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Contribution from the Department of Chemistry,
Wichita State University, Wichita, Kansas 67208

Hydrolysis of Bidentate Triphosphate Coordinated to Tetraamminecobalt(III)

RICHARD D. CORNELIUS

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The rate of hydrolysis of bidentate triphosphate in $Co(NH_3)_4H_nP_3O_{10}$ has been studied by phosphomolybdate analysis and by phosphorus-31 NMR and has been found to proceed at two-thirds the rate of that for the free ligand. At 40 °C in 1 M perchloric acid the rate constant for hydrolysis is 1.4×10^{-4} s^{-1}, and the rate is approximately linearly dependent upon the concentration of hydrogen ion in the range 0.1–1.0 M. When the hydrolysis is allowed to proceed in frozen solution, the phosphorus-31 NMR of the thawed solution reveals the existence of the species $Co(NH_3)_4(H_nPO_4)(H_nP_2O_7)$ and $Co(NH_3)_4H_nP_2O_7$ as intermediate products of hydrolysis. The former corresponds to hydrolysis of the phosphate linkage in the six-membered chelate ring and the latter to hydrolysis between the terminal, uncoordinated phosphate group and the chelate ring. Hydrolysis proceeds at essentially equal rates at the two sites.

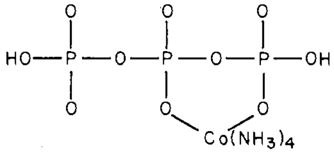
Introduction

The importance of phosphates for energy metabolism in biological systems has long provided stimulus for research designed toward revealing the mechanistic intricacies of phosphoryl transfer and phosphate hydrolysis. Metal ions,[1] through their association with enzymes that catalyze these reactions, are part of the mechanistic detail. Thus, the interaction of metal ions with phosphates has been the subject of considerable study. Cooperman[2] has summarized the problems which often cloud the interpretation of studies on the catalysis of phosphoryl transfer reactions by metal ions. These problems include variable stoichiometry of the metal–phosphate complexes involved as well as unknown and constantly changing binding modes (e.g., monodentate and bidentate). The application of presently available techniques to systems containing the labile metal ions commonly utilized by enzymes does not reveal the species in solutions to which catalysis should be attributed or what fraction of the species present exists in that active form. Without such information conclusions concerning the extent of catalysis by metal ions have only limited application.

Such difficulties may be overcome by the use of substitution-inert metal ions. In complexes of these metal ions, species of specific configurations, which can be prepared in pure form, remain intact in solution for sufficient time that their individual rates of reaction can be examined. Cobalt(III) has been the metal ion of choice in several studies, and phosphorus-31 NMR studies of its complexes have recently been used by several workers to provide definitive characterization of the mode of coordination of phosphates to the metal.

An early application[3] of substitution-inert metal ions to the study of phosphate hydrolysis led to the discovery of a significant rate enhancement for the hydrolysis of methyl phosphate in the coordination sphere of triethylenetetraaminecobalt(III), although the mechanism of catalysis in this system is uncertain. The largest increase in rate observed by using cobalt(III) complexes[4] involved the complex ((*p*-nitrophenyl)phosphato)bis(trimethylenediamine)cobalt(III) for which a rate enhancement of 10^9 over the rate for the free ester was found. This effect was attributed predominantly to charge neutralization and the strain inherent in the four-membered chelate ring of the bidentate phosphate group. A much smaller effect has been observed[5] for the hydrolysis of ATP (adenosine triphosphate) in the presence of a diethylenetriaminecobalt(III) complex. Most recently Milburn[6] has found an increase in rate of 10^5 for cobalt(III) complexes with a 3:1 metal:pyrophosphate stoichiometry as inferred from phosphorus-31 NMR spectra. The existence of four-membered chelate rings, known to exist in the (*p*-nitrophenyl)phosphato complex, was postulated to be important for the catalyzed hydrolysis of pyrophosphate.

The present study focuses on the hydrolysis of a single metal–phosphate complex of a substitution-inert complex for which the stoichiometry is known and fixed and for which the binding mode has been definitively determined both in the crystal and in solution. Both the X-ray crystal structure[7] and the phosphorus-31 NMR spectrum[8] of the complex $Co(NH_3)_4H_2P_3O_{10}$ (**1**) show that the center phosphate and one terminal phosphate group of the triphosphate ligand are coordinated, each through one oxygen atom, to the cobalt atom. The remaining terminal phosphate residue is not bonded to the metal atom. Although the strain in a four-membered ring appears to increase the rate of hydrolysis, the effect of metal ions on the rate of hydrolysis of a six-membered phosphate chelate ring[8,9] has not been investigated. The complex $Co(NH_3)_4H_2P_3O_{10}$ contains phosphate linkages both in a six-membered chelate ring and independent of a chelate ring. It thus provides a unique opportunity to study, in a single complex, the effects on the rate of hydrolysis produced by different modes of coordination of triphosphate to a metal ion.



