Res. **1978,** *11,* 1–7.
(3) Hine, J. "Structural Effects on Equilibria in Organic Chemistry"; Wiley-Interscience: New York, 1975; Chapter 8.
(4) Cf. (a) Hine, J.; Via, F. A.; Gotkis, J. K.; Craig, J. C., Jr. *J. Am. Chem. Soc.* **1970,** *92,* 5186–5193; (b) Hine, J.; Cholod, M. S.; Chess, W. K., Jr. *J. Am. Chem. Soc.* **1973,** *95,* 4270–4276.
(5) Hine, J.; Dempsey, R. C.; Evangelista, R. A.; Jarvi, E. T.; Wilson, J. M. *J. Org. Chem.* **1977,** *42,* 1593–1599.
(6) Hine, J.; Mulders, J. *J. Org. Chem.* **1967,** *32,* 2200–2205.
(7) Wiig, E. O. *J. Phys. Chem.* **1928,** *32,* 961–981.
(8) Franke, W.; Brathuhn, G. *Justus Liebigs Ann. Chem.* **1931,** *487,* 1–52.
(9) Pedersen, K. J. *J. Phys. Chem.* **1934,** *38,* 559–571. *J. Am. Chem. Soc.* **1938,** *60,* 595–601. *Acta Chem. Scand.* **1954,** *8,* 710–722.
(10) Miller, J. G.; Kilpatrick, M. *J. Am. Chem. Soc.* **1931,** *53,* 3217–3224.
(11) Westheimer, F. H.; Cohen, H. *J. Am. Chem. Soc.* **1938,** *60,* 90–94.
(12) Westheimer, F. H.; Jones, W. A. *J. Am. Chem. Soc.* **1941,** *63,* 3283–3286.
(13) Hamilton, W. C. "Statistics in Physical Science"; Ronald Press: New York, 1964; Section 5-3.
(14) Cf. Brown, H. C.; Brewster, J. H.; Shechter, H. *J. Am. Chem. Soc.* **1954,** *76,* 467–474.
(15) Silver, S. M.; Sayer, J. M. *J. Am. Chem. Soc.* **1973,** *95,* 5073–5075.
(16) Rosenberg, S.; Silver, S. M.; Sayer, J. M.; Jencks, W. P. *J. Am. Chem. Soc.* **1974,** *96,* 7986–7998.
(17) Hine, J.; Via, F. A. *J. Am. Chem. Soc.* **1972,** *94,* 190–194.
(18) Hine, J.; Cholod, M. S.; King, R. A.; *J. Am. Chem. Soc.* **1974,** *96,* 835–845.
(19) Leonard, N. J.; Paukstelis, J. V. *J. Org. Chem.* **1963,** *28,* 3021–3024.
(20) Buhle, E. L.; Moore, A. M.; Wiselogle, F. Y. *J. Am. Chem. Soc.* **1943,** *65,* 29–32.
(21) Stetter, H.; Spangenberger, H. *Chem. Ber.* **1958,** *91,* 1982–1988.
(22) This compound was analyzed for C, H, N, and halogen. Only in the case noted did any analysis deviate from the theoretical by more than 0.25%.
(23) King, H. *J. Chem. Soc.* **1942,** 432–433.
(24) Hine, J.; Kaufmann, J. C.; Cholod, M. S. *J. Am. Chem. Soc.* **1972,** *94,* 4590–4595.
(25) Davies, C. W. *J. Chem. Soc.* **1938,** 2093–2098.
(26) Brass, H. J.; Edwards, J. O.; Fina, N. J. *J. Chem. Soc., Perkin Trans 2* **1972,** 726–729.
(27) Gravitz, N.; Jencks, W. P. *J. Am. Chem. Soc.* **1974,** *96,* 499–506.
(28) See paragraph at end of paper regarding supplementary material.

Thermochemical Identification of the Structural Factors Responsible for the Thermodynamic Instability of 3′,5′-Cyclic Nucleotides

John A. Gerlt,*[1a,2] Neal I. Gutterson,[1a] Pradip Datta,[1a] Bernard Belleau,[1b] and Christopher L. Penney[1b]

Contribution from the Departments of Chemistry, Yale University, New Haven, Connecticut 06520, and McGill University, Montreal, Quebec, Canada H3A 2K6. *Received February 5, 1979*

Abstract: The enthalpies of hydrolysis of several cyclic phosphate diesters which can be considered to be structural analogues of the trans-fused trimethylene phosphate–ribofuranoside ring system of adenosine 3′,5′-cyclic phosphate have been determined by microcalorimetric techniques using the metal-dependent phosphohydrolase from *Enterobacter aerogenes* as catalyst. At pH 7.3 and 25 °C we have obtained the following values (kcal/mol) for sodium salts: *trans*-2-hydroxytetrahydrofuranmethanol cyclic phosphate, −10.6; *trans*-2-hydroxycyclopentanemethanol cyclic phosphate, −7.9; *cis*-2-hydroxycyclopentanemethanol cyclic phosphate, −2.5; 5-methoxytrimethylene phosphate, −4.9; 5-methyltrimethylene phosphate, −3.8. From these values and those determined previously, we can make the following conclusions: (1) the trans-fused trimethylene phosphate–tetrahydrofuran structure is responsible for the 8 kcal/mol more exothermic enthalpy of hydrolysis which cyclic AMP displays relative to trimethylene phosphate; (2) about 5 kcal/mol of the excess enthalpy of hydrolysis of cyclic AMP is the result of geometric distortion due to the trans-ring fusion; (3) about 3 kcal/mol of the excess enthalpy of hydrolysis of cyclic AMP cannot be accounted for by intramolecular effects, suggesting that solvation effects play an important role in the thermodynamic stability of cyclic AMP.

