

# THE RATES OF HYDROLYSIS OF THYMIDYL-3', 5'-THYMIDINE-H-PHOSPHONATE: THE POSSIBLE ROLE OF NUCLEIC ACIDS LINKED BY DIESTERS OF PHOSPHOROUS ACID IN THE ORIGINS OF LIFE

JOHN R. PEYSER<sup>1</sup> and JAMES P. FERRIS<sup>2\*</sup>

<sup>1</sup> Department of Chemistry, Cleveland State University, Cleveland, Ohio 44115, U.S.A.;

<sup>2</sup> NY Center for Studies on the Origins of Life and the Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY 12180-3590, U.S.A.

(\* e-mail: ferrij@rpi.edu, web page: www.origins.rpi.edu, phone: 518:276-8493)

(Received 20 November 2000; accepted in revised form 30 January 2001)

**Abstract.** Thymidyl-3',5'-thymidine H-phosphonate undergoes acid, base, and water-catalyzed hydrolysis. The products were 3'-thymidine H-phosphonate, 5'-thymidine H-phosphonate, and thymidine in a ratio of 1:1:2. The rate constants are  $1.8 \times 10^{-3} \text{ M}^{-1} \text{ sec}^{-1}$ ,  $7.2 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ , and  $1.5 \times 10^{-6} \text{ sec}^{-1}$  for acid, base and water catalysis, respectively. These values are comparable with previous reports for the rates of hydrolysis of simple dialkyl esters of phosphorous acids. The Arrhenius activation energy for the base-catalyzed reaction is 20 kcal/mol. and the enthalpy and entropy of activation are 19 kcal/mol and  $-14 \text{ eu.}$ , respectively. The Gibbs free energy of activation is 23 kcal/mol. The rate constants suggest that nucleic acids linked by diesters of phosphorous acid hydrolyze too rapidly in aqueous solution to have accumulated in useful amounts on the primitive Earth.

**Keywords:** hydrolysis rates, nucleotide hydrolysis, phosphorus-3-bonds, phosphite hydrolysis, pre-RNA, phosphorous acid diesters

## 1. Introduction

The low solubility of phosphate salts in water has led to the postulate that higher concentrations of phosphorous acid salts were used in the prebiotic synthesis of nucleic acid and other phosphorus-containing compounds on the primitive Earth (Gulick, 1955). In this scenario RNA linked by diesters of phosphorous acid preceded the prebiotic synthesis of RNA linked by phosphodiester bonds because of the greater solubility, and thus availability, of the salts of phosphorous acids. In addition, phosphorous acid derivatives are more reactive than those of phosphoric acid, so the diester link between two nucleosides may have formed more readily in aqueous solution (Glindemann *et al.*, 1999; De Graaf and Schwartz, 2000).

Mono- and di-esters of phosphorous acid are named H-phosphonates because they contain a P-H group (see for example structure **8**). This structure is in equilibrium with the tautomeric phosphite ester form in which the proton on phosphorous



*Origins of Life and Evolution of the Biosphere* **31**: 363–380, 2001.

© 2001 Kluwer Academic Publishers. Printed in the Netherlands.

is bound to the phosphoryl oxygen. The equilibrium between the two tautomers is strongly in favor of the H-phosphonate tautomer.

A problem with the scenario that phosphorous acid esters linked the first RNA is that these bonds undergo rapid hydrolysis. Nylen (1937) prepared a series of these esters and observed that they decomposed rapidly in both acidic and basic aqueous solution to give H-phosphonate monoesters and the corresponding alcohol. The calculated half-lives, for example, at 25.0 °C and pH 7.0, ranged from 720 min for the 2-propyl diester to 5.5 minutes for the dimethyl ester. As the solution pH decreased, the diesters presumably hydrolyzed by an acid-catalyzed mechanism. At pH 1.0 the half-lives ranged from 7.2 min for the methyl ester to 60 min for the 2-propyl ester at 25.0 °C. Nylen observed that the stability of the diesters to hydrolysis generally increased with increasing size of the ester substituent, and that the secondary 2-propyl diester was particularly stable to base hydrolysis. In addition to the acid and base catalyzed hydrolysis of the H-phosphonate diesters, these compounds are also decomposed by direct hydrolysis with water. The stability of the esters reached a maximum at pH 4, but even at this pH, the comparatively stable 2-propyl diester had a half-life of about 40 days neglecting water hydrolysis. These hydrolysis rates indicate the rapid degradation of H-phosphonate diesters in aqueous solution. It was proposed that these hydrolyses proceed by a two step mechanism (Westheimer *et al.*, 1988)

The increasing stability to hydrolysis of Nylen's diesters with increasing chain length suggested that the bulky nucleoside diesters of phosphorous acid might have greater stability than the simple diesters studied previously. We sought to investigate the proposal that the nucleoside diesters of phosphorous acid may have been an alternative to, or precursor of, RNA on the primitive Earth.

## 2. Experimental

### 2.1. GENERAL PROCEDURES

Proton magnetic resonance spectra were recorded on Varian model XL-200. Chemical shifts are reported in parts per million (ppm) from tetramethylsilane (TMS) (delta scale). The <sup>1</sup>H NMR data are presented in the following form: delta value of the signal in ppm, multiplicity, coupling constants (where applicable), integrated number of protons, and assignments (where known). Spectra were collected using deuteriochloroform as solvent with a tetramethylsilane (TMS) internal standard except where otherwise noted. Water soluble products were dissolved in 2 mL of deuterium oxide (Aldrich), and the solvent was removed at oil pump pressure. The process was repeated twice, and the residue was redissolved in fresh deuterium oxide for NMR analysis. The residual water resonance was used as an internal reference.

Spectra of diastereomeric mixtures of products are presented in the following form: Where resolution permitted, multiplicity of the signal is prefaced by the

number of such signals which may be assigned to a single proton in the diastereomeric mixture, and the reported chemical shift of the multiple signals represents the average value in ppm of the multiple signals.

Assignments for resonances arising from corresponding hydrogens in each of two monomer subunits in dimeric compounds are distinguished from each other by the arbitrary assignment of a subscript 'a' to one of the two corresponding resonances and is not intended to distinguish between monomer subunits.

All glassware used in synthetic reactions and all glassware used to contain reaction products were base washed in a solution of sodium hydroxide and ethanol, rinsed thoroughly with distilled water, and air dried.

Reaction solvents were of reagent grade and were used without further purification unless otherwise noted. Methylene chloride was high performance liquid chromatography grade (Baker). Pyridine and acetonitrile were continuously distilled from calcium hydride under a nitrogen atmosphere in apparatus fitted with rubber septa to allow removal of solvent by syringe.

All nucleoside and nucleotide starting materials and reaction products were handled at temperatures below 40 °C. Solvent removal from reaction products was typically done at oil pump pressure using a rotary evaporator fitted with a dry ice condenser and an argon-filled balloon to restore atmospheric pressure. All reactions were conducted in flasks fitted with rubber septa under an atmosphere of argon at room temperature unless otherwise noted. Analytical thin layer chromatography (TLC) was done on silica gel (Kieselgel 60 F254, 0.2 mm, Merck EM) using a developer of 5% methanol in methylene chloride, and preparative column chromatography was done using Merck EM Kieselgel 230–400 mesh packed in methylene chloride.