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Experimental Section

Materials. The complexes $Co(NH_3)_4HP_2O_7$ and $Co(NH_3)_5HP_2O_7$ were prepared as previously described.[8] A standard procedure was used for the preparation of $[Co(NH_3)_4CO_3]NO_3$.[10] Solutions of uncoordinated triphosphate ion free of metal ion were prepared by passing solutions of $Na_5P_3O_{10}$ solutions through cation-exchange columns in the H^+ form.

The previous procedure[8] for $Co(NH_3)_4H_2P_3O_{10}$ was modified to give a better chromatographic separation and to decrease the possibility

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(1) A. S. Mildvan and C. M. Grisham, *Struct. Bonding (Berlin)*, **20**, 1 (1974).
(2) B. S. Cooperman, *Met. Ions Biol. Syst.*, **5**, 79 (1976).
(3) F. J. Farrell, W. Kjellstrom, and T. G. Spiro, *Science*, **164**, 320 (1969).
(4) B. Anderson, R. M. Milburn, J. M. Harrowfield, G. B. Robertson, and A. M. Sargeson, *J. Am. Chem. Soc.*, **99**, 2652 (1977).
(5) S. Suzuki, T. Higashiyama, and A. Nakahara, *Bioinorg. Chem.*, **8**, 277 (1978).
(6) P. W. A. Hubner and R. M. Milburn, *Inorg. Chem.*, in press.
(7) E. A. Merritt, M. Sundaralingam, R. D. Cornelius, and W. W. Cleland, *Biochemistry*, **17**, 3274 (1978).
(8) R. D. Cornelius, P. A. Hart, and W. W. Cleland, *Inorg. Chem.*, **16**, 2799 (1977).
(9) F. Seel and G. Bohnstedt, *Z. Anorg. Allg. Chem.*, **435**, 257 (1977); **441**, 237 (1978).
(10) G. Schlessinger, *Inorg. Synth.*, **6**, 173 (1960).

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of the formation of turbid solutions during the preparation. Ten percent excess 1 M HCl is used to generate the aquo complex from $[Co(NH_3)_4CO_3]NO_3$. A solution of $Na_5P_3O_{10}$ adjusted to pH 5 using 1 M HCl is mixed with a solution of the aquo complex to give a solution 0.01 M in triphosphate ion and in the cobalt complex. The solution is brought to pH 3.0 with 1 M HCl and is heated 10 min at 80 °C. The cooled solution is passed through a column of AG1-X2 anion-exchange resin, 100–200 mesh, pretreated with 1 M ethylenediamine–HCl, pH 8.0. Chromatography with 0.1 M HCl adjusted to pH 5.0 with pyridine drives off several extraneous products and slowly moves the desired red tetraammine complex down the column ahead of the lavender triammine complex. As the solution containing the tetraammine comes off the column, it is adjusted to pH 7.0 with 5 M NaOH, charged onto a second pretreated column of anion-exchange resin, and eluted with 0.05 M HCl. One volume of ethanol is added to the product solution and crystals form after several weeks of storage at −20 °C. The crystals are filtered, washed with ethanol and diethyl ether, and dried over P_4O_{10}. The dry product is stored desiccated at −20 °C to retard phosphate hydrolysis, which occurs slowly during storage at room temperature.

Kinetic Measurements. Solutions of the appropriate phosphate or phosphato complex were prepared by careful weighing of the corresponding solid. Where necessary, a solution of tetramethylammonium chloride was added to maintain the ionic strength at 1.0 M. This salt was used in order that no other metal ions be introduced into the reaction solutions. Hydrochloric acid rather than perchloric acid was used to establish the acidity in most experiments due to the insolubility of tetramethylammonium perchlorate. The phosphate solutions were incubated in 5- or 10-mL volumetric flasks at 40.0 ± 0.1 °C for 10 min at which time the appropriate volume of standardized acid was added. Water was added to bring the solutions to volume, the solutions were mixed, and the first samples were taken immediately. The concentration of orthophosphate ion produced was determined as described below. Due to the recognized existence of consecutive reactions which could produce orthophosphate ion, measurements on the hydrolysis of triphosphate ion or its complexes obtained by the phosphomolybdate method were made only up to the point that the concentration of orthophosphate ion was equal to one-third the initial concentration of triphosphate or its complex. For the reaction examined by NMR, the concentration of the reactant could be measured directly, and data up to 90% of the reaction were used. Data from each reaction were fit[11] to a first-order rate law to give an observed first-order rate as defined by k_{obsd} in the equation