3′,5′-Cyclic nucleotides, e.g., adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and guanosine 3′,5′-cyclic monophosphate (cyclic GMP), are involved in the regulation of many biochemical and biological processes, including hormonal regulation of metabolism, the action of neurotransmitters at synapses, cellular differentiation and malignant transformation, and immunological processes such as the graft vs. host reaction.[3] In essentially all of these processes, the mechanism by which a cyclic nucleotide influences a biochemical reaction is apparently the same: in the case of cyclic AMP, adenylate cyclase catalyzes the formation of cyclic AMP from ATP in response to an extracellular stimulus; the cyclic AMP then activates a cyclic AMP-dependent protein kinase. The activated (and often dissociated) form of protein kinase catalyzes the phosphorylation of an enzyme, thereby altering its catalytic activity and producing the desired cellular response. Cyclic AMP is removed from the system by its hydrolysis to 5′-AMP, which is catalyzed by a specific phosphodiesterase.

Cyclic AMP has been demonstrated to be a "high-energy" phosphate,[4] i.e., the free energy of its hydrolysis to yield 5′-AMP is −11.9 kcal/mol at pH 7.3, pMg 3, and 25 °C. Thermochemical studies reported by Gerlt, Westheimer, and Sturtevant[5] demonstrated that this thermodynamic instability is the result of an unusually exothermic enthalpy of hydrolysis (−12.1 kcal/mol) as compared to those measured for "strain-free" diesters, diethyl phosphate (−2.5 kcal/mol) and trimethylene phosphate[6] (−3.8 kcal/mol); the entropies of hydrolysis of cyclic AMP and "low-energy" phosphate esters appear to be similar and approximately equal to *zero* cal/mol·deg. These studies did not permit an explanation for the enthalpic behavior of cyclic AMP but did suggest that the structural origin of the unusual enthalpic effect was a property of the ribofuranoside–cyclic phosphate portion of the molecule. Also, these studies revealed that the enthalpy of hydrolysis of methyl α-D-glucopyranoside 4,6-cyclic phosphate (−6.9 kcal/mol) was similar to that of ethylene phosphate, a strained five-membered ring phosphodiester; subsequent structural characterization of the glucoside cyclic phosphate[7] revealed no evidence for geometric distortion, i.e., strain. Thus, two

0002-7863/80/1502-1655$01.00/0

questions remained to be answered as a result of the previous studies: (1) Why is the hydrolysis of cyclic AMP so much more exothermic than that of the methyl glucoside cyclic phosphate? (2) Why is the hydrolysis of the methyl glucoside cyclic phosphate more exothermic than that of trimethylene phosphate?

In this paper, we describe the synthesis of additional structural analogues of ribofuranoside cyclic phosphates and the results of thermochemical measurements made possible by the availability of the metal requiring nonspecific phosphohydrolase from *Enterobacter aerogenes* for use as catalyst. Our results clearly demonstrate that both strain arising from the trans-ring fusion and an effect related to the presence of the ribofuranoside endocyclic oxygen atom (O_4') of 3',5'-cyclic nucleotides are responsible for their unusual thermodynamic instability. In the following papers, a detailed explanation for the enthalpy effect caused by the oxygen atom which is based on the results of molecular mechanics and ab initio calculations[8] and the solution conformational properties of various substituted trimethylene phosphates[9] is proposed.

Experimental Section

Melting points were measured in open capillaries with a Hoover melting point apparatus and were corrected. ^{31}P NMR spectra were obtained at ambient temperature with a Varian CFT-20 spectrometer equipped with a phosphorus probe. High-resolution ^1H NMR spectra were obtained at 270 MHz using the Bruker spectrometer of the Southern New England High Resolution NMR Facility at Yale University. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

The syntheses of the benzylammonium salts of several of the cyclic esters examined in this study have been reported by Penney and Belleau.[10] Since these salts cannot be conveniently converted to the corresponding sodium salts in quantitative yield, the crystalline free acids of most of these esters were prepared according to procedures similar to those previously reported. Also, since samples of enzymatic hydrolysis products are necessary to obtain the enthalpies of hydrolysis of monoanionic diesters to yield monoanionic monoesters, the cyclohexylammonium salts of some of these materials which had a single resonance in their ^{31}P spectrum when proton decoupled were also prepared.

5-Methoxytrimethylenephosphoric Acid. A mixture of the cis and trans phenyl esters of 5-methoxytrimethylenephosphoric acid was prepared by the addition of 0.10 mol of phenyl phosphorodichloridate to a stirred ice-cold pyridine solution of 0.10 mol of 2-methoxy-1,3-propanediol.[11] After 16 h at room temperature, the solvent was removed by rotary evaporation and the residue was partitioned between chloroform and dilute aqueous hydrochloric acid. The chloroform solution was washed with dilute acid, water, and 7.5% aqueous sodium bicarbonate. The dried chloroform solution was evaporated to dryness and the resulting residue was crystallized from ethanol–water. A portion of the crystalline mixture of phenyl esters was dissolved in absolute ethanol, and the phenyl groups were removed by hydrogenolysis in a Parr apparatus at 60 psi, using platinum oxide as the catalyst. After removal of the catalyst and rotary evaporation of the solvent, the residue was crystallized from dioxane to yield colorless plates, mp 101.5–104.0 °C. Anal. Calcd for $C_4H_9O_5P$: C, 28.58; H, 5.40; P, 18.43. Found: C, 28.66; H, 5.50; P, 18.53.

2-Methoxy-3-hydroxypropane-1-(biscyclohexylammonium phosphate). An excess of 2-methoxy-1,3-propanediol was phosphorylated with diphenyl phosphorochloridate in dry pyridine. After isolation and hydrogenolysis of the oily diphenyl ester of the desired phosphate ester by procedures analogous to those described for the cyclic diester of the diol, the ethanolic solution of product was neutralized with cyclohexylamine. After removal of the solvent by rotary evaporation, the residue was crystallized from absolute ethanol, mp 165–172 °C. Anal. Calcd for $C_{16}H_{37}N_2O_6P$: C, 49.99; H, 9.70; N, 7.29; P, 8.06. Found: C, 50.26; H, 9.71; N, 7.42; P, 8.19.