High performance liquid chromatography (HPLC) was performed on a Waters HPLC system consisting of two model M45 solvent delivery pumps controlled by a programmable automated gradient controller and a model 481 variable wavelength spectrophotometric detector. Output was recorded on a Spectral Physics model SP 4290 integrator. The HPLC system employed a Waters  $\mu$ Bondpak octadecyl silica gel (C18) 0.2  $\times$  30 cm analytical column (P/N 27324). Sample elution was performed employing a solvent gradient of buffers 'A' and 'B'. Buffer 'A' was an aqueous solution which was 5.00 mmolar in  $\text{KH}_2\text{PO}_4$  (Fisher). Buffer 'B' was a 30% (v/v) acetonitrile-water solution which was 10.0 mmolar in  $\text{KH}_2\text{PO}_4$ . The buffers were adjusted to a pH of 3.5 with a solution of 0.25 M phosphoric acid. Sample elution employed a 1.0 mL/min gradient which was 100% buffer 'A' for 1 min and then the concentration of 'A' decreased along a linear gradient to 0% in 8 min. The final buffer composition was maintained for 1 min, and then the concentration of 'A' was increased from 0–100% from 9–10 min.

A preparative intermediate pressure reverse phase (C18) column was employed for final nucleotide product purification. The column was prepared by dry-packing an Altex 20  $\times$  200 mm glass column with octadecyl silica gel (Waters, 37–55 micron, P/N 30632). The column was fitted to a Fluid Metering Inc. (FMI) solvent

delivery pump model RP5Y. Product elution was monitored with an LKB Uvicord type 4701A optical detector equipped with a LKB Uvicord model 4701A control unit. Output was recorded on a Sargent-Welsh model SRLG chart recorder. The column was prepared by sequential rinsing of the dry-packed gel with 500 mL portions of water, methanol, acetonitrile, methylene chloride, acetonitrile, methanol, and water. In typical use, the column was operated at 100 PSI. The column was cleaned after each use by rinsing with 500 mL of acetonitrile, and the column was routinely stored after use in this solvent.

### 3. Kinetic Studies

#### 3.1. HYDROLYSIS OF THYMIDYL-3',5'-THYMIDINE-H-PHOSPHONATE (**8**)

19.2 g (0.100 mol) of anhydrous citric acid (Fisher) were added to each of nine 100 mL volumetric flasks and dissolved in *ca.* 50 mL of double distilled water from an all glass still. The pH of each solution was measured with a glass electrode (Fisher #13-620-252) and adjusted with a saturated solution of KOH to yield nine solutions from pH 1.5 to 7.5 in 0.5 pH unit increments. The solutions were diluted to 100.0 mL with double distilled water and stored at 4 °C between uses. Aliquots of buffer solution at each pH were diluted in polystyrene centrifuge tubes with water using an Eppendorf pipette to give solutions which were 1.0 M, 0.10 M, and 0.010 M in citrate for each pH increment to give a total of 27 solutions. The solutions were allowed to equilibrate to 26 °C (within 0.5 degrees) on a drybath (Thermolyne Model DB-17615). Dilution resulted in noticeable pH drift and the pH of each reaction mixture was measured to an accuracy of 0.05 pH units with a glass electrode standardized at each pH with freshly prepared standard buffer solutions (Microessential Laboratory). The glass electrode response was particularly slow with the 1.0 M solutions, and the sealed centrifuge tubes were allowed to equilibrate with the electrode until no change in pH was observed for 20 min. Equilibration routinely was achieved in *ca.* 1 h for the 1.0 M solutions and within 10–15 min for the 0.10 and the 0.010 M solutions.

To each of the solutions was added 20  $\mu$ L of a 20 mg/mL solution of dimer **8** to give a final dimer concentration of 0.30 mmolar. The decomposition reactions were monitored by HPLC for a minimum of 2 half lives. The final pH of each experimental sample was measured and showed no pH change after hydrolysis. Experimental results were plotted using a Cricket Graph program on a Macintosh II computer.

The decomposition products were identified by HPLC coinjection with samples of 5'-thymidine-H-phosphonate and 3-thymidine-H-phosphonate prepared by literature methods (Chen and Benkovic, 1953). Thymidine was identified as by coinjection with an authentic sample (Sigma).

#### 4. Determination of Activation Energy

The activation energy for the decomposition of **8** was determined at pH 6.0 in 0.01 M solutions of citrate buffer at 0.00°, 22.2° and 40.5 °C within 0.5 °C, by conducting the reactions under same conditions as described above in ice, at ambient temperature, and in a constant temperature bath respectively.

#### 5. Synthesis of Thymidyl-3',5'-Thymidine-H-Phosphonate (**8**)

##### 5.1. 5'-DIMETHOXYTRITYLTHYMIDINE (**1**) (Michelson and Todd, 1953)

12.1 g (50 mmol) of thymidine (**3**) (Sigma) were dried in a 500 mL round-bottomed flask by coevaporation three times with 75 mL of pyridine continuously distilled from CaH<sub>2</sub> on a rotary evaporator fitted to an oil pump. The evacuated system was brought to atmospheric pressure by bleeding argon into the system with the aid of a large balloon. After the last coevaporation the reaction flask was stoppered with a rubber septum, and 250 mL of continuously distilled pyridine were added from a syringe to yield a colorless solution. 25.0 g (75 mmol) of dimethoxytrityl chloride (Aldrich) and 10.5 mL (75 mmol) of triethylamine (Fisher) were added, and the pale yellow slurry was swirled until most of the solid had dissolved. 150 mg (1.25 mmol) 4-N,N-dimethylaminopyridine (Aldrich) recrystallized from diethyl ether were added, and the flask was quickly resealed and swirled to yield a dark brown syrupy solution containing a colorless granular precipitate. The reaction mixture was allowed to stand at room temperature overnight. TLC of the crude mixture using a developing solution of 15% methanol in methylene chloride on silica gel indicated only the slightest faded trace of the nucleoside starting material. The reaction was quenched by the addition of 15 mL of dry methanol and allowed to stir at room temperature for 15 min. The resulting dark turbid solution was poured into 1.5 L of distilled water and stirred magnetically for 30 min to precipitate the product as a dark gum. The aqueous phase was decanted, saturated with sodium chloride, and extracted with four 200 mL portions of ethyl acetate. The gummy product was dissolved in the combined organic extracts, and the darkly colored organic solution was washed with three 100 mL portions of saturated sodium bicarbonate solution and one 300 mL portion of a saturated solution of sodium chloride. The solvent was removed from the organic phase on a rotary evaporator to yield a dark gum. The gum was dissolved in 100 mL of benzene, and the product spontaneously precipitated as a tan powder. The powder was collected on a Buchner funnel and rinsed with three 25 mL portions of benzene to yield an off-white solid. The solid was dissolved in 500 mL of a refluxing solution of benzene and acetone (2:1), and the yellow solution was boiled to a volume of *ca.* 300 mL. The solution was seeded with a trace of the crude product and allowed to cool slowly to room temperature. A visibly growing network of fine crystalline material formed

over a period of several minutes, and the mixture was allowed to stand at room temperature for 2 h. The crystals were collected by vacuum filtration on a glass sintered funnel, rinsed with three 25 mL portions of benzene, and dried in *vacuo* to yield 25.89 g (95%) of **1** as white crystals, m.p. 125–135 °C (lit 128 °C) which gained mass slowly on the balance.