$$-d[\text{reactant}]/dt = k_{obsd}[\text{reactant}]$$

Phosphate Analysis. The majority of kinetic experiments were monitored by using a phosphomolybdate technique to determine the concentration of free orthophosphate ion. In a typical experiment to measure the rate of hydrolysis of phosphate in cobalt complexes, a 0.1-mL aliquot was taken from a 0.01 M cobalt complex solution held in a water bath thermostated at 40.0 ± 0.1 °C and was pipetted into 1 mL of water. Next 0.1 mL of a 0.1 M vanadium(II) solution freshly prepared by passing through a Jones reductor 0.05 M V_2O_5 in 2 M HCl was added, and the solution was allowed to stand for 2 min. This procedure reduces the cobalt(III) to cobalt(II) and thereby releases all bound phosphate ligands. Because the vanadium(II) was found to strongly interfere with the phosphate analysis, the vanadium(II) and other metal ions were removed by passing the solution through a 0.6-mL column of AG50W-X2 cation-exchange resin. The column was washed with several portions of water, and the solution coming from the column was diluted to 5.00 mL. A freshly prepared solution made by mixing one volume 6 N H_2SO_4, one volume 2.5% ammonium molybdate, two volumes water, and then one volume 10% ascorbic acid has been described[12] for use in orthophosphate analyses. For analyses in the present study, 5.00 mL of this mixture was added to the phosphate solution to be analyzed, and the absorbance at 820 nm was recorded after 20.0 min. The heating procedure reported[12] was avoided in order not to induce further phosphate hydrolysis. The procedure used for the uncoordinated phosphates was identical except for the omission of the vanadium(II) solution and of the ion-exchange treatment. For all analyses the absorbance values were compared with those of standard phosphate solutions treated in an identical manner on the same day.

Figure 1. Phosphorus-31 NMR spectra at 40.5 MHz showing quenched samples (pH 7.5) for hydrolysis of $Co(NH_3)_4H_nP_3O_{10}$ in 1 M $HClO_4$ at 40 °C. From bottom to top, times for hydrolysis are 8, 35, 135, and 265 min.

The rate of hydrolysis of $Co(NH_3)_4H_2P_3O_{10}$ was also monitored by using phosphorus-31 NMR. For these experiments a 2.00-mL aliquot of 0.1 M $Co(NH_3)_4H_2P_3O_{10}$ in 50% D_2O held at 40.0 ± 0.1 °C was pipetted into a test tube cooled in ice, and 0.2 mL of 0.2 M Na_2EDTA was added. The pH was adjusted to 7.5 with 5 M NaOH, and the solution was kept cooled in ice until the spectrum could be taken the same day. Phosphorus-31 NMR spectra were recorded at 10 °C by using a Varian XL-100 spectrometer operating at 40.5 MHz in the pulsed mode with operating parameters of a 15-μs pulse width and a 7-s delay time. No decoupling was used, and 500 transients were collected for each spectrum. Chemical shifts are reported as parts per million downfield from 85% phosphoric acid. A violet precipitate found in some of the quenched reaction solutions gave the following analysis: Calcd for $Co(NH_3)_4(H_2O)PO_4$: Co, 24.55; P, 12.90; N, 23.34; H, 5.88. Found: Co, 24.93; P, 13.13; N, 23.68; H, 5.49.

Results

A potentiometric pH titration of $Co(NH_3)_4H_2P_3O_{10}$ at 1.0 M ionic strength (maintained by tetramethylammonium chloride) gave a pH curve with two inflection points. An analysis[13] assuming only two protonation steps yielded values

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[11] Fits to equations were accomplished by using the nonlinear least-squares program FIT kindly supplied by Professor Gilbert Gordon, Miami University. A description of the algorithm of the program is given in the Los Alamos publication LA-2367 and addenda.