5-Methyltrimethylenephosphoric Acid. A mixture of the cis and trans phenyl esters of this cyclic acid was prepared by a procedure analogous to that described for the 5-methoxy cyclic acid by phosphorylating 2-methyl-1,3-propanediol with phenyl phosphorodichloridate. After hydrogenolysis, the solvent was removed and the residue was crystallized from ethyl acetate, mp 112.5–114 °C. Anal. Calcd for $C_4H_9O_4P$: C, 31.59; H, 5.96; P, 20.37. Found: C, 31.35; H, 6.18; P, 20.42.

2-Methyl-3-hydroxypropane-1-(bisbenzylammonium phosphate). This material was prepared by a procedure analogous to that described for the corresponding 2-methoxy compound. Both ^1H and ^{31}P NMR spectra were in accord with the assigned structure.

***trans*-2-Hydroxycyclopentanemethanol Cyclic Phosphoric Acid.** *trans*-2-Hydroxycyclopentanemethanol, obtained by acid-catalyzed addition of formaldehyde to cyclopentene,[12] was phosphorylated with phenyl phosphorodichloridate as described for the previous two cyclic esters. The resulting mixture of phenyl esters was purified by crystallization from ethanol–water. The phenyl groups were removed by hydrogenolysis using platinum oxide as catalyst, and the product was crystallized from ethanol–diethyl ether, mp 138.6–139.6 °C. Anal. Calcd for $C_6H_{11}O_4P$: C, 40.46; H, 6.22; P, 17.39. Found: C, 40.54; H, 6.29; P, 17.29.

***trans*-2-Hydroxycyclopentanemethanol-α-(biscyclohexylammonium phosphate).** Preparation of this monoester by phosphorylation of *trans*-2-hydroxycyclopentanemethanol with diphenyl phosphorochloridate in dry pyridine was not successful. Rather, this material was prepared by phosphorylation of the diol with phosphorus oxychloride in triethyl phosphate as solvent.[13] After the dichloride was quenched with water, the product was absorbed to Dowex-1-X8 (formate) and eluted with 0.1 N formic acid. After removal of the formic acid by rotary evaporation, the residue was dissolved in absolute ethanol and neutralized with cyclohexylamine. The product was crystallized from ethanol several times to give colorless, crystalline material, mp 195.8 °C. Anal. Calcd for $C_{18}H_{39}N_2O_5P{\cdot}H_2O$: C, 52.41; H, 10.02; N, 6.79; P, 7.51. Found: C, 52.50; H, 10.28; N, 6.78; P, 7.55.

***cis*-2-Hydroxycyclopentanemethanol Cyclic Phosphate.** This compound was prepared as described by Penney and Belleau.[10] Prior to use in the calorimetric experiments, this compound was converted to the sodium salt by ion exchange on Amberlite IR-120 (Na^+).

(1*R*,2*S*)-2-Hydroxytetrahydrofuranmethanol-α-(biscyclohexylammonium phosphate). 3,5-Di-*p*-toluyl-2-deoxy-D-ribosyl chloride was prepared according to the literature procedure.[14] Then 5.88 g (ca. 15 mmol) was dissolved in dry tetrahydrofuran and added dropwise to 3.3 g of lithium aluminum hydride dissolved in 200 mL of dry tetrahydrofuran; the mixture was refluxed overnight. The excess lithium aluminum hydride was decomposed by addition of 12 mL of ethyl acetate followed by 150 mL of water. The precipitated metal salts were removed by centrifugation and the supernatant was concentrated by rotary evaporation. The concentrate was washed with chloroform and neutralized by percolation through an Amberlite IR-120 (H^+) column. The acidic effluent was concentrated to yield the crude diol. Without further purification, the diol was phosphorylated preferentially at the primary alcohol functionality with phosphorus oxychloride in triethyl phosphate.[13] The hydrolyzed dichloride was purified on Dowex-1-X8 as previously described. An ethanolic solution of the acidic product was neutralized with cyclohexylamine. The precipitated salt was recrystallized from ethanol to give colorless material, mp 222.1–224.1 °C after preliminary softening at 174.5 °C. Anal. Calcd for $C_{17}H_{37}N_2O_6P{\cdot}H_2O$: C, 49.26; H, 9.48; N, 6.76; P, 7.47. Found: C, 50.63; H, 10.10; N, 6.93; P, 7.60. Despite the poor values for the C and H analyses of this compound, both ^1H and ^{31}P NMR were consistent with the assigned structure. This material was converted to the corresponding analytically pure cyclic phosphate, suggesting that this acyclic material is the indicated compound. We have experienced considerable difficulty in obtaining satisfactory elemental analyses for other biscyclohexylammonium salts.

(1*R*,2*S*)-2-Hydroxytetrahydrofuranmethanol Cyclohexylammonium Cyclic Phosphate. Four millimoles of the acyclic phosphate described in the previous paragraph was converted to the free acid by percolation through an Amberlite IR-120 (H^+) column. The acidic solution was neutralized with pyridine and rotary evaporated to dryness. After the solution was dried by repeated evaporation with pyridine, 4 mmol of *N*-morpholinodicyclohexylcarboxamidine was added and the pyridine solution of the acyclic phosphate was added dropwise to a refluxing solution of 8 mmol of dicyclohexylcarbodiimide in pyridine.[15] After the 2.5-h addition was complete, reflux was continued for an additional 2 h. After evaporation of the pyridine, the residue was partitioned between water and diethyl ether; the aqueous layer was filtered, concentrated, and applied to a DEAE-cellulose (carbonate) column. After an initial water wash, the column was eluted with a 2-L gradient

Table I. Protonation Data and Enthalpies of Hydrolysis for Hydrolysis Products

monoester	pK_a[a]	proton released[b]	ΔH_{prot}, kcal/mol[c]	ΔH_{H_2O}, kcal/mol[d]
2-methyl-3-hydroxypropyl phosphate	6.68	0.81	0.07 ± 0.05	
2-methoxy-3-hydroxypropyl phosphate	6.37	0.89	0.57 ± 0.05	0.27 ± 0.17
trans-2-hydroxycyclopentanemethanol α-phosphate	6.61	0.83	0.33 ± 0.05	+0.08 ± 0.08
trans-2-hydroxytetrahydrofuranmethanol α-phosphate	6.38	0.89	0.51 ± 0.05	−0.06 ± 0.11