<sup>1</sup>H NMR:

d 9.36(s, 1, CONH), 7.60 (s, 1, H6), 7.45-7.20 (m, 9, aryl), 6.82 (d, J=8.9 Hz, 4, aryl), 6.43 (t, J=6.0 Hz, 1, H1'), 4.62–4.51 (m, 1, H3'), 4.12–4.05, (m, 1, H4'), 3.77 (s, 6, OCH<sub>3</sub>), 3.51–3.22 (m, 2, H5',5'), 3.03 (d, J=4.4 Hz, 1, OH), 2.52–2.25 (m, 2, H2',2''), 1.43 (s, 3, CH<sub>3</sub>).

## 5.2. 5'-DIMETHOXYTRITYLTHYMIDINE-3'-H-PHOSPHONATE (**2**) (Froehler *et al.*, 1986)

600 mL of HPLC grade methylene chloride (Baker) from a freshly opened bottle and 82.6 mL (76 g, 750 mmol) of N-methylmorpholine (Aldrich) were added to a vacuum-dried, magnetically stirred 1 L round-bottomed flask fitted with a rubber septum. 10.0 g (6.35 mL, 75 mmol) of phosphorous trichloride were added to the flask dropwise by syringe with vigorous stirring to yield a yellow fuming solution. 17 g (250 mmol) of 1,2,4-triazole (Aldrich) which had been dried at oil pump pressure for 30 min, were added in one portion, and the fuming yellow slurry was stirred for 30 min and then cooled in an ice bath. 8.16 g (15 mmol) of 5'-dimethoxytritylthymidine (**1**) were dried by a single coevaporation with 150 mL of dry acetonitrile and dissolved in 250 mL of HPLC grade methylene chloride. The solution was added to the cold reaction mixture dropwise from an addition funnel over 30 min. After addition was complete, the resulting yellow emulsion was stirred for 10 min and poured into 600 mL of a cold 1 M triethylammonium bicarbonate (TEABC) solution (pH 8.5) with vigorous stirring. The milky colorless organic layer was separated, and the aqueous layer was extracted with a single 200 mL portion of methylene chloride. The solvent was removed from the turbid organic layer, containing a considerable amount of water, on a rotary evaporator at water aspirator pressure to yield a wet yellow gum. Most of the water was removed from the gum by rotary evaporation of the residue at oil pump pressure. Drying was completed by repeated coevaporation of the residue with dry methylene chloride at water aspirator pressure until a methylene chloride solution of the product was not turbid. Solvent removal from the yellow solution yielded 13 g of the crude product as a yellow foam. The foam was dissolved in 20 mL of methylene chloride and purified by chromatography on a 4.5 × 30 cm column of silica gel by elution with a 0–50% step gradient in 5% steps of methanol in methylene chloride containing 2 drops triethylamine per liter to a total solvent volume of *ca.* 4 L. The product-containing fractions were combined, and the solvent was removed on a rotary evaporator to yield 10.28 g of a brittle yellow foam. The sample was redissolved in 500 mL of methylene chloride, and the solution was washed with three 100 mL

portions of 1 M TEABC solution (pH 8.5). The turbid organic layer was filtered through a pad of Celite and the solvent was removed on a rotary evaporator to yield a thick, pale yellow oil. The oil was dissolved in 150 mL of methanol, and the turbid solution was again filtered through a pad of Celite. The solvent was removed from the clear pale yellow filtrate on a rotary evaporator to yield 9.4 g (89%) of the triethylamine salt of the product **2** as a brittle yellow foam.

<sup>1</sup>H NMR (D<sub>2</sub>O):

d 8.56 (s, 1, CONH), 7.62 (s, 1, H6), 7.42–7.22 (m, 9, aryl), 6.90 (d, J=617 Hz, 1, P-H), 6.82 (d, J=8.9 Hz, 4, aryl), 6.46 (m, 1, H1'), 5.05–4.95 (m, 1, H3'), 4.28 (m, 1, H4'), 3.78 (s, 6, OCH<sub>3</sub>), 3.51–3.35 (m, 2, H5',5''), 3.04 (q, J=7.3 Hz, 6, CH<sub>2</sub>-N), 2.65–2.22 (m, 1, H2'), 2.48–2.32 (m, 1, H2''), 1.36 (s, 3, CH<sub>3</sub>), 1.33 (t, 9, CH<sub>3</sub>-CH<sub>2</sub>-N).

### 5.3. 3'-DIMETHOXYTRITYLTHYMIDINE (**6**) (Ogilvie and Letsinger, 1967)

5.0 g (20.5 mmol) of thymidine (**3**) (Aldrich) were dried in a 250 mL round-bottomed flask by coevaporation with three 30 mL portions of continuously distilled pyridine, and the flask was stoppered with a rubber septum. An additional 125 mL of dry pyridine were added followed by 2.85 mL (21.5 mmol) of isobutylchloroformate (**4**) (Aldrich). The clear yellow solution of carbonate (**5**) which bleached in 30 min was allowed to stand at room temperature overnight, and 10.14 g (30 mmol) of DMT-Cl were added in one portion. The resulting orange solution was heated on a boiling water bath for 2 h, cooled to room temperature, and 5 mL of methanol were added. After 5 min the solvent was removed on a rotary evaporator at oil pump pressure to yield an orange granular residue. The residue was dissolved in 150 mL of ether, and the orange slurry was filtered on a sintered glass filter funnel. The precipitate was rinsed with three 10 mL portions of ether, and the solvent was removed from the turbid orange filtrate on a rotary evaporator. The residue was freed of pyridine by coevaporation with three 40 mL portions of acetonitrile, redissolved in 150 mL of ether, and *ca.* 5 g of Celite were added to the turbid solution. The solution was filtered through a pad of Celite to rid of the solution of an insoluble oil, and the solvent was removed from the clear yellow filtrate on a rotary evaporator to give crude 3'-dimethoxytritylthymidine-5'-isobutyl carbonate as a yellow gum. The gum was dissolved in 50 mL of dioxane, 50 mL of 1 M sodium hydroxide solution were added, and the dark purple solution was brought to reflux temperature for 30 min to hydrolyze the carbonate. The resulting two phase mixture was cooled to room temperature and reduced in volume to 50 mL on a rotary evaporator. 150 mL of water were added, and the mixture was extracted with five 50 mL portions of ethyl acetate. The organic portions were combined and washed with two 50 mL portions of water. The solvent was removed from the organic layer on a rotary evaporator, and the residue was dissolved in methylene chloride and applied to a 4.5 × 30 cm column of silica gel. The column was eluted with a 0–4% step gradient of methanol dissolved in methylene chloride to a total

solvent volume of *ca.* 8 L. Fractions were collected and analyzed by TLC. Product-containing fractions which eluted in two bands were combined, and the solvent was removed on a rotary evaporator to yield 10.08 g (90%) of **6** as a brittle yellow foam.

