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Nucleotide hydrolysis in the presence of μ-hydroxo-bridged-cobalt(III) complexes

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Two cobalt(III) complexes with tridentate ligands, the acyclic diethylenetriamine and macrocyclic 1,4,7-triazacyclononane, were examined as potential catalysts for the hydrolysis of adenosine 5'-triphosphate (ATP). Studies were performed primarily at pH 4.5, where the two complexes were in the binuclear di-μ-hydroxo forms. For both complexes, a rapid initial hydrolysis with first order dependence on the concentration of ATP was observed, k_{obs} of approximately 0.20 min^{-1}, followed by very slow hydrolysis. Deuterium isotope studies done in D_2O showed a normal isotope effect with k_H/k_D = 1.8. Spectral investigations and ^{59}Co NMR studies indicated that the biphasic nature of the hydrolysis reaction may be due to the formation of a complex in which inorganic phosphate is coordinated to the cobalt, effectively poisoning the catalyst.

INTRODUCTION

Phosphate chemistry, so interwoven in complex metabolic reaction sequences, has been the subject of intensive study for a number of years. The required presence of metal ions in most of the biological processes involving nucleotidyl and phosphoryl transfer has generated interest in understanding the role of the metal ions in these systems.[1,2–3] In recent years the desirable properties of cobalt(III) complexes, namely their chemical inertness and diamagnetism, have led to the use of this ion as a probe for mechanistic aspects of nucleotide and phosphate hydrolysis reactions.[2–5] As a result of these studies several factors have surfaced. First, binuclear metal ion intermediates are involved in phosphate hydrolysis reactions. Secondly, the presence of several vacatable coordination sites on the metal ions facilitates the hydrolyses. Finally, the proximal coordination of a hydroxide capable of nucleophilic attack is required.

In consideration of these factors, we sought to examine the hydrolytic aptitude of *preformed* binuclear complexes with coordinated hydroxides, while maintaining potential coordination sites for incoming phosphates. Hence, a study of cobalt(III) complexes with triaza ligands was undertaken. These complexes maintain three vacatable coordination sites in their monomeric forms, yet they readily condense to form hydroxo-bridged dimers.[4] Two triaza ligands were chosen for this study, the acyclic diethylenetriamine (dien) and its macrocyclic analog 1,4,7-triazacyclononane (tcn). Complexes of both of these ligands are readily accessible in dimeric cobalt (III)-μ-hydroxo-bridged forms, while the geometries of the macrocyclic complexes are more limited in the necessity for forming only *fac* isomers.

EXPERIMENTAL

Physical Measurements. Electronic spectra were obtained on a Hewlett-Packard Model 8450A diode array spectrophotometer from 800 to 230 nm. The NMR spectra were recorded on a Varian XL-300 spectrometer operating at 121.4 MHz for ^{31}P and at 71.86 MHz scanning in increments of 0.05 MHz in both directions for ^{59}Co. Solutions 10% (v/v) D_2O were used with phosphoric acid (85%) as an external standard for the ^{31}P, and potassium hexacyanocobaltate(III) as standard for ^{59}Co. Deuterium isotope studies were done entirely in commercially available D_2O (0.5% H_2O). All pH measurements were made at 22°C on a Corning digital pH meter using a combination microelectrode. Microanalyses were made by Dr. Tho Nguyen at the Microanalytical Laboratory, University of Kansas. Mass spectra were obtained from a Ribermag R10-R10 spectrometer by Dr. Charles Judson at the Mass Spectrometer Laboratory, University of Kansas.

Synthesis. Commercially available chemicals were of reagent or analytical grade and were used without further purification. The sodium salts of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) were purchased either from Aldrich or Sigma. The ligand 1,4,7-triazacyclononane was synthesized according to previ-

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ously published methods,[5] as were the complexes trichlorodiethylenetriamine cobalt(III)($Co(dien)Cl_3$)[6] and trichloro-1,4,7-triazacyclononanecobalt(III)($Co(tcn)Cl_3$).[4]