[a] From titrations in 0.126 M KCl. [b] Calculated for pH 7.3 at 25 °C. [c] Measured by mixing HCl with excess dianionic monoester. [d] Enthalpy of hydrolysis at pH 7.3 after correcting for buffer protonation.

from 0 to 0.1 M triethylammonium bicarbonate, pH 7.5. Fractions containing the cyclic phosphate (as assessed by cellulose strip chromatography using 2-propanol-NH_4OH-water (7:1:2) as solvent) were pooled and rotary evaporated. The resulting triethylammonium salt was converted to the barium salt with barium hydroxide followed by removal of the volatile amine; the barium salt was converted to the cyclohexylammonium salt by passage through an Amberlite IR-120 (cyclohexylammonium) column. The salt was recrystallized from ethanol-diethyl ether, mp 197.3–198.3 °C. Anal. Calcd for $C_{11}H_{22}NO_5P$: C, 47.31; H, 7.94; N, 5.02; P, 11.09. Found: C, 46.95; H, 7.89; N, 4.95; P, 11.29.

Enzymes. The divalent metal ion requiring phosphohydrolase from *Enterobacter aerogenes* (ATCC 13048) was isolated according to the published procedure.[16] Alkaline phosphatase from *Escherichia coli* was purchased from Sigma.

Calorimetric Methods. Solutions of phosphohydrolase and alkaline phosphatase were dialyzed against 0.05 M sodium piperazine-*N,N*-bis(ethanesulfonate) (sodium Pipes), pH 7.3, prior to use. For the hydrolyses of all diesters except the cyclic phosphate of *trans*-2-hydroxycyclopentanemethanol, the concentration of the phosphohydrolase solution prior to mixing in the calorimeter was 0.04 mM (based on a subunit molecular weight of 29 000); for the trans-fused cyclopentane ester, the enzyme concentration before mixing was 0.2 mM. The concentration of alkaline phosphatase used to catalyze all monoester hydrolyses was 0.08 mM (based on a subunit molecular weight of 43 000) prior to mixing.

The sodium salts of cyclic phosphodiester acids were prepared by neutralization with sodium hydroxide, and the sodium salts of cyclohexylammonium salts were obtained by treatment with sodium hydroxide followed by lyophilization. For calorimetry the sodium salts were prepared as 1 mM solutions in 0.05 M sodium Pipes, pH 7.3.

Measurements of the enthalpies of hydrolysis of the esters were made at 25 °C in flow calorimeters using a stopped-flow procedure.[5] In this procedure, the enzyme and substrate solutions are flowed into the mixing chamber and reaction tubing at known flow rates for a period of time less than that required to completely fill the reaction tubing. The total heat evolved during the reaction is measured and the viscous heating during the flow period is subtracted to obtain the observed gross enthalpies of hydrolysis. This stopped-flow procedure rather than a flow procedure was used to reduce the amount of enzyme required for these experiments. We have determined that the enthalpies of hydrolysis of trimethylene and tetramethylene phosphates measured with the stopped-flow procedure are identical, within error, with those values determined using the flow procedures,[5] establishing the validity of the stopped-flow technique.

The enthalpies of dilution of the substrates and enzymes were measured, and only in the case of the concentrated phosphohydrolase solution used for the hydrolysis of the *trans*-cyclopentane cyclic phosphate was a small but significant enthalpy of dilution observed; this value was used to correct the observed gross enthalpies of hydrolysis for this compound.

The relative flow rates of the enzyme and substrate solutions were such that the concentrations of substrates in the hydrolysis reactions were between 0.25 and 0.5 mM in total reaction volumes of 0.3–0.5 mL. For the hydrolysis of the cyclic phosphate of *trans*-2-hydroxycyclopentanemethanol, the phosphohydrolase concentration was about 0.13 mM; for the hydrolyses of the other diesters the phosphohydrolase concentration was about 0.02 mM. For the hydrolyses of the monoesters, the final alkaline phosphatase concentration was about 0.04 mM.

For the hydrolyses of all of the esters, heat evolution was observed to be complete in 20 min or less. Since it was not possible to isolate the reaction products generated in the calorimeter, that the various reactions had proceeded to stoichiometric completion in the calorimeter was determined in parallel hydrolyses conducted at 25 °C using concentrations of enzyme and substrate identical with those employed in the calorimetry. For the diester hydrolyses, product analysis was performed in two ways. First, the reactions were monitored in a pH stat (using enzyme dialyzed against 0.126 M KCl and conducted in a solution which was 0.126 M in KCl; this solution is equal in ionic strength to 0.05 M sodium Pipes at pH 7.3) and in all cases the reactions were complete in 8 min or less. Second, the amount of inorganic phosphate produced by an alkaline phosphatase catalyzed hydrolysis of the products generated by the phosphohydrolase both in the pH stat reactions and in buffered solutions was quantitated; the reactions attained stoichiometric completion within the 20-min duration of the hydrolysis reactions conducted in the calorimeter.

Analysis of the inorganic phosphate produced by action of the phosphohydrolase alone was also measured, since this enzyme is capable of catalyzing the (slow) hydrolysis of monoesters.[16] The results of these determinations revealed that significant production of inorganic phosphate occurred in the hydrolyses of *trans*-2-hydroxycyclopentanemethanol cyclic phosphate, *trans*-2-hydroxytetrahydrofuranmethanol cyclic phosphate, and 5-methoxytrimethylene phosphate, with the values being 15, 50, and 40% of the starting diester, respectively; the hydrolyses of the *cis*-2-hydroxycyclopentanemethanol cyclic phosphate and 5-methyltrimethylene phosphate resulted in production of less than 3% inorganic phosphate. Since inorganic phosphate was produced in some of the hydrolyses, the enthalpies of hydrolysis of the primary phosphate monoester products of the trans-fused bicyclic cyclic phosphates and of 2-methoxy-3-hydroxypropane 1-phosphate were determined using alkaline phosphatase as catalyst. That these hydrolysis reactions proceeded to stoichiometric completion in the calorimeter was also determined in parallel hydrolyses in which the amount of inorganic phosphate produced was quantitated. The values for the enthalpies of hydrolysis of the monoesters at pH 7.3 corrected for the enthalpy of buffer (de)protonation by the fractional proton uptake are compiled in Table I. Given the errors of these determinations (due to the low heat evolution), the further hydrolysis of the monoester products to inorganic phosphate was assumed to have a negligible effect on the measured enthalpies of hydrolysis of the diesters.