<sup>1</sup>H NMR:

d 8.92 (s, 1, CONH), 7.5–7.0 (m, 10, H<sub>6</sub>, aryl), 6.85–6.65 (d, J=8.7 Hz, 4, aryl), 6.13 (m, 1, H1'), 5.27 (s, CH<sub>2</sub>Cl<sub>2</sub>), 4.35 (m, 1), 3.95 (m, 1), 3.77 (s, 6, OCH<sub>3</sub>), 3.70–3.55 (m, 2), 3.4–3.2 (m, 1), 2.65 (m, 1), 2.15 (s, 0.3, imp), 2.00–1.80 (m, 1, H2'), 1.82 (s, 3, CH<sub>3</sub>), 1.75–1.60 (m, 1, H2'')

#### 5.4. 5'-DIMETHOXYTRITYLTHYMIDINE-(3,5)-H-PHOSPHONYL-3'-DIMETHOXYTRITYLTHYMIDINE (**7**) (Regberg *et al.*, 1988)

3.54 g (5.00 mmol) of 5'-dimethoxytrityl thymidine-3'-H-phosphonate **2** and 2.72 g (5.00 mmol) of 3'-dimethoxytritylthymidine **6** were dried in a 250 mL round-bottomed flask by coevaporation with three 50 mL portions of continuously distilled pyridine on a rotary evaporator at oil pump pressure. The flask was fitted with a rubber septum, and the residue was dissolved in an additional 50 mL of dry pyridine. The clear yellow solution was cooled in an ice bath and 2.35 mL (2.30 g, 20 mmol, 4 eq) of trimethylacetyl chloride (Aldrich) were added dropwise by syringe over 5 min with vigorous magnetic stirring. When addition was complete, the yellow solution containing a granular precipitate was allowed to stir an additional 10 min. The solution was poured into a 400 mL of a cold 1 M TEABC solution (pH 7.0) and extracted with three 175 mL portions of methylene chloride. The organic portions were combined and dried with a single 100 mL portion of a saturated solution of NaCl. The solvent was removed from the clear yellow organic layer on a rotary evaporator to yield a yellow oil. The oil was dissolved in 5 mL of methylene chloride and applied to a 4.5 × 30 cm column of silica gel. The column was eluted with a 0–4% step gradient of methanol in methylene chloride in 1% steps to a total solvent volume of *ca.* 4 L. Fractions were collected and analyzed by TLC using ethyl acetate as a developing solvent. The solvent was removed from the two bands of diastereomeric mixtures of the product-containing fractions to yield 1.25 g of **7** as a yellow gum from the first band and 3.21 g of a brittle, pale yellow foam of pure from the second fraction (total 77%).

<sup>1</sup>H NMR:

d 9.65–8.90 (s, 2, CONH), 7.65–7.15 (m, 20, H<sub>6</sub>, aryl), 6.81 (d, J=8.7 Hz, 8, aryl), 6.79 (d, J=718 Hz, 0.6, P-H), 6.70 (d, J=720 Hz, 0.4 P-H), 6.48–6.21 (m, 2, H1', 1a'), 5.18 (m, 1, H3'), 4.40–3.83 (m, 3), 3.76 (s, 6, OCH<sub>3</sub>), 3.75, 3.74 (2s, 6, OCH<sub>3</sub>), 3.85–3.25 (m, 4, H5',5''), 2.6–2.2 (m, 2), 1.80–1.50 (m, 2), 1.98, 1.97 (2s, 3, CH<sub>3</sub>), 1.84, 1.82 (2s, 1.5, CH<sub>3</sub>), 1.38, 1.37 (2s, 1.5, CH<sub>3</sub>)

#### 5.5. THYMIDYL-3',5'-THYMIDINE-H-PHOSPHONATE (**8**) (Kume *et al.*, 1984)

1.24 g (1.1 mmol) of 5'-Dimethoxytrityl thymidine-(3,5)-H-phosphonyl-3'-dimethoxytrityl thymidine (**7**) were placed in a 250 mL round-bottomed flask and

150 mL of a 2% solution of dichloroacetic acid dissolved in methylene chloride were added. The resulting red solution was swirled at room temperature 2.0 min, and the solution was quickly titrated with triethylamine to a yellow color. The solution was transferred to a 500 mL separatory funnel and 200 mL of ether were added. The solution was extracted with five 150 mL portions of water, and the aqueous fractions were combined and adjusted to a pH of 3.5–4.0 with a 0.1 M solution of KOH. The aqueous solution was reduced in volume on a rotary evaporator at oil pump pressure to *ca.* 20 mL. The slightly turbid colorless solution was applied to a 20 × 500 mm reverse phase chromatography column, and the column was eluted with 2 L of water followed by a 0–20% linear gradient of acetonitrile dissolved in water (1 L each) at a flow rate of *ca.* 2 mL/min. Fractions were collected and analyzed by HPLC. Fractions containing the product in excess of 95% purity were combined and the solvent was removed on a rotary evaporator to yield a colorless glass. The glass was rendered anhydrous by coevaporation with three 10 mL portions of acetonitrile to yield 211 mg (39%) of **8** as a white powder.

<sup>1</sup>H NMR (D<sub>2</sub>O):

δ 7.45 (s, 1, H6a), 7.37 (s, 1, H6b), 6.10 (m, 2, H1', 1a'), 5.08–4.93 (m, 1, H3'a), 4.65 (s, D<sub>2</sub>O), 4.45–3.95 (m, 5, H3'b, H4'a, 4'b, H5'a, 5''a), 3.62 (m, 2, H5'b, 5''b), 2.5–2.2 (m, 4, H2'a, 2''a, 2'b, 2''b)

## 6. Results and Discussion

The thymidine H-phosphonate diester **8** was prepared by adaptation of literature methods shown in Figure 1. Details for its preparation are described in the experimental section. Hydrolysis of dimer **8** gave three products identified as 3'-thymidine H-phosphonate, 5'-thymidine H-phosphonate, and thymidine by comparison with authentic standards.

The rate of hydrolysis of dimer **8** was determined in aqueous solution within a pH range of 2–7.5 in 0.5 unit increments at a temperature of 26.0 ± 0.5 °C in citrate buffer. To determine the rate of buffer hydrolysis, studies were performed at each pH in 1.0 M, 0.10 M and 0.010 M buffer solutions. Dilution of each 1.0 M solution resulted in noticeable pH changes. The decomposition reactions were monitored by HPLC for a minimum of two half lives. Measurement of the final pH of each experimental solution showed no noticeable pH drift during hydrolysis. A least squares plot of the natural log (ln) of the percent integrated area of dimer **8** against time gave a straight line for all samples with linear correlation coefficients of 0.998 or higher.