Kinetic Measurements. Stock solutions of the nucleotide or polyphosphate substrates (0.100 M) and cobalt complexes (0.200 M) were kept at 4°C to avoid decomposition. Stock solutions of $Co_2(dien)_2(OH)_2^{4+}$ and $Co_2(tcn)_2(OH)_2^{4+}$ were prepared by dissolving the monomeric trichloride in 5 M NaOH followed by adjusting the pH to 4.5. All metal instruments were excluded during the preparation of the solutions. In a typical kinetic run the pH of 2.5 mL aliquots of both substrate and cobalt complex (after dilution to give a final substrate concentration of 0.01 M) were adjusted prior to mixing. After mixing the solutions were placed in a thermostated water bath equilibrated to the desired temperature. Aliquots (0.4 mL) were withdrawn at intervals from the reaction mixture and transferred to a 5 mm NMR tube that contained 70 μL of 5 M NaOH in order to quench the reaction. Solutions quenched in this manner showed no further hydrolysis after several hours at room temperature. Acquisition of proton decoupled ^{31}P NMR spectral data (450 scans, 6 min) was obtained at time intervals depending on the reaction rate. Dephosphorylation was monitored by the disappearance and appearance of the various phosphorus signals.

RESULTS AND DISCUSSION

While considerable evidence has pointed to the importance of (1) 2:1 metal:ATP ratios and (2) the presence of coordinated hydroxide ion in accelerating dephosphorylation reactions, little information is available on the catalytic capabilities of preformed dimers with associated hydroxides. The binuclear cobalt(III) complexes with the tridentate amine ligands form readily under basic conditions, and are tri-μ-hydroxo-bridged. In the presence of acid, one of the bridges is broken, forming aquo di-μ-hydroxo species and *cis-trans* isomerization can occur.

The rates of hydrolysis of ATP in the presence of the μ-hydroxo-bridged dimers were examined at various ratios of metal ion:ATP at 60°C (Table 1, Figure 1). Large rate enhancements were observed at pH 4.5 as long as at least a 2:1 metal:ATP ratio was maintained, indicating the importance of a dimeric species. The first order rate plots are biphasic, with an apparent saturation point followed by a considerably slower rate of dephosphorylation. Rates in Table 1 are given only for the initial fast reaction, which appears to be first order in ATP. These rates are almost comparable for the two complexes, e.g. 0.210 compared to 0.198 min^{-1} for a 1:1 dimer:ATP mixture of the dien and tcn complexes,

Table 1 First order rate constants for the hydrolysis of ATP and ADP (0.01 M) in the presence of cobalt(III) complexes at 60°C.

Substrate	*Complex*	*Dimer:Substrate*	*pH*	$k_{obs} \times 10^2$ *min*$^{-1}$
ATP	$Co_2(dien)_2(OH)_2$	0.5:1	4.5	0.2
ATP	$Co_2(dien)_2(OH)_2$	1:1	4.5	21.0
ATP	$Co_2(dien)_2(OH)_2$	2:1	4.5	22.2
ATP	$Co_2(dien)_2(OH)_2$*	1:1	7.0	3.8
ATP	$Co_2(dien)_2(OH)_3$*	1:1	10.0	0.3
ADP	$Co_2(dien)_2(OH)_2$	1:1	4.5	7.8
ATP	$Co_2(tcn)_2(OH)_2$	0.5:1	4.5	7.0
ATP	$Co_2(tcn)_2(OH)_2$	1:1	4.5	19.8
ATP	$Co_2(tcn)_2(OH)_2$	2:1	4.5	26.3
ATP	$Co_2(tcn)_2(OH)_2$*	1:1	7.0	1.0
ATP	$Co_2(tcn)_2(OH)_3$*	1:1	10.0	<0.1
ADP	$Co_2(tcn)_2(OH)_2$	1:1	4.5	7.1

*At higher pH's considerable tri-μ-hydroxo-bridged species is formed.

respectively, and 0.0078 and 0.0071 min^{-1} for ADP in the analogous study. The rates of hydrolysis of the 0.5:1 dimer:ATP ratio, i.e. one metal ion per ATP, are extremely slow for both ligand systems, 0.002 and 0.070 min^{-1} for dien and tcn, respectively.

The biphasic nature of the reaction is such that the reaction slows dramatically after approximately 10 min. This behavior suggests "poisoning" or decomposition of the catalyst. The most plausible explanation to account for this observation is the formation of an inert phosphate complex between the cobalt and inorganic phosphate. Additional aliquots of the dien dimer added during the slow portion of the reaction resulted in an increase in hydrolysis rate to 0.24 min^{-1}. To the contrary, hydrolysis is essentially halted if the starting solution has been incubated with a 1:1 molar ratio of inorganic phosphate:dimer before adding ATP. Both of these observations point to the formation of a substitutionally inert cobalt (III) phosphate complex. Further evidence for such a species was obtained from NMR and UV-vis spectroscopic data, as discussed below.