[12] P. S. Chen, T. Y. Toribara, and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

Table I. Concentration of Phosphate Species from Phosphorus-31 NMR Spectra of $Co(NH_3)_4H_nP_3O_{10}$ Hydrolysis[a]

time, min	species concns, M			
	H_nPO_4	$H_nP_2O_7$	$Co(NH_3)_4$-$H_nP_2O_7$	$Co(NH_3)_4$-$H_nP_3O_{10}$
3		−0.004[b]	0.004	0.100
8		0.003	0.007	0.094
15	0.007	0.002	0.002	0.095
25	0.012	0.009	0.006	0.086
35	0.022	0.005	0.015	0.080
50	0.032	0.009	0.018	0.071
65	0.035	0.016	0.010	0.061
105	0.062	0.021	0.038	0.040
135	0.123	0.021	0.037	0.021
205	0.121	0.020	0.056	0.009
265	0.139	0.024	0.057	
385	0.164	0.017	0.053	
505	0.188	0.009	0.047	
1080	0.251		0.025	

[a] 40 °C, 1 M $HClO_4$, 0.1 M $Co(NH_3)_4H_nP_3O_{10}$. [b] Concentrations determined by difference in peak integrations; the resulting large experimental error is responsible for this negative value.

$pK_1 = 2.2 \pm 0.1$ and $pK_2 = 5.7 \pm 0.1$. Titration of a solution of the complex containing excess acid revealed that any protonation steps at lower pK values are too acidic to be observed by the titration technique used. Due to the many different possible states of protonation which may not be accurately known under a given set of conditions, general formulas[6] of the type $H_nP_3O_{10}$ and $Co(NH_3)_4H_nP_3O_{10}$ without a charge shown are used to indicate all of the species from a given parent phosphate without regard to the state of protonation.

NMR. Figure 1 presents 4 of 14 phosphorus-31 NMR spectra which show the relative concentrations of several species as they appear and/or disappear during the hydrolysis of $Co(NH_3)_4H_nP_3O_{10}$ at 40 °C in 1 M perchloric acid. The species that produce the peaks in these spectra can be readily identified from previous studies.[8,9] The bottom spectrum, which was obtained after 8 min of heating before quenching, has as major peaks the two doublets of the phosphorus atoms in the terminal phosphates at 4 and −5 ppm, and the double doublet of the middle phosphorus atom of $Co(NH_3)_4H_nP_3O_{10}$ at −9 ppm. This pattern clearly shows the large downfield shift upon coordination of the phosphates to cobalt(III). The other species apparent in these spectra can be discerned most readily in the top spectrum in Figure 1 which arises after heating for 265 min. The largest peak, at 3 ppm, represents the ultimate hydrolysis product, orthophosphate ion. The other large peak, at 5 ppm, is $Co(NH_3)_4H_nP_2O_7$, and the small peak at −6 ppm is $H_nP_2O_7$. These last two species are present only as intermediates, neither one being present in the original reaction mixture or after complete hydrolysis. Two species, $Co(NH_3)_4(H_2O)H_nPO_4$ and uncoordinated triphosphate ion, are conspicuous by their absence from these spectra. The orthophosphato complex is identified by elemental analysis as being present in a precipitate from the solution used to prepare spectral samples; its insolubility prevents it from giving an NMR spectrum. The absence of uncoordinated triphosphate ion confirms that the reaction being monitored is indeed the hydrolysis of coordinated triphosphate ion rather than the dissociation of the cobalt complex followed by the hydrolysis of the uncoordinated species.

The lines in these NMR spectra are too closely spaced to yield reliable integrations of peaks for individual species. Instead, integrations of four groups of peaks were obtained, and the amounts of individual species present were calculated by appropriate combinations of the results of the integrations.

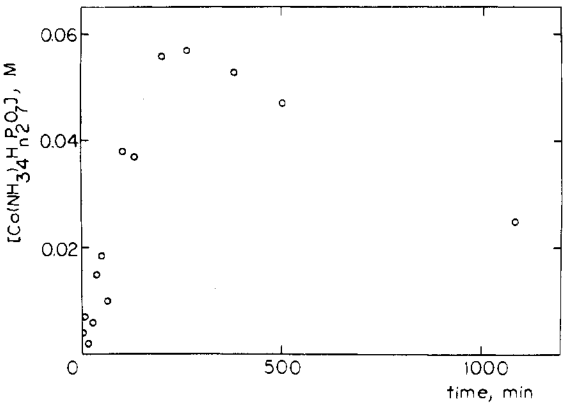

Figure 2. Appearance and subsequent disappearance of $Co(NH_3)_4H_nP_2O_7$ during hydrolysis of $Co(NH_3)_4H_nP_3O_{10}$ at 40 °C in 1 M $HClO_4$.