A sample plot of the data for the decomposition of **8** in 0.01 M buffer at pH 4 is shown in Figure 2. The slope of the line in Figure 2 gave minus the value of the pseudo first order rate constant (*k*<sub>obs</sub>) for the decomposition reaction at pH 4, and similar determinations of *k*<sub>obs</sub> were measured at each pH. The results are presented in Table I. The log<sub>10</sub> (log) of the rate constants from Table I were plotted against pH

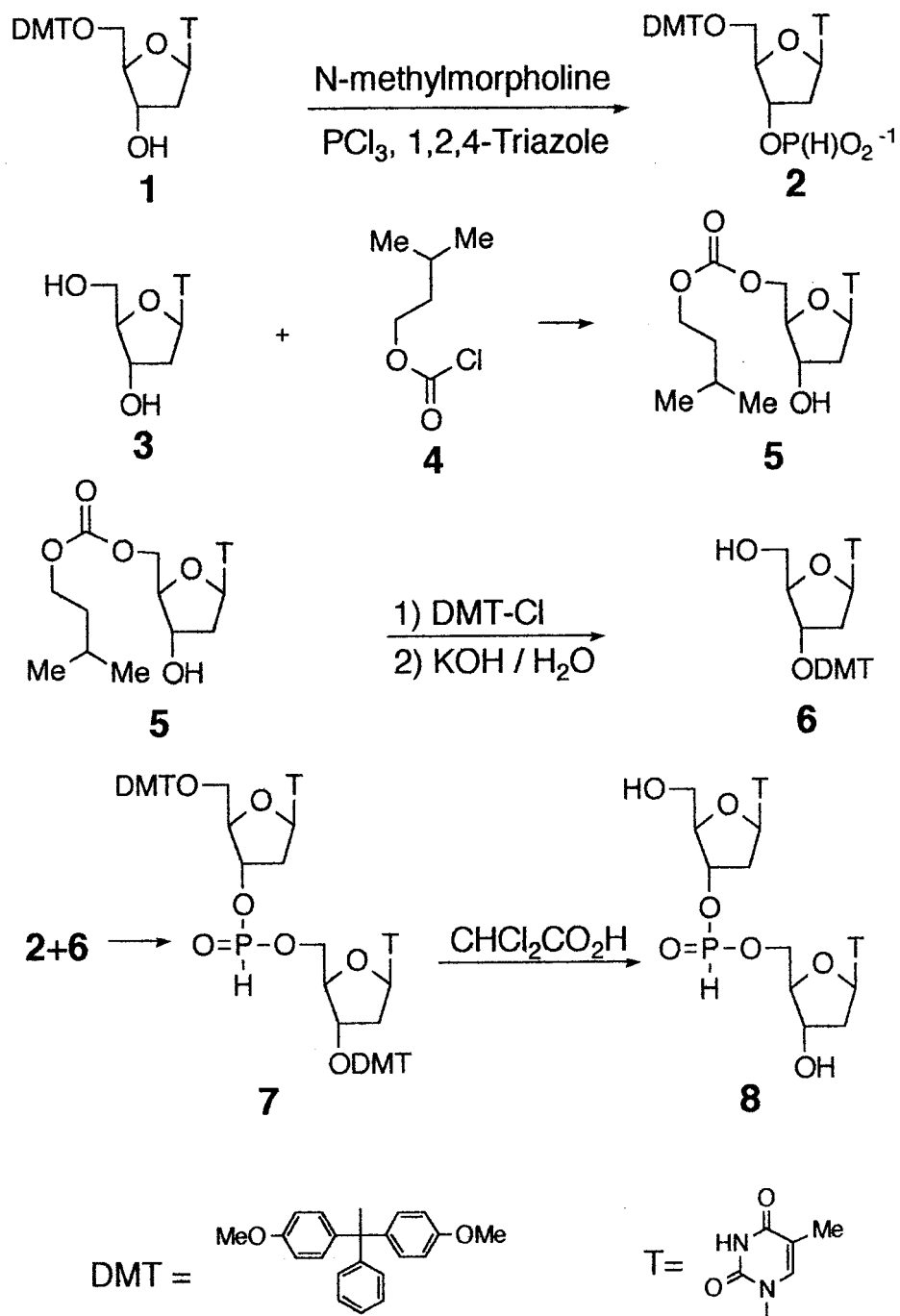


Figure 1. The synthesis of thymidyl-3',5'-thymidine H-phosphonate.

TABLE I

Pseudo first order rate constants for the hydrolysis of thymidyl-3',5'-thymidine H-phosphonate (dimer)

1.0 M Citrate		0.10 M Citrate		0.010 M Citrate	
pH	$k_{\text{obs}}$ ( $\text{sec}^{-1}$ )	pH	$k_{\text{obs}}$ ( $\text{sec}^{-1}$ )	pH	$k_{\text{obs}}$ ( $\text{sec}^{-1}$ )
7.21	7.5 E-4	7.30	1.5 E-3	7.37	1.9 E-3
6.75	2.8 E-4	6.86	4.0 E-4	6.99	6.2 E-4
6.00	9.7 E-5	6.12	9.0 E-5	6.41	1.7 E-4
5.59	6.2 E-5	5.75	3.9 E-5	6.01	7.0 E-5
4.55	2.5 E-5	4.68	6.3 E-6	4.89	6.5 E-6
3.60	1.6 E-5	3.72	3.3 E-6	3.89	1.8 E-6
3.01	1.4 E-5	3.20	3.5 E-6	3.41	2.0 E-6
2.22	2.1 E-5	2.48	7.4 E-6	2.90	3.4 E-6
1.51	3.6 E-5	2.02	1.3 E-5	2.62	4.5 E-6

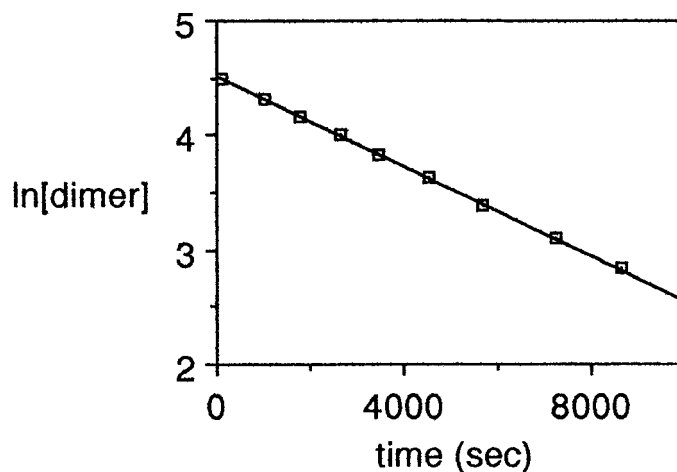


Figure 2. The hydrolysis of thymidyl-3',5'-thymidine H-phosphonate in 0.010 M buffer.

for each buffer concentration, and the data were computer curve-fitted as presented in Figure 3.