In addition to inorganic phosphate, AMP, ADP, and ATP resonances, several other downfield ^{31}P signals were observed for the quenched samples during the hydrolysis (Figure 2). Two are consistently present at +15 and +18 ppm. The two peaks at +15 and +18 ppm can be assigned to coordinated inorganic phosphate. This assignment is further substantiated by the observance of analogous resonances when the μ-hydroxo-bridged dimers are reacted with inorganic phosphate in the absence of ATP. The resonance at +15 ppm is assigned to monodentate coordinated phosphate, and agrees well with the +14 ppm observed for $(NH_3)_5CoOPO_3$.[8,9] These peaks disappear over a period of time in the presence of concentrated hydroxide ion. The resonance at +18 ppm is possibly due to a μ-bridging phosphate based on +17.5 ppm observed for $[(NH_3)_5Co_2(O_3POH)]^{4+}$.[8] These observations, in conjunction with the afore-cited spectral changes, serve

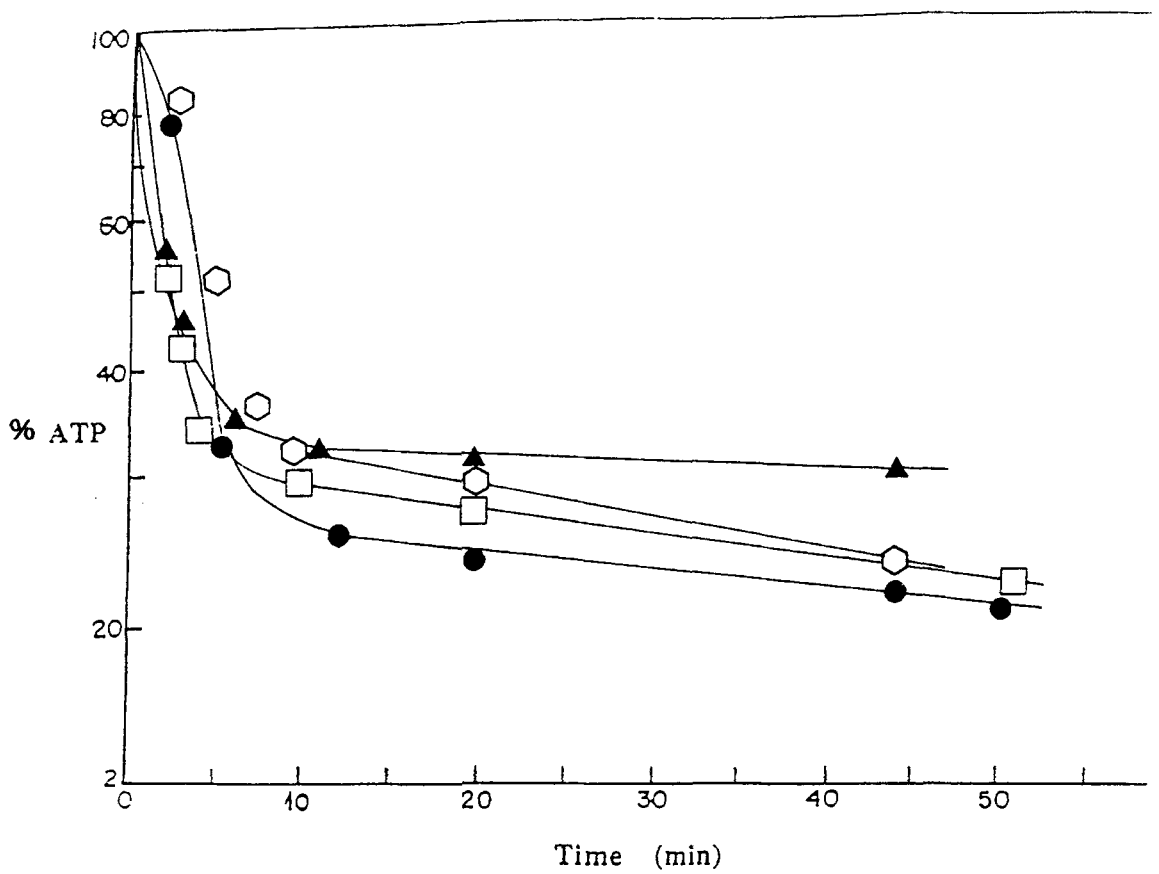

Figure 1. Hydrolysis of ATP (0.01) M by the cobalt dimers in different dimer:ATP mole ratios at pH 4.5 and 60 °C: (▲) $Co_2(dien)_2$:ATP, 1:1; (□) $Co_2(dien)_2$:ATP, 2:1; (○) $Co_2(tcn)_2$:ATP, 1:1; (●) $Co_2(tcn)_2$:ATP, 2:1.

to confirm that the potential catalyst poison is the hydrolysis product, P_i. No resonances were observed farther downfield (+20 to +40 ppm). Attempts to monitor the NMR spectra of unquenched samples were not successful primarily due to line broadening from trace amounts of cobalt(II). Similar findings have been cited by other workers.[2d] ^{59}Co NMR spectra of solutions of P_i and the Co(dien) dimer contain several signals indicating several different complexes are present in solution in agreement with the ^{31}P data.

