or on charge development in the transition state. These parameters are essentially unchanged from reactions of *p*-nitrophenyl diesters that do not form a cyclic product.

The specific base (carbonate buffer) reaction differs in that the ^{15}k isotope effect is unity within experimental error, and the $^{18}k_{\text{bridge}}$ isotope effect is somewhat reduced from its value in the other reactions. The lack of a ^{15}k isotope effect implies a lack of charge development on the leaving group in this reaction, which led us to consider the possibility that it may have a different rate-limiting step, such as formation of a phosphorane intermediate. Since no charge would be developed on the nitrophenol group in formation of a phosphorane, there should be no ^{15}k isotope effect in this mechanism. Because such a species would be able to pseudorotate before expelling the leaving group, the stereochemical outcome of the reaction could reveal whether such an intermediate lies on the reaction pathway of the specific base reaction. Therefore, with a mixture of diastereomers of uridine 3′-*p*-nitrophenyl phosphate in known proportion, a concerted reaction would yield diastereomeric products in the same ratio as that of the starting diester. A reaction proceeding via a phosphorane intermediate provides the opportunity for epimerization at the phosphorus atom.

Stereochemical experiments. A 2:1 diastereomeric mixture of the substrate **4a** and **4b** was prepared as the 2′,5′-bis(tert-butyldiphenylsilyl) protected compound. Following deprotection, the compound was subjected to cyclization to cUMP in imidazole buffer and in carbonate buffer. The ^{31}P NMR (Fig. 2, center) shows the signals for the unlabeled and the ^{18}O-labeled cUMP; the diastereomers of the labeled

compound cannot be distinguished. Following methylation using methyl iodide, ^{31}P NMR (Figure 2, right) can distinguish the diastereomers of the resulting triester.

The stereochemical analysis of the two base-catalyzed reactions shows that both proceed with the same stereochemical outcome. The results do not determine whether the reactions proceed with inversion or with retention. However, since the general base reaction is concerted, the results imply that the specific base one is concerted as well. The most likely explanation for the reduced value for ^{15}k in the specific base-catalyzed reaction is that it proceeds with an earlier transition state, with P–O bond cleavage less advanced and less negative charge is present on the leaving group. This may be rationalized by the more powerful nucleophile in the specific base-catalyzed reaction, which is the fully deprotonated 2′-alkoxide. In the general base-catalyzed reaction the 2′-hydroxyl group is deprotonated simultaneously with nucleophilic attack, and the nucleophile is essentially neutral in the transition state for the reaction. Figure 3 shows schematic diagrams of the transition states of the two reactions.

A concerted mechanism is consistent with linear free energy experiments by Williams and co-workers that indicate that for phosphodiesters with good (i.e., aryl) leaving groups, phosphoryl transfer is concerted with no intermediate (*17*). The Brønsted $\beta_{\text{leaving group}}$ value for diester reactions is much smaller than that for monoesters, indicating that reactions of diesters are less dissociative and bond cleavage to the leaving group in the transition state is less advanced, but that nevertheless leaving group departure is concerted with nucleophilic attack. Similarly, the $^{18}k_{\text{bridge}}$ isotope effects in the reactions studied here are all considerably smaller than those found for reactions of the monoester *p*-nitrophenyl phosphate.

The ribonuclease reaction. Ribonuclease A catalyzes the cleavage of the P–O(5′) bond of RNA (*1*). In the generally accepted mechanism, the side chain of His-12 acts as a base that abstracts a proton from the 2′-hydroxyl group, facilitating its attack on the phosphoryl group. In the same step the side chain of His-119 acts as an acid to protonate the leaving group (for a review including references to other mechanistic proposals see Ref. *1*). The reported Brønsted dependencies indicate that considerably less negative charge develops on the leaving group than in the general base-catalyzed reaction with imidazole buffer (*18*). The reaction of the substrate **1** with RNase A has been shown to give an atypical response to mutation of the enzymatic general acid H119. Specifically, while k_{cat}/K_m for H119A is reduced by three to four orders of magnitude with the substrates poly(c) and UpA, with **1** the rate is only slightly

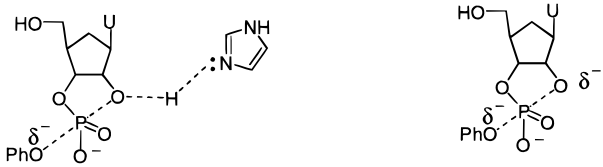

FIG. 3. A diagram of the transition state for the general base (left) and specific base (right) reactions of **5**. In the general base-catalyzed reaction, proton abstraction by imidazole from the nucleophilic 2′ hydroxyl group is concerted with phosphoryl transfer. In the specific base-catalyzed reaction, proton transfer occurs before phosphoryl transfer. The extent of P–O bond cleavage in the transition state is less in the specific base-catalyzed reaction.

reduced (*19*). This is most likely a result of the stability of *p*-nitrophenolate anion, which allows it to function as a good leaving group even in the absence of protonation.

The ^{15}k isotope effects for the RNase-catalyzed reaction of **1** at the pH optimum and at pH 7.6 are smaller than for the imidazole-catalyzed reaction, indicating that less negative charge develops on the leaving group in the enzymatic reaction in agreement with the Brønsted studies (*18*). The isotope effect at pH 6.2 is consistent with protonation of the leaving group in the transition state. The larger isotope effect at pH 7.6 may result from a significant amount of reaction occurring from an incorrectly protonated form of the enzyme.[4] Specifically, if H119 is unprotonated this residue cannot protonate the leaving group in the transition state. The kinetic data from the H119A mutant indicate that *p*-nitrophenolate anion is a sufficiently good leaving group that it can depart as the anion (*19*), in contrast to the natural substrates for RNase where this group is a nucleoside. If the rate of catalysis by the H119A mutant is a fair estimate of the rate of the incorrectly protonated form of the enzyme, then with the nitrophenyl substrate these rates will be similar. This would permit a significant fraction of reaction to proceed via the incorrectly protonated form of the enzymatic reaction of substrate **1** at pH values above its typical pH optimum.

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[4] The $^{18}k_{bridge}$ isotope effects were not measured for the ribonuclease reaction. Since this isotope effect varies very little in the different hydrolysis reactions of 1 in solution, it is unlikely that this isotope effect would yield additional information about the transition state for the ribonuclease reaction.