The rate of decomposition of **8** was the slowest between pH 3–4, and the rate was effected by the strength of the buffer solutions (Figure 3). The effect of buffer catalysis was most pronounced at pH 3.5 to 4 where the rate of decomposition of **8** was almost an order of magnitude faster in the 1.0 M solutions compared with the 0.01 M buffer. Above pH 6.5 the presence of concentrated citrate buffer appeared to slightly retard the hydrolysis of **8**.

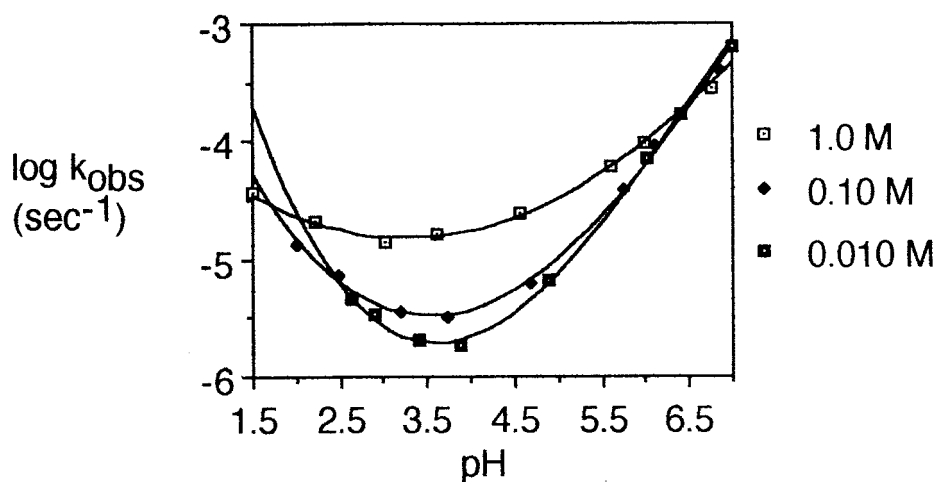


Figure 3. The hydrolysis of thymidyl-3',5'-thymidine H-phosphonate at all buffer strengths.

TABLE II

Calculated pseudo first order rate constants for the hydrolysis of thymidyl-3',5'-thymidine H-phosphonate at exact pH values

pH	1.0 M Citrate	0.10 Citrate	0.010 M Citrate
	$k_{\text{calc}}$ ( $\text{sec}^{-1}$ )	$k_{\text{calc}}$ ( $\text{sec}^{-1}$ )	$k_{\text{calc}}$ ( $\text{sec}^{-1}$ )
2.50	1.8 E-5	6.4 E-6	6.1 E-6
3.00	1.5 E-5	3.9 E-6	2.7 E-6
3.50	1.5 E-5	3.3 E-6	1.9 E-6
4.00	1.7 E-5	3.8 E-6	2.2 E-6
4.50	2.2 E-5	5.6 E-6	3.5 E-6
5.00	3.3 E-5	1.0 E-5	7.6 E-6
5.50	5.4 E-5	2.3 E-5	2.0 E-5
6.00	1.0 E-4	6.3 E-5	6.3 E-5
6.50	2.1 E-4	1.9 E-4	2.1 E-4
7.00	4.8 E-4	6.6 E-4	7.3 E-4

Since dilution of the original samples caused noticeable pH change in the reaction solutions, rate constants at each exact pH were calculated by curve fitting the original data from Table I and evaluation of rate constants at each exact pH. The calculated rate constants ( $k_{\text{calc}}$ ) at each exact pH are shown in Table II.

The rate of decomposition of dimer **8** in the absence of buffer was calculated by plotting the rate of decomposition at 1.0 M, 0.10 M, 0.010 M buffer concentrations

TABLE III

Calculated pseudo first order rate constants for the hydrolysis of thymidyl-3',5'-thymidine H-phosphonate in the absence of buffer

pH	$k_{(\text{calc})} \text{ sec}^{-1}$	$t_{1/2} \text{ (sec)}$	pH	$k_{(\text{calc})} \text{ sec}^{-1}$	$t_{1/2} \text{ (sec)}$
2.50	5.6 E-6	1.2 E5	5.00	7.5 E-6	9.2 E4
3.00	2.6 E-6	2.7 E5	5.50	2.0 E-5	3.5 E4
3.50	1.9 E-6	3.6 E5	6.00	6.1 E-5	1.1 E4
4.00	2.1 E-6	3.3 E5	6.50	2.1 E-4	3.3 E3
4.50	3.5 E-6	2.0 E5	7.00	7.1 E-4	9.8 E2

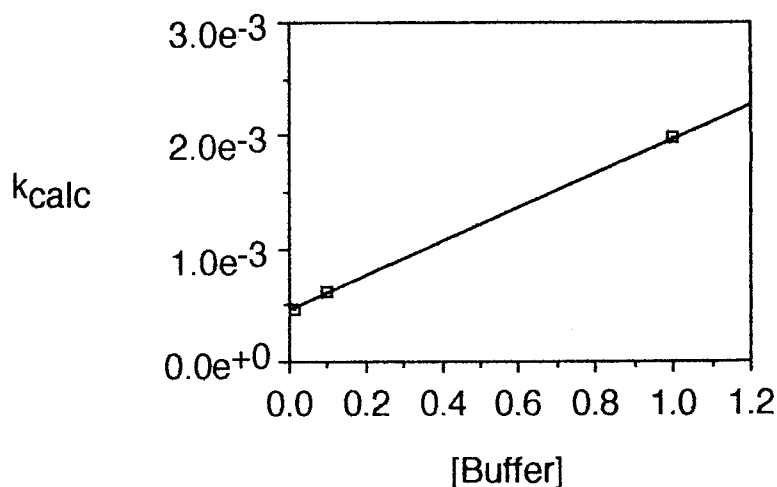


Figure 4. The extrapolated rate of hydrolysis of thymidyl-3',5'-thymidine H-phosphonate in the absence of buffer at pH 5.00.

for each pH value in Table II and extrapolation to zero buffer concentration. A sample plot for extrapolation to zero buffer concentration for pH 5 is shown in Figure 4. Similar plots were obtained at each pH in Table II and the calculated rate constants and half-lives for the decomposition of **8** in the absence of buffer are listed in Table III. The  $\log_{10}$  of these rates were plotted against pH in Figure 5.

The H-phosphonate dimer **8** is most stable in the absence of buffer at pH 3.5 (Figure 3). At lower pH the rate of decomposition, presumably by acid catalysis, rapidly increases. At pH levels above 6, the log of the rate of decomposition of **8** increases in a nearly linear fashion with pH.