The changes in the UV-visible spectra also provide strong indications pointing to cobalt-phosphate complexes. All of the metal complexes in the presence of either inorganic phosphate or nucleotide undergo a distinct color change from red to purple during the course of the reaction. The visible spectra of both dimers exhibit a broad absorption band with a λ_{max} between 520 and 530 nm, depending on the ligand. When the complexes are mixed in a 1:1 mole ratio with inorganic phosphate at a pH of 4.5 and heated at 50°C for 4 h, the absorption maximum in each case shifts to 534 nm. Under the same conditions with ATP, the absorption maxima exhibit shifts to 534 and 538 nm, for the tcn and dien complexes, respectively. The general spectral features of solutions of complex containing P_i and ATP are, therefore, quite similar.

The rates decrease at higher pH's. At pH 4.5 predominantly the di-μ-hydroxo species is in solution. At pH 7.0, however, considerable amounts of the tri-μ-hydroxo species is present; and at pH 10.0 the tri-μ-hydroxo complex predominates.[4] Rate retardations at higher pH can at least in part be attributed to the presence of more of the tri-μ-hydroxo species, which contains no easily accessible coordination sites.

A plausible mechanistic sequence for the hydrolysis reaction in the presence of μ-hydroxo-cobalt(III) complexes can thus be suggested which takes into account the known solution chemistry of these bridged complexes (Figure 3).

The apparent first order dependence on ATP of the fast initial reaction can be explained by immediate and complete complexation of ATP with the cobalt complex. Similar findings have been reported for other cobalt(III) complexes,[2d] as well as for polyammonium macrocycle-catalyzed hydrolysis of ATP.[10]

Once the initial complex of ATP and the cobalt dimer, **1**, is formed, *cis-trans* isomerization follows to place the nucleophile in position for attack; or, if the complex is