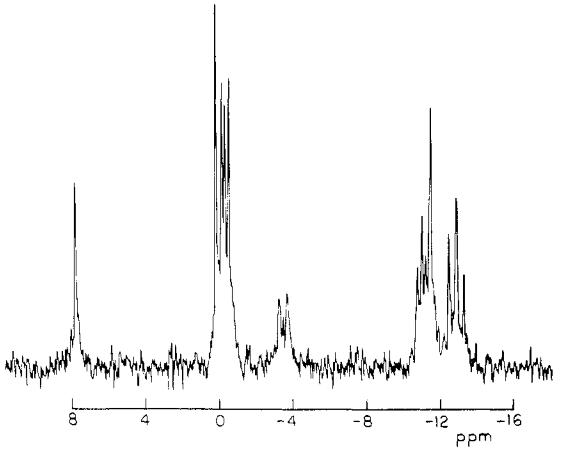

Figure 3. Phosphorus-31 NMR spectrum at 40.5 MHz of a solution of $Co(NH_3)_4H_nP_3O_{10}$ kept frozen in 1 M $HClO_4$ for 1 week.

Thus, for example, the amount of $Co(NH_3)_4H_nP_3O_{10}$ could be determined from the area of the double doublet of the center phosphorus atom, but the area under the peak for $H_nP_2O_7$ could not be measured except as the total of this peak with the doublet of the free terminal phosphorus atom in $Co(NH_3)_4H_nP_3O_{10}$. The amount of phosphorus present as $H_nP_2O_7$ was calculated from the difference between the area of this combination of peaks and the double doublet of $Co(NH_3)_4H_nP_3O_{10}$. The species concentrations thus obtained are given in Table I, and the concentrations of $Co(NH_3)_4H_nP_3O_{10}$ were used to obtain a rate constant of $(1.44 \pm 0.11) \times 10^{-4}$ s^{-1} for the hydrolysis of $Co(NH_3)_4H_nP_3O_{10}$. The low concentrations of products listed in the table for the reaction quenched after 3 min of heating confirm that the quenching procedure used does not induce hydrolysis. Figure 2 shows the appearance and subsequent disappearance of $Co(NH_3)_4H_nP_2O_7$ during the hydrolysis. From the last four points on this figure an approximate value of 2×10^{-5} s^{-1} is calculated for the hydrolysis of $Co(NH_3)_4H_nP_2O_7$ in 1 M perchloric acid at 40 °C.

During the preliminary stages of this research an attempt to quench samples at different times during the hydrolysis of $Co(NH_3)_4H_nP_3O_{10}$ in 1 M perchloric acid by freezing aliquots in liquid nitrogen resulted in the finding that the hydrolysis occurs, albeit slowly, even in frozen solutions. Figure 3 shows the phosphorus-31 NMR spectrum of the first sample which was not heated at all but was quickly frozen by immersion in liquid nitrogen after the $Co(NH_3)_4H_nP_3O_{10}$ solution was prepared at 0 °C. During the next week the solution was thawed once briefly to allow conventional phosphate analysis but was otherwise kept frozen at −20 °C. The sample was then

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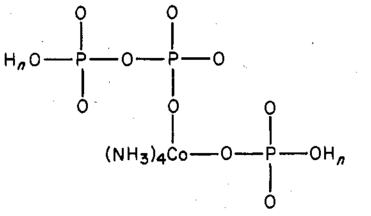
(13) G. M. Fleck, "Equilibria in Solution", Holt, Rinehart, and Winston, New York, 1966, p 105.

Table II. Rate Constants for Hydrolysis of Free and Coordinated Triphosphate Ion[a]

acid concn, M	10^4k_{obsd} for $Co(NH_3)_4$-$H_nP_3O_{10}$, s^{-1}	10^4k_{obsd} for $H_nP_3O_{10}$, s^{-1}
1.0 ($HClO_4$)	1.44[b]	
1.0 ($HClO_4$)	1.39	
1.0 (HCl)	2.02	2.62
0.5 (HCl)	1.02	1.26
0.2 (HCl)	0.35	0.58
0.1 (HCl)	0.17	0.32

[a] 40 °C, 0.010 M $Co(NH_3)_4H_nP_3O_{10}$; see text for error estimate. [b] 0.10 M $Co(NH_3)_4H_nP_3O_{10}$ from NMR spectra of quenched solutions; all other results are obtained by phosphomolybdate analysis.