The activation parameters for the base-catalyzed decomposition of dimer **8** were determined by measuring the rate of decomposition of the dimer at three temperatures. Samples of **8** were prepared at pH 6.00 in 0.010 M citrate buffer solutions

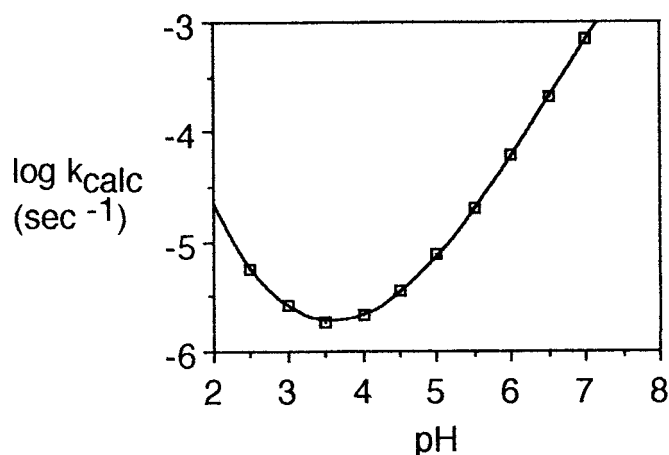


Figure 5. The hydrolysis of thymidyl-3',5'-thymidine H-phosphonate in the absence of buffer.

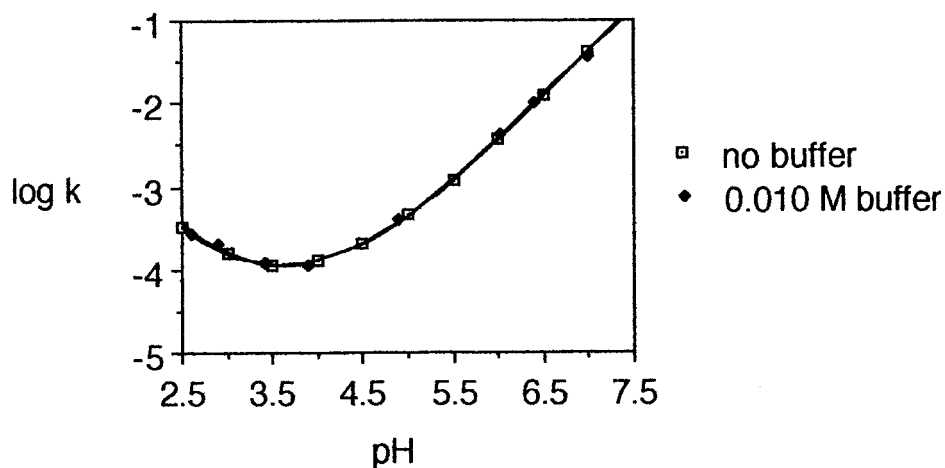


Figure 6. The hydrolysis of thymidyl-3',5'-thymidine H-phosphonate in 0.010 M buffer and without buffer.

since the effects of 0.010 M citrate buffer on the hydrolysis rate of **8** are negligible (Figure 6).

Reactions were performed at 0.0, 22.2, and 40.5 °C. No change in pH was observed as a result of the temperature differences consistent with the observation that the pH of citrate buffers do not vary significantly in the temperature range studied (Staple and Bates, 1969). The decomposition reactions were monitored by HPLC for a minimum of two half lives, and a plot of the natural log of the integrated percent area of starting dimer **8** against time gave a straight line with linear correlation of 0.999 or greater for each of the three samples. The slope of each line gave the pseudo first order rate constants for the decomposition of **8** to

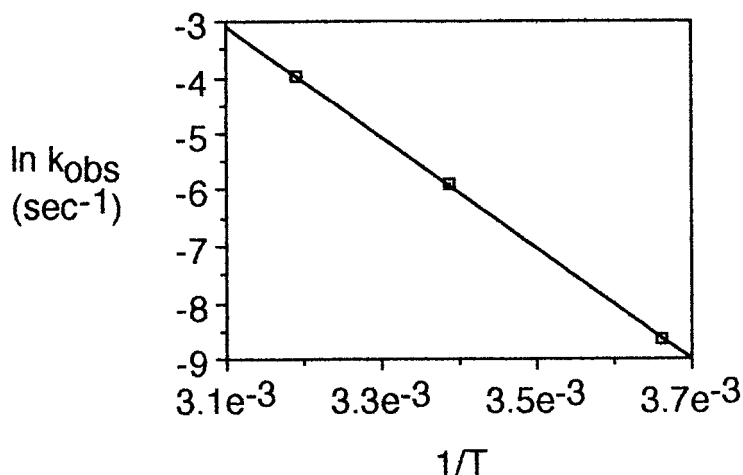


Figure 7. Arrhenius plot: hydrolysis of thymidyl-3',5'-thymidine H-phosphonate at pH 6.00.

be  $3.0 \times 10^{-6} \text{ sec}^{-1}$ ,  $4.6 \times 10^{-5} \text{ sec}^{-1}$ , and  $3.1 \times 10^{-4} \text{ sec}^{-1}$  for reactions at 0.00, 22.2, and 40.5 °C, respectively.

The Arrhenius activation energy was found by plotting the natural log of the rate constants against the reciprocal absolute temperature (Figure 7). Multiplication of the negative of the slope of the plot by the gas constant gave an activation energy of 19.5 kcal/mol for the base catalyzed hydrolysis of **8**. Taking the antilog of the y-intercept in gave the value of the Arrhenius frequency factor as  $1.30 \times 10^{10} \text{ sec}^{-1}$ .

The Gibbs free energy and entropy of activation were calculated from the relationship: (Cagle and Eyring, 1951)

$$\ln(k/T) = \ln(K_b/h) + \Delta S_{\text{act}}/R - \Delta H_{\text{act}}/RT$$

where  $k$  is the observed rate constant,  $K_b$  is Boltzman's constant, and  $h$  is Planck's constant. A plot of the natural log of the rate constant divided by the absolute temperature gave a straight line with a slope equal to minus the enthalpy of activation divided by the gas constant and a y-intercept equal to the sum of the natural log of the quotient of Boltzman's constant divided by Planck's constant and the entropy of activation divided by the gas constant. Multiplication of the negative of the slope of the line by the gas constant gave an enthalpy of activation of 19 kcal/mol. Subtraction of the natural log of the quotient Boltzman's constant divided by Planck's constant from the y-intercept and multiplication of the result by the gas constant gave a value for the entropy of activation of  $-14 \text{ kcal/mol K}$ . Combination of the entropy and enthalpy terms for 298 K gave the Gibbs free energy of activation for the hydrolysis of **8** of 23 kcal/mol.