The observed gross enthalpies of hydrolysis of the diesters were corrected for the enthalpies of protonation of the buffer by the fractional proton released in each hydrolysis (Table I). This correction was determined from the fractional proton values calculated at pH 7.3 from the pK values of the products measured in 0.126 M KCl and the heat of protonation of disodium Pipes, −2.55 ± 0.03 kcal/mol at 25 °C.[5]

Although about 40 mol % phosphohydrolase was necessary for the rapid hydrolysis of the cyclic phosphate of *trans*-2-hydroxycyclopentanemethanol, no correction was applied for the heat of mixing of product with the enzyme. That such a correction would not be necessary was suggested by the fact that the primary phosphate hydrolysis product of this cyclic ester is a poor inhibitor of the enzyme and verified by mixing the product with the concentrated enzyme.

Measurements of the enthalpies of protonation of the hydrolysis products (Table I) were made so that the enthalpies of hydrolysis could be calculated for reactions yielding either the mono- or dianionic monoester product.

Although only a single product can be obtained by the phosphohydrolase catalyzed hydrolyses of 5-methyl- and 5-methoxytrimethylene phosphates, each of the bicyclic phosphate esters could yield either a primary or a secondary phosphate monoester or a mixture of the two upon hydrolysis. For these esters, the identity of the monoester products derived from each of the bicyclic esters was determined by ^{31}P NMR spectra of product mixtures obtained using higher concentrations of the diesters (20 mM) than those used in the calorimetric studies. In each of these hydrolyses, both primary and secondary phosphate monoesters (identified in proton-coupled spectra) were

Table II. Enthalpies of Hydrolysis of Phosphate Diesters

diester	$-\Delta H_{obsd}$[a]	$-\Delta H_{-OPO_3^{2-}}$[b]	$-\Delta H_{-PO_3^-}$[c]
trimethylene phosphate[d]	3.82 ± 0.13	3.95 ± 0.16	3.01 ± 0.16
5-methyltrimethylene phosphate	3.75 ± 0.16	3.76 ± 0.19	3.69 ± 0.19
5-methoxytrimethylene phosphate	4.90 ± 0.17	4.96 ± 0.20	4.39 ± 0.20
methyl α-D-glucopyranoside 4,6-cyclic phosphate[d]	6.87 ± 0.10	6.92 ± 0.14	6.26 ± 0.14
cyclic AMP (3′-ester bond)[d]	12.09 ± 0.14	12.15 ± 0.20	11.14 ± 0.20
methyl β-D-ribofuranoside 3,5-cyclic phosphate[d]	11.74 ± 0.20	11.81 ± 0.22	11.12 ± 0.22
trans-2-hydroxytetrahydrofuranmethanol cyclic phosphate	10.57 ± 0.38	10.62 ± 0.42	10.11 ± 0.42
trans-2-hydroxycyclopentanemethanol cyclic phosphate	7.86 ± 0.59	7.95 ± 0.62	7.59 ± 0.62
cis-2-hydroxycyclopentanemethanol cyclic phosphate	2.50 ± 0.15	2.59 ± 0.18	2.23 ± 0.18

[a] In kcal/mol for enthalpy measured at pH 7.3 after correction for buffer protonation. [b] In kcal/mol for hydrolysis yielding dianionic monoester. [c] In kcal/mol for hydrolysis yielding monoanionic monoester. [d] Reference 5.

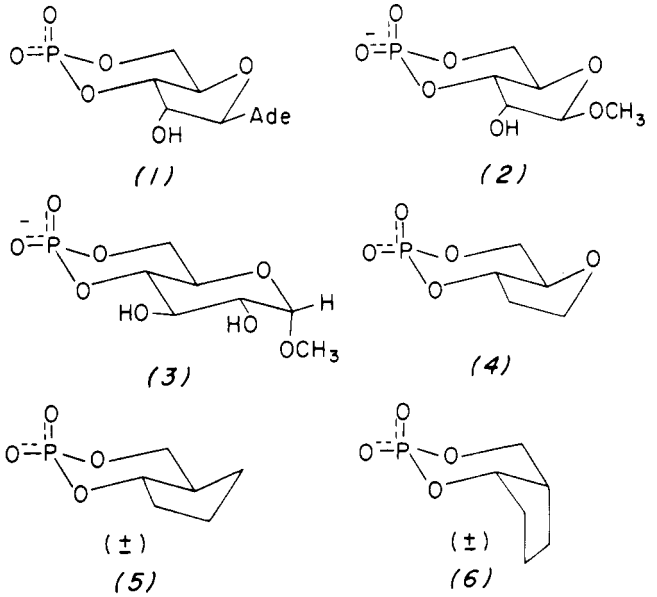

Figure 1. Structures of bicyclic phosphate diesters studied by calorimetry. The compounds shown: 1, cyclic AMP; 2, methyl β-D-ribofuranoside 3,5-cyclic phosphate; 3, methyl α-D-glucopyranoside 4,6-cyclic phosphate; 4, *trans*-2-hydroxytetrahydrofuranmethanol cyclic phosphate; 5, *trans*-2-hydroxycyclopentanemethanol cyclic phosphate; 6, *cis*-2-hydroxycyclopentanemethanol cyclic phosphate.

produced in a ratio of about 5:1, with the primary ester being predominant. In determining the ionization properties of the monoester products derived from the hydrolyses, we have chemically synthesized only the primary monoester products and assumed that the enthalpies of protonation and pK_as of the primary and secondary esters would have similar values; given the pronounced excess of the primary phosphate product, this assumption would not be expected to introduce significant errors in our reported values.