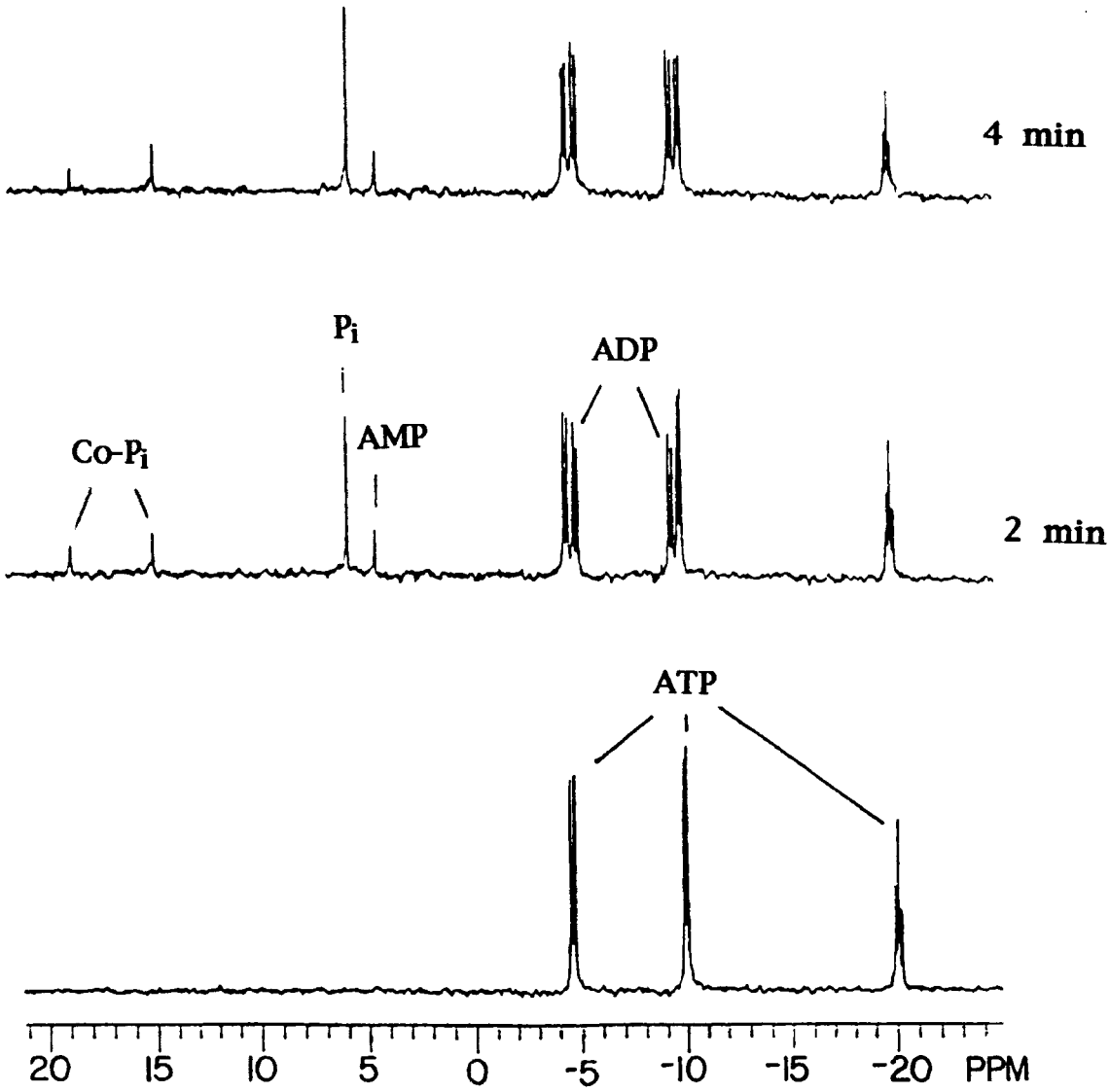

Figure 2. ^{31}P NMR of solutions of 0.01 M ATP and 0.01 M $Co_2(dien)_2$ at pH 4.5 and 60 °C.

already in the *cis* configuration of **3**, this step is not necessary. Previous studies have indicated that the *trans* form of the complex may be preferred in solution as it appears to be in the solid state.[4] The tcn complex shows a slight inductive period, which is over after the first two minutes, followed by the rapid linear disappearance of ATP, while the reaction starts essentially immediately for the dien dimer. The short induction period observed for the tcn complex could be due to a slower rate of isomerization, because of complex stabilization via the macrocyclic effect. An examination of the kinetics of the *cis-trans* isomerization reactions of these complexes has revealed that when progressing from monodentate to tridentate ligands, the rates of cobalt-hydroxo bond cleavage and formation increase considerably.[4] Furthermore, the macrocyclic tcn complex is 30 to 40 times slower in cleavage than the acyclic dien complex.

Rate studies performed in D_2O for a 1:1 complex:ATP mixture using the Co(tcn) dimer give a $k_H/k_D = 1.8$, which constitutes a normal isotope effect. The magnitude is consistent with the proposed deprotonation of coordinated water of **3** to give **4** prior to nucleophilic attack of the ATP. This is reasonable since most acids are weaker in D_2O than in H_2O by a factor of 3.[11] While deprotonation during the rate determining step is possible, a larger isotope effect would be expected (3.0–10.0) were that to be the case.