Evaluation of the first derivative of the third order polynomial curve fit from Figure 5 at pH 7.00 where acid catalysis was presumably minimal showed a slope

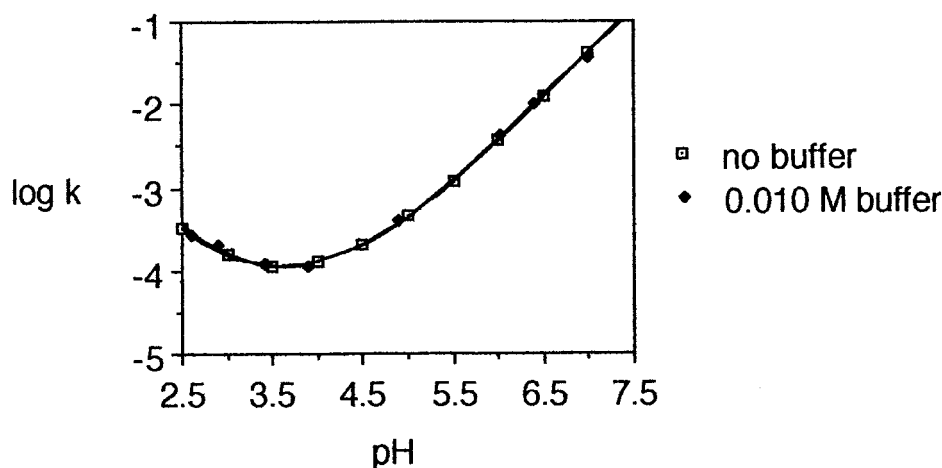


Figure 8. Hydrolysis of thymidyl-3',5'-thymidine H-phosphonate by water.

of 1.05 which is within experimental error of the expected value of 1.00 for simple hydroxide catalysis (Jencks, 1969). Similar evaluation of the curve at pH 2.50 gave a slope of  $-0.92$ . This value was slightly less than the theoretical value of  $-1.00$  for purely acid catalysis which is presumably the result of a small base and water catalysis at this pH.

The second order rate constant for catalysis by hydroxide was calculated from the linear plot of the pseudo first order rate constants from Figure 5 against the hydroxide ion concentration from pH 6–7. The slope of this plot,  $7.2 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ , corresponds to the second order rate constant for the base catalysis ( $k_b$ ) of dimer **8** by the hydroxide ion. This value is about three times larger than those reported for the dibutyl and dipropyl ester, and it is about twice as large as the rate constant for the decomposition of the diethyl ester (Nylen, 1937).

The second order rate constant for catalysis by acid,  $k_a$ , was estimated by division of the pseudo first order rate constant at pH 2.50 by the concentration of the hydrogen ion at this pH to give a value of  $1.77 \times 10^{-3} \text{ M}^{-1} \text{ sec}^{-1}$ . The value of this number is somewhat uncertain since at pH 2.50, it includes some contribution by base catalysis and water hydrolysis. This value is about the same as that for the acid-catalyzed decomposition of di-2-propyl H-phosphonate (Nylen, 1937).

A value for rate of catalysis by water was estimated by plotting the log of the rate of catalysis by acid and by base against pH (Figure 8) (Jencks, 1969).

Subtraction of the antilog of the intersection point of the acid and base catalysis lines from the antilog of the observed rate of catalysis at the intersection pH value gave a pseudo first order value of for catalysis by water ( $k_w$ ) of  $1.5 \times 10^{-6} \text{ sec}^{-1}$ . Considering the method by which this value was obtained, it is in excellent agreement with the value reported by of  $4.4 \times 10^{-6} \text{ sec}^{-1}$  for the hydrolysis of the simple dialkyl esters of phosphorous acid (Nylen, 1937).

## 7. Conclusions

The hydrolysis of diester **8** is rapid and subject to both acid and base catalysis at rates comparable to those reported for simple alkyl diesters (Nylen, 1937). The dimer displayed its greatest stability at a pH of *ca.* 3.5. At neutral pH the thymidine dimer had a half-life of only sixteen minutes at 25 °C. This rapid degradation would presumably have prevented accumulation of significant amounts of phosphorous acid oligomers on the primitive earth. For phosphorous acid diesters to have played an important role in the prebiotic formation of oligomeric nucleotide chains, they would have had to have been rapidly converted to a more stable form perhaps by oxidation to phosphate compounds.

## Acknowledgements

This research was supported by NSF grant CHE-0076281, A NASA Graduate Student Researchers Award (NGT-50601) to JRP and NASA NSCORT grant NAG5-9607.

## References

- Cagle, F. W. and Eyring, H.: 1951, Application of absolute reaction rate theory to the racemization of certain sterically hindered compounds, *J. Am. Chem. Soc.* **73**, 5628–5630.
- Chen, J. T. and Benkovic, S. J.: 1983, Synthesis and separation of diastereomers of deoxynucleoside 5'-O-(thio) triphosphates, *Nucleic Acid Res.* **11**, 3737–3751.
- De Graaf, R. M. and Schwartz, A. W.: 2000, Reduction and activation of phosphate on the primitive Earth, *Origins Life Evol. Biosphere* **30**, 405–410.
- Froehler, B. C., Ng, P. G., and Matteucci, M. D.: 1986, Synthesis of DNA via deoxynucleoside H-phosphonate intermediates, *Nucleic Acids Res.* **14**, 5399–5407.
- Glindemann, D., De Graaf, R. M., and Schwartz, A. W.: 1999, Chemical reduction of phosphate on the primitive Earth, *Origins Life Evol. Biosphere* **29**, 551–553.
- Gulick, A.: 1955, Phosphorus as a factor in the origin of life, *Amer. Scientist* **43**, 479–489.
- Jencks, W. P.: 1969, *Catalysis in Chemistry and Enzymology*, McGraw-Hill Inc., New York, New York, Chapter 11.
- Kume, A., Fujii, M., Sekine, M., and Hata, T.: 1984, Acyl phosphonates. 4. Synthesis of dithymidine phosphonate: A new method for generation of the phosphonate function via aroylphosphonate intermediates, *J. Org. Chem.* **49**, 2139–2143.
- Michelson, A. M. and Todd, A. R.: 1953, Nucleotides. Part XX. Mononucleotides derived from thymidine. Identity of thymidylic acid from natural sources with thymidine-5' phosphate, *J. Chem. Soc.*, 951–956.
- Nylen, P.: 1937, Die kinetik der verseifung von dialkylphosphiten. I. Die verseifung durch wasserstoff und hydroxylion, *Svensk Kem. Tid.* **49**, 29–59.
- Ogilvie, K. K. and Letsinger, R. L.: 1967, Use of isobutyloxycarbonyl as a blocking group in the preparation of 3'-O-*p*-monomethoxytritylthymidine, *J. Org. Chem.* **32**, 2365–2366.
- Regberg, T., Stawinski, J., and Stromberg, R.: 1988 Nucleoside H-phosphonates IX. Possible side-reactions during hydrogen phosphonate diester formation, *Nucleosides Nucleotides* **7**, 23–35.

- Staples, B. R. and Bates, R. G.: 1969, Two new standards for the pH scale, *J. Res. NBS* **73A**, 37–41.
- Westheimer, F. H., Huang, S., and Covitz, F.: 1988, Rates and mechanisms of hydrolysis of esters of phosphorous acid, *J. Am. Chem. Soc.* **110**, 181–185.