However, a potential problem associated with the fact that the phosphohydrolase-catalyzed hydrolyses yield mixtures of products is that the enthalpies of hydrolysis of a given ester to yield the primary and secondary products may not be identical. In our previous study of the enthalpies of hydrolysis of the 3′- and 5′-ester bonds of cyclic AMP itself,[5] the enthalpies of hydrolysis to yield each product were found to be identical within experimental error; in fact, this determination was possible because the phosphohydrolase used in the present study catalyzes the hydrolysis of cyclic AMP to yield a mixture of products (in which the 3′ product predominates). Although this single example of identical enthalpies of hydrolysis to yield the primary and secondary ester products does not prove that such will always be true, it is supportive of our assumption that such is the case in the studies we report on this paper. The most crucial comparisons of enthalpies of hydrolysis which we make in this study are those of the *cis*- and *trans*-2-hydroxycyclopentanemethanol cyclic phosphates and of the trans-fused esters of cyclopentanemethanol and tetrahydrofuranmethanol. The difference between the two trans-fused esters is the smaller of the two and is equal to 2.7 kcal/mol. Calculations of the enthalpies of hydrolysis of these two phosphodiesters to yield either the primary or secondary monoester product are reported in the following paper;[8] differences in enthalpies of hydrolysis of 1–2 kcal/mol were estimated with the hydrolysis yielding the secondary monoester giving the more exothermic enthalpy. Given our previous observation concerning the enthalpies of hydrolysis of cyclic AMP and the fact that one product is predominantly produced, we do not believe that this consideration would indicate that a large uncertainty is associated with any comparisons of enthalpy values which we have determined. It should be emphasized that no other catalyst, either enzymatic or nonenzymatic, is known that can catalyze the hydrolyses of phosphate diesters at rates sufficiently rapid for calorimetric studies. Given this severe limitation and the hydrolytic specificity of the enzyme, the values we report in this paper are the best that can be determined.

Results

The enthalpies of hydrolysis measured in this study are tabulated in Table II. Also included in the table for the purpose of comparison are the previously reported values for the hydrolyses of trimethylene phosphate, methyl β-D-ribofuranoside 3,5-cyclic phosphate, methyl α-D-glucopyranoside 4,6-cyclic phosphate, and the 3′-ester bond of cyclic AMP. The structures of the bicyclic compounds for which enthalpies of hydrolysis have now been measured are shown in Figure 1. In our discussion of these data, we will primarily refer to the values calculated for hydrolysis of diester to yield monoanionic monoester so that differences in solvation caused by differing anionic charge in the product will be minimized.

Discussion

The previous calorimetric study of the enthalpies of hydrolysis of phosphodiesters did not yield thermochemical data which would allow a complete explanation of the thermodynamic instability of 3′,5′-cyclic nucleotides in terms of their structures.[5] The results of that study suggested that the nature of the substituent on C_1 of the ribofuranoside ring did not influence the enthalpy of hydrolysis and that the enthalpies of hydrolysis of compounds having a trans-fused ribofuranoside–trimethylene phosphate ring system were about 8 kcal/mol more exothermic than that of unsubstituted trimethylene phosphate. In the present study, we have confirmed the earlier suggestion that the substituent on C_1 of the glycoside ring has little influence on the enthalpy of hydrolysis of a trans-fused trimethylene phosphate ring and further demonstrated that the presence of a hydroxyl group on C_2 of the five-membered ring also has essentially no influence on the enthalpy of hydrolysis, since the *trans*-2-hydroxytetrahydrofuranmethanol cyclic phosphate has nearly the same enthalpy of hydrolysis as does cyclic AMP. Thus the thermodynamic instability of the cyclic phosphate rings in the 3′,5′-cyclic nucleotides is determined by the tetrahydrofuran–cyclic phosphate portion of the structure.

The explanation for why the enthalpy of hydrolysis of the trans-fused tetrahydrofuran cyclic phosphate is 7–8 kcal/mol more exothermic than that for trimethylene phosphate was obtained by measuring the enthalpies of hydrolysis of the cis-

and trans-fused 2-hydroxycyclopentanemethanol cyclic phosphates. The cis-fused cyclic phosphate displays an enthalpy of hydrolysis which is similar to that found for trimethylene phosphate. This observation is certainly true despite the fact that we were not able to chemically prepare a sample of the major enzymatic hydrolysis product, *cis*-2-hydroxycyclopentanemethanol α-phosphate, owing to limited availability of the diol and difficulty in obtaining a sample which had a single proton decoupled ^{31}P NMR resonance. In calculating the thermochemical values for the hydrolysis of this cyclic phosphate which are listed in Table II, we have assumed that the ionization properties of the *trans*-2-hydroxycyclopentanemethanol α-phosphate are a good approximation of those which would be obtained for the cis isomer. In contrast to the low exothermicity of the cis isomer, the *trans*-cyclopentanemethanol cyclic phosphate had an enthalpy of hydrolysis of −7.6 kcal/mol, 4.6 kcal/mol more exothermic than trimethylene phosphate but 2.7 kcal/mol less exothermic than the tetrahydrofuran cyclic phosphate. We believe that the 4.6 kcal/mol increase in the exothermicity of hydrolysis of a six-membered ring cyclic ester which is produced by its trans fusion to the cyclopentane ring is the result of geometric destabilization (strain). This behavior was initially unexpected in view of the strain energies for cycloalkanes, in which trans fusion of a cyclohexane ring to a cyclopentane ring (*trans*-hydrindan) does not result in the generation of a ring fusion strain.[17] In fact, for the cycloalkanes, *cis*-hydrindan is *more* strained than the trans isomer by 1.0 kcal/mol. Presumably, the sp^2-hybridized oxygen atoms in cyclic phosphate rings cannot be as easily accommodated in the trans-fused system as in the cis-fused system. Also, we would not necessarily expect the relative order of strain for the cis- and trans-fused cyclic phosphates to be that observed for the analogous bicycloalkanes, since in the cyclic phosphates the presence of oxygen atoms in the phosphate ring will lead to a smaller 1,3-diaxial repulsion term in the conformational energy of the cis-fused ester than would be expected for the cis-fused alkane.