thawed and diluted fourfold with water, and its spectrum was taken at 10 °C. A comparison of the spectrum in Figure 3 with the various spectra in Figure 1 serves to identify many of the peaks, although the pH difference between the solutions used for the spectra in Figures 1 and 3 does result in measurable chemical shift differences.[6,9] The tallest group of four lines at 0 ppm consists of singlets for orthophosphate and $Co(NH_3)_4H_nP_2O_7$ and a doublet for the phosphorus atom in the coordinated terminal phosphate of $Co(NH_3)_4H_nP_3O_{10}$. The triplet at −13 ppm arises from the center phosphorus atom of $Co(NH_3)_4H_nP_3O_{10}$. The remaining group of four lines at −11 ppm includes the two taller peaks which are a doublet from the phosphorus atom in the free terminal phosphate of $Co(NH_3)_4H_nP_3O_{10}$ and two shorter lines which are a doublet in the correct position[6,8,9] for a free terminal phosphate. Also in the spectrum are a singlet at 8 ppm in the position found[6,8,9] for bound orthophosphate ion and a doublet at −4 ppm with the same coupling constant as the small doublet in the group of four lines at −11 ppm and which has the chemical shift found for a terminal coordinated phosphate group. Integration of the areas under the singlet and the doublet at −4 ppm which is free from other peaks gives a ratio of 1.05:1.00. All of these observations are consistent with the assignment of the species responsible for the singlet and the two doublets as $Co(NH_3)_4(H_nPO_4)(H_nP_2O_7)$ (**2**). Thus in frozen solution the hydrolysis of $Co(NH_3)_4H_nP_3O_{10}$ gives not only all the products which are seen at higher temperatures but also a species which corresponds to hydrolysis between the coordinated phosphate groups. Analysis of the integrals of the peaks in Figure 3 indicates that 51% of the $Co(NH_3)_4H_nP_3O_{10}$ remained intact, 26% hydrolyzed to $Co(NH_3)_4(H_nPO_4)(H_nP_2O_7)$, and 23% hydrolyzed to $Co(NH_3)_4H_nP_2O_7$.



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Other Kinetic Studies. The rate of hydrolysis of $Co(NH_3)_4H_nP_3O_{10}$ in other studies was monitored spectrophotometrically by phosphomolybdate analysis. As for the NMR studies, very low concentrations of products at the first time intervals used provide assurance that the analysis procedure does not induce hydrolysis. The rate constants from these studies are presented in Table II, and the value in 1 M perchloric acid is in excellent agreement with the result obtained from NMR spectra. Also given in Table II are comparable rate constants for uncoordinated triphosphate ion for comparison. Standard deviations averaged 3% but are not presented lest they imply an unrealistic degree of accuracy. The presence of consecutive reactions an order of magnitude slower than the reaction of interest could alone introduce an error of 3% after one-third of reaction. The standard deviation of 8% found for the reaction followed to 90% by NMR probably gives a reasonable estimate of the reliability that should be assigned to all of the values presented.

For both species given in Table II the observed first-order rate constants increase as the acid concentration is increased. Results for both species have been fitted to an equation of the form $k_{obsd} = k[H^+]^n$. For the cobalt complex the fitted values are $k = (2.06 \pm 0.07) \times 10^{-4}$ and $n = 1.09 \pm 0.02$, and for free triphosphate ion $k = (2.49 \pm 0.12) \times 10^{-4}$ and $n = 0.90 \pm 0.03$.

Preliminary experiments at 40 °C and 1 M perchloric acid have resulted in the following observed first-order rate constants for several other complexes: $Co(NH_3)_3H_nP_3O_{10}$, 1×10^{-4} s^{-1}; $Co(NH_3)_5H_nP_2O_7$, 2×10^{-5} s^{-1}; $Co(NH_3)_4H_nP_2O_7$, 4×10^{-5} s^{-1}; and uncoordinated pyrophosphate ion, 1×10^{-4} s^{-1}.

Discussion

Perhaps the most striking observation to be made in an examination of the results presented here is that the rate of hydrolysis of triphosphate in $Co(NH_3)_4H_nP_3O_{10}$ is actually slightly less than that for the free ligand at the same acid concentration. The values in Table II show small differences in the rate constants for the two species over the range of 0.1–1.0 M hydrochloric acid, with the rate constant for the free ligand averaging 1.5 times the rate constant for the cobalt complex over the acid range studied.