The hydrolysis step, **4**-**5**, is depicted as resulting from nucleophilic attack of a non-bridging coordinated hydroxide. Cleavage of one of the hydroxo bridges, followed by attack on the terminal phosphate appears unlikely, since the tcn complex undergoes bridge cleavage so much more slowly than the dien complex. A significantly greater rate differential between these two

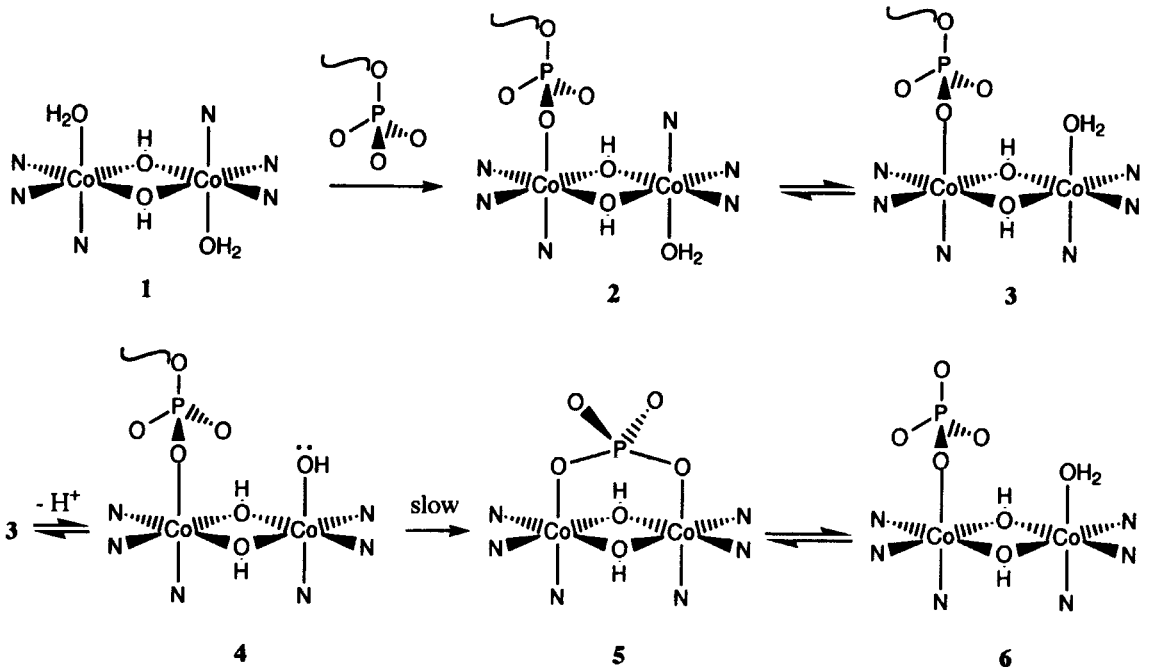

Figure 3. Proposed mechanistic pathway for hydrolysis of nucleotides by the binuclear cobalt(III) complexes. Charges and specific amine ligands have been omitted for clarity.

complexes would be anticipated if bridge cleavage were involved in the rate determining step.

The NMR spectra of the products, which indicate both bridging, **5**, and non-bridging, **6**, phosphates, is evidence in support of the last step of the Scheme. While the phosphate complex, **6**, can coordinate an additional ATP molecule, hydrolysis is essentially halted until the coordinated phosphate is replaced by a water molecule. Further breakdown to the monomeric species may occur at this point at pH 4.5, but after quenching, conversion should occur to the dimeric tri-μ-hydroxo-bridged forms.

A number of similarities are observed for these reactions compared to other monomeric cobalt(III) complex-ATP studies. These include cobalt(III) studies with preformed cobalt(III)-ATP or polyphosphate complexes to which additional aliquots of the same or different cobalt (III) complexes were added.[2] Rapid initial rates followed by rate retardation were observed.

In conclusion, therefore, the use of dimeric cobalt(III) species has provided additional insight to the chemistry of ATP hydrolysis. Most importantly, preformed dimers are indeed more efficient than their monomer analogs in catalyzing hydrolysis as long as readily accessible coordination sites are available. Furthermore, the deuterium isotope findings indicate that deprotonation is not involved in the rate determining step. Finally, the differences between the macrocyclic and acyclic ligand complexes point to the possibility of achieving fine-tuned steric controls in the hydrolysis of nucleotides.

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