The 2.7 kcal/mol difference between the enthalpies of hydrolysis of the *trans*-tetrahydrofuran and the *trans*-cyclopentane cyclic phosphates cannot be so easily explained by intramolecular geometric destabilization considerations. The only difference in structure in these two compounds is the interchange of a methylene group and an oxygen atom; perhaps it could be argued that in the tetrahydrofuran cyclic ester the presence of C–O bonds rather than the C–C bonds found in the cyclopentane cyclic ester leads to additional strain, since C–O bonds are 0.08 Å shorter than C–C bonds. This suggestion is not supported by the molecular mechanics and ab initio calculations reported in the following paper.[8]

We have also found that the enthalpies of hydrolysis of 5-methyl- and 5-methoxytrimethylene phosphates differ by only 0.9 kcal/mol. That this difference is 1.8 kcal/mol smaller than that found for the tetrahydrofuran and cyclopentane cyclic esters indicates that intramolecular effects cannot account for the observed differences.

The 2.7 kcal/mol difference in enthalpies which we observed for the five-membered ring containing bicyclic esters is very similar to the 3.2 kcal/mol difference in the enthalpies of hydrolysis of methyl α-D-glucopyranoside cyclic phosphate and trimethylene phosphate. In the crystal structure of the glucoside cyclic phosphate, no evidence for intramolecular destabilization was found.[7] We have tried to measure the enthalpy of hydrolysis of *trans*-2-hydroxycyclohexanemethanol cyclic phosphate, but this cyclic ester is *not* a good substrate for the phosphohydrolase from *E. aerogenes;* several attempts to determine its enthalpy of hydrolysis have lead to uncertain values owing to the long times necessary for its complete hydrolysis, but the data we have do suggest that its hydrolysis is not very exothermic. We are still attempting to find conditions which will allow a quantitatively precise value to be determined. However, the ab initio and molecular mechanics calculations carried out on this compound predict that the enthalpy of hydrolysis will be similar to that found for trimethylene phosphate.[8] We assume, therefore, that the similar differences observed between the tetrahydrofuran and cyclopentane esters and the glucoside ester and trimethylene phosphate (or cyclohexanemethanol cyclic phosphate) share a common explanation.

An explanation for the differences between the enthalpies of hydrolysis of the tetrahydrofuran derived cyclic phosphates and the *trans*-cyclopentane cyclic phosphate and between the enthalpies of hydrolysis of the methyl glucoside cyclic phosphate and trimethylene phosphate cannot be obtained from these thermochemical data but can be formulated on the basis of the NMR studies reported in an accompanying paper.[9] NMR studies on 5-alkoxy substituted trimethylene phosphates, e.g., 5-methoxytrimethylene phosphate, and 5-alkyl substituted trimethylene phosphates, e.g., 5-methyltrimethylene phosphate, have revealed that the conformational behavior of the 5-alkoxy esters but not the 5-alkyl esters is solvent dependent, with the alkoxy group preferring an axial orientation in D_2O and an equatorial orientation in less polar solvents, e.g., acetone-d_6. This behavior can be described in the conformationally flexible monocyclic esters as the more polar conformation, i.e., with the alkoxy group axial, being favored in polar solvents and disfavored in nonpolar solvents. In the *trans*-tetrahydrofuran derived cyclic esters, e.g., cyclic AMP, and in the methyl glucoside cyclic phosphate, the analogous oxygen substituent of the cyclic phosphate ring (O_4' in cyclic AMP and O_5 in the glucoside cyclic phosphate) is constrained to be in an equatorial orientation, owing to the trans ring fusion which leads to conformational rigidity. Perhaps half of the excess enthalpies of hydrolysis of the oxygen substituted bicyclic cyclic esters relative to the analogous cycloalkane cyclic esters can be accounted for by this *solvent-dependent destabilization.*

We do not believe that this is the only factor which contributes to the excess enthalpy but believe, on the basis of some preliminary NMR experiments on the conformational preferences of phosphate monoesters,[18] that the hydrolysis products derived from the rigid 5-alkoxy cyclic esters experience a *solvent-dependent stabilization* relative to the product obtained from trimethylene phosphate. Evidence for this statement is provided by the observation that the rotameric distribution about the exocyclic C–C bond in the phosphate monoester of either tetrahydrofuranmethanol or tetrahydropyranmethanol is solvent dependent.

We attempted to gain calorimetric evidence for these solvent-dependent contributions to enthalpies of hydrolysis by measuring the enthalpy of hydrolysis of a conformationally rigid ester which would be expected to have an alkoxy group as an axial substituent of a six-membered cyclic phosphate ring. As an example of such a conformationally rigid diester, methyl α-D-galactopyranoside 4,6-cyclic phosphate was prepared and we found by examination of its ^1H NMR spectrum that O_5 was, indeed, an axial substituent of the cyclic phosphate ring (this ester should, in principle, be conformationally flexible since it is a cis-fused system, but the oxygen-equatorial conformer is presumably disfavored because of unfavorable 1,3-diaxial interactions in addition to the solvation reasons already discussed). This glycoside cyclic phosphate ester is *not* a substrate for the phosphohydrolase.

Thus, we believe that the thermodynamic instabilities of trans-fused glycoside cyclic phosphates have significant contributions from solvation effects in addition to geometric destabilization which can be present as a result of the trans ring fusion. Further discussion of these solvent-dependent effects is presented in an accompanying paper,[9] as is a hypothesis as

to the importance of solvation effects in the biochemical activity of 3′,5′-cyclic nucleotides.

Acknowledgments. We thank Professor Julian M. Sturtevant for use of his microcalorimetric equipment. This research was supported by the Research Corporation and the National Institutes of Health (GM-23026).