A detailed examination of the results allows a conclusion regarding the site of hydrolysis. The hydrolysis reactions at the two possible sites are represented by 1 and 2. The first

$$Co(NH_3)_4H_nP_3O_{10} + H_2O \rightarrow Co(NH_3)_4H_nP_2O_7 + H_nPO_4 \quad (1)$$

$$Co(NH_3)_4H_nP_3O_{10} + H_2O \rightarrow Co(NH_3)_4(H_nPO_4)H_nP_2O_7 \quad (2)$$

reaction represents hydrolysis of $Co(NH_3)_4H_nP_3O_{10}$ (**1**) between the uncoordinated phosphate group and the chelate ring. The resulting cobalt complex contains bidentate pyrophosphate. Equation 2 shows the product of hydrolysis of the phosphate linkage which is part of the chelate ring. This product, **2**, contains orthophosphate and monodentate pyrophosphate. Two pieces of evidence indicate that reactions 1 and 2 proceed at comparable rates. The first evidence comes from the rate of appearance of pyrophosphate in the series of spectra in Figure 1 and the second from the existence of the species containing both pyrophosphate and orthophosphate as revealed by the spectrum in Figure 3. The spectra in Figure 1 and the data in Table I show the parallel appearance of $Co(NH_3)_4H_nP_2O_7$ and pyrophosphate ion. The lack of an induction period in the formation of pyrophosphate ion indicates that the ion is not formed primarily from the pyrophosphato complex; that is, reaction 3 does not occur to a

$$Co(NH_3)_4H_nP_2O_7 + 2H_2O \rightarrow Co(NH_3)_4(H_2O)_2^{3+} + H_nP_2O_7 \quad (3)$$

significant extent. Also, the spectra taken after the longer heating times show that pyrophosphate disappears faster than does the pyrophosphato complex. This observation lends further support to the contention that the free pyrophosphate ion is formed not by eq 3 but from $Co(NH_3)_4H_nP_3O_{10}$. The pyrophosphate must then arise from hydrolysis of the phosphate linkage in the chelate ring in the triphosphato complex as shown in eq 2. Dissociation of the product of this reaction could then yield free pyrophosphate ion and $Co(NH_3)_4(H_2O)H_nPO_4$, a complex not seen in the NMR spectra but rather found as a precipitate due to its insolubility under the conditions used to quench the reaction. This dissociation reaction is shown in eq 4.

$$\mathrm{Co(NH_3)_4(H_nPO_4)(H_nP_2O_7)} \rightarrow \mathrm{Co(NH_3)_4(H_2O)H_nPO_4 + H_nP_2O_7} \quad (4)$$

A complex with two adjacent phosphate groups in the coordination sphere of cobalt(III) finds precedent in a study[14] involving $\mathrm{Co(NH_3)_4H_nATP}$ in which ATP is a β,γ-bidentate ligand. This ATP complex is a substrate for several enzymes, and its reaction with hexokinase has been studied in detail. When the other substrate for hexokinase is glucose, the product of enzyme catalysis is $\mathrm{Co(NH_3)_4}$ADP(glucose-6-phosphate) (ADP = adenosine diphosphate). This product dissociates rapidly, breaking down at the rate of about 10%/h at 25 °C and pH 7. Interestingly, ADP is lost from the coordination sphere faster than is glucose-6-phosphate,[14] a result which is paralleled in the present study by the dissociation of pyrophosphate more rapidly than orthophosphate ion from $\mathrm{Co(NH_3)_4(H_nPO_4)(H_nP_2O_7)}$, as shown in eq 4.

Direct evidence to support the existence of $\mathrm{Co(NH_3)_4(H_nPO_4)H_nP_2O_7}$ comes from the phosphorus-31 NMR spectrum of a sample for which $\mathrm{Co(NH_3)_4H_nP_3O_{10}}$ hydrolysis occurred in frozen solution. Analysis of the integral of the peaks in Figure 3 indicates that nearly equal amounts of $\mathrm{Co(NH_3)_4H_nP_2O_7}$ and $\mathrm{Co(NH_3)_4(H_nPO_4)(H_nP_2O_7)}$ were formed. Thus the rates of hydrolysis at the two phosphate linkages as shown in eq 1 and 2 are essentially the same under the conditions employed despite the participation of one linkage in a six-membered chelate ring.