References and Notes

(1) (a) Yale University; (b) McGill University.
(2) NIH Career Development Awardee, CA-00499, 1978–1983.
(3) Current review articles describing the biochemical and biological functions of cyclic AMP and cyclic GMP may be found in the continuing series *Adv. Cyclic Nucleotide Res.* (P. Greengard and G. A. Robison, Eds.).
(4) Y. Kurashira, K. Takai, C. Suzuki-Hori, H. Okamoto, and O. Hayaishi, *J. Biol. Chem.*, **249**, 4824 (1974).
(5) J. A. Gerlt, F. H. Westheimer, and J. M. Sturtevant, *J. Biol. Chem.*, **250**, 5059 (1975).
(6) According to IUPAC rules, trimethylenephosphoric acid is 2-hydroxy-2-oxo-1,3,2-dioxaphosphorinane.
(7) C. L. Coulter, *J. Am. Chem. Soc.*, **98**, 4997 (1976).
(8) F. J. Marsh, P. Weiner, J. Douglas, P. A. Kollman, G. L. Kenyon, and J. A. Gerlt, *J. Am. Chem. Soc.*, following paper in this issue.
(9) J. A. Gerlt, N. I. Gutterson, R. E. Drews, and J. Sokolow, *J. Am. Chem. Soc.*, **102**, 1665 (1980).
(10) C. Penney and B. Belleau, *Can. J. Chem.*, **56**, 2396 (1978).
(11) H. S. Hill, M. S. Whelen, and H. Hibbert, *J. Am. Chem. Soc.*, **50**, 2235 (1928).
(12) K. Odon, S. Janos, and S. Gyula, *Magy. Kem. Foly.*, **71**, 93 (1965).
(13) M. Yoshikawa, T. Kato, and T. Takenishi, *Bull. Chem. Soc. Jpn.*, **42**, 3505 (1969).
(14) M. Hoffer, *Chem. Ber.*, **93**, 2777 (1960).
(15) M. Smith, G. I. Drummond, and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 698 (1961).
(16) J. A. Gerlt and G. J. R. Whitman, *J. Biol. Chem.*, **250**, 5053 (1975).
(17) C. C. Browne and F. D. Rossini, *J. Phys. Chem.*, **64**, 927 (1960).
(18) J. A. Gerlt, A. V. Youngblood, C. Bigler, and W. Apfeldorf, unpublished observations.

Theoretical Calculations on the Geometric Destabilization of 3′,5′- and 2′,3′-Cyclic Nucleotides[1]

Frederick J. Marsh,[2a] Paul Weiner,[2a] John E. Douglas,[2a] Peter A. Kollman,*[2a,d] George L. Kenyon,*[2a,b,e] and John A. Gerlt*[2c,f]

Contribution from the Department of Pharmaceutical Chemistry, School of Pharmacy, Department of Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, California 94143, and the Department of Chemistry, Yale University, New Haven, Connecticut 06520. *Received February 5, 1979*

Abstract: We present molecular mechanics and ab initio quantum mechanical calculations on some cyclic nucleotides and analogues with the goal of accounting for the unusually exothermic heats of hydrolysis of the six membered ring containing phosphodiester, 3′,5′-cyclic adenosine monophosphate (AMP), and of the five membered ring containing phosphodiester, 2′,3′-cyclic AMP. We attribute the greater exothermicity for hydrolysis of 3′,5′-cyclic AMP and the corresponding simple model compounds relative to that for trimethylene phosphate (8 kcal/mol) to the following effects: (1) the trans ring fusion in 3′,5′-cyclic AMP, which leads to 4–5 kcal/mol of strain energy in this molecule, and (2) the presence in the 3′,5′-cyclic nucleotide of an unfavorable O-C-C-O interaction which is relieved upon phosphate ring cleavage. This second interaction, which is solvent dependent, can account for about 1–2 kcal/mol of the difference in hydrolysis energy. (3) This leaves approximately 1–2 kcal/mol unaccounted for, and this may arise from differential solvation energies of the reactant and product. Our calculations overestimate the exothermicity of hydrolysis of the five membered ring containing phosphodiester, 2′,3′-cyclic AMP, but find, in agreement with experiment, that its exothermicity is much closer to the simple model phosphodiester, ethylene phosphate, than found in the corresponding six membered ring phosphodiesters.

Introduction

One of our laboratories has recently obtained thermochemical evidence that the unusually exothermic enthalpy of hydrolysis of 3′,5′-cyclic adenosine monophosphate (AMP) is determined by the trans-fused trimethylene phosphate–tetrahydrofuran portion of the structure, i.e., the substituents on C1′ (the heterocyclic base) and on C2′ (a hydroxyl group) are not required to produce the 8 kcal/mol more exothermic enthalpy of hydrolysis which cyclic AMP (and analogues which have this structure) display relative to trimethylene phosphate.[3] However, the enthalpy of hydrolysis of a structural analogue having trans-fused trimethylene phosphate–cyclopentane rings was about 3 kcal/mol less exothermic than those with tetrahydrofuran rings, suggesting that both the trans ring fusion and some uncharacterized effect resulting from the tetrahydrofuran oxygen were responsible for the total heat effect. Presumably, the trans ring fusion effect is caused by geometric distortion (strain), but the origin of the effect associated with the oxygen atom was not clear. Perhaps this latter effect could also be due to geometric distortion, since C–O bonds are about 0.08 Å shorter than C–C bonds and this shorter bond length could introduce additional strain.

In this paper we describe the results of molecular mechanics and ab initio calculations which support the notion of considerable strain being associated with the trans ring fusion in the 3′,5′-cyclic AMP, but which suggest that *inter*molecular considerations (solvation) are necessary to provide the complete explanation for the enthalpy of hydrolysis of 3′,5′-cyclic AMP. Results on calculations of hydrolysis energies on 2′,3′-cyclic nucleotides are also presented for comparison purposes. In the following paper,[4] experimental data from one of our laboratories are presented which implicate differential solvation of the reactants and products of the hydrolysis reaction as the source of the unaccounted for enthalpy of hydrolysis in the 3′,5′-cyclic AMP case.

Methods of Procedure

For the quantum-mechanical calculations, we employed an ab initio SCF approach using the program GAUSSIAN 70[5] and an STO-3G basis set.[6] The molecular mechanics calculations

0002-7863/80/1502-1660$01.00/0