This result of the similarity in rates for chelated and nonchelated phosphate linkages is also shown in the preliminary results given for the rates of hydrolysis of the monodentate and bidentate pyrophosphato complexes. The rate of hydrolysis for $\mathrm{Co(NH_3)_4H_nP_2O_7}$, in which pyrophosphate forms a six-membered chelate ring with the cobalt atom, is nearly the same as the rate for $\mathrm{Co(NH_3)_5H_nP_2O_7}$ in which no chelate ring is present.

The rate of hydrolysis of $\mathrm{Co(NH_3)_4H_nP_3O_{10}}$ exhibits an apparent order of 1.09 ± 0.02 with respect to the concentration of hydrogen ion. A plausible explanation for this order, which appears to be significantly different from unity, is that the predominant pathway for the hydrolysis reaction is through the activated complex $[\mathrm{Co(NH_3)_4H_3P_3O_{10}}]^+$. The protonation step at $\mathrm{p}K_1 = 2.2$ is incomplete in the lower range of acid concentration used in this study and thus contributes to an apparent reaction order of greater than unity with respect to the hydrogen ion concentration. If K_0 is the dissociation constant for a protonation step that occurs at too high an acid concentration to be observed by potentiometric titration, then the expression for k_{obsd} assuming $[\mathrm{Co(NH_3)_4H_3P_3O_{10}}]^+$ to be the sole reactive species would be

$$k_{\mathrm{obsd}} = \frac{k[\mathrm{H^+}]^2}{K_0K_1 + K_0[\mathrm{H^+}] + [\mathrm{H^+}]^2}$$

In this equation k is the microscopic rate constant for the hydrolysis of $[\mathrm{Co(NH_3)_4H_3P_3O_{10}}]^+$. The larger magnitude of $[\mathrm{H^+}]$ over K_1 prevents a fit of k_{obsd} to this equation from being a useful method for the determination of the value of K_0; however, this equation could readily yield an order with respect to the concentration of hydrogen ion of slightly more than unity when $K_0[\mathrm{H^+}] > K_0K_1 \gg [\mathrm{H^+}]^2$. In the absence of evidence to the contrary, a mechanism involving $[\mathrm{Co(NH_3)_4H_3P_3O_{10}}]^+$ is regarded as most probable.

The technique of allowing a reaction to proceed in frozen solution may have general applicability. The effect of freezing the solution in this study is to greatly retard the rate of dissociation of ligands from the coordination sphere of cobalt(III) while allowing the hydrolysis of the phosphate chain to proceed at a modest rate. The result is the buildup of the intermediate $\mathrm{Co(NH_3)_4(H_nPO_4)(H_nP_2O_7)}$ which in liquid solution at 40 °C dissociates too rapidly for detection. The relevant difference between the dissociation and hydrolysis reactions would seem to lie in the volume of activation, $\Delta V^\ddagger$. The value of $\Delta V^\ddagger$ for the dissociative loss of a phosphate from $\mathrm{Co(NH_3)_4(H_nPO_4)(H_nP_2O_7)}$ should be much greater than for the hydrolysis of the phosphate chain which involves nucleophilic attack of a water molecule and is an associative process. The effect of freezing the solution may be compared to the effect of raising the pressure on the solution inasmuch as both processes place a volume constraint on the system. Thus the reaction with the greater $\Delta V^\ddagger$, viz., the dissociation reaction, is retarded by freezing much more than the reaction with the smaller $\Delta V^\ddagger$, viz., the hydrolysis reaction. This freezing technique may find application as a probe of mechanism in other reactions where under ordinary conditions intermediates dissociate too rapidly for their concentration to reach measurable values.

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Registry No. $\mathrm{Co(NH_3)_4H_2P_3O_{10}}$, 63915-32-2; $[\mathrm{Co(NH_3)_4CO_3}]\mathrm{NO_3}$, 15040-52-5; $\mathrm{Na_5P_3O_{10}}$, 13845-36-8; $\mathrm{Co(NH_3)_4(H_2O)(PO_4)}$, 73173-83-8; $\mathrm{H_5P_3O_{10}}$, 10380-08-2; $\mathrm{Co(NH_3)_5(P_2O_7)^-}$, 63915-21-9; $\mathrm{Co(NH_3)_4(P_2O_7)^-}$, 63915-34-4; $\mathrm{Co(NH_3)_4(PO_4)(P_2O_7)^{4-}}$, 73104-91-3.

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[14] R. D. Cornelius and W. W. Cleland, *Biochemistry*, **17**, 3279 (1978).