Heavy-Atom Isotope Effects on Reactions of Co(III)-Bound *p*-Nitrophenyl Phosphate: Nucleophilic Displacements of *p*-Nitrophenol and Dissociation of *p*-Nitrophenyl Phosphate

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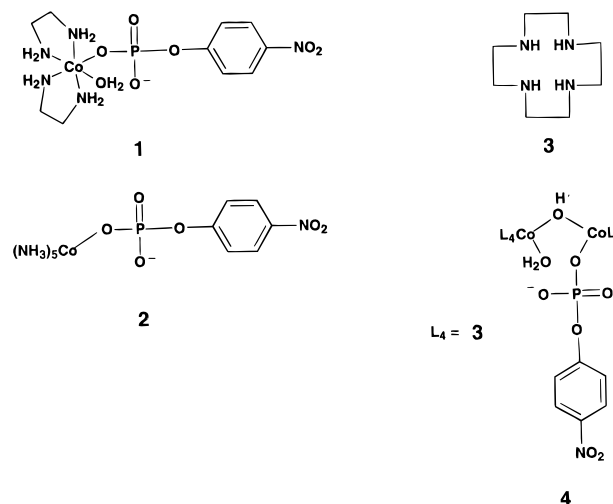
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Abstract: Heavy-atom isotope effects have been measured for the reactions of *p*-nitrophenyl phosphate (pNPP) in the stable Co complexes *cis*-[Co(en)$_2$(OH)pNPP] (**1**), Co(NH$_3$)$_5$pNPP (**2**), and a Co(cyclen) complex of pNPP (**4**) which forms reversibly in solution. The isotope effects in the nonbridge oxygen atoms ($^{18}k_{nonbridge}$), in the bridge oxygen atom ($^{18}k_{bridge}$), and in the nitrogen atom of pNPP (^{15}k) were measured. Complexes **1** and **2** undergo dissociation of pNPP in competition with nucleophilic attack to liberate *p*-nitrophenolate. The kinetic isotope effects on both processes were measured, and the theory and equations for the determination of isotope effects in parallel reactions are presented. With complex **4** the kinetic isotope effects for the nucleophilic reaction were measured, as well as the equilibrium isotope effects on formation of the complex between Co(cyclen) and pNPP. The isotope effects sensitive to bond cleavage, ^{15}k and $^{18}k_{bridge}$, for the nucleophilic reactions were respectively 1.0021 ± 0.0002 and 1.0213 ± 0.0012 for **1**, 1.0012 ± 0.0001 and 1.0098 ± 0.0010 for **2**, and 1.0016 ± 0.0002 and 1.0207 ± 0.0006 for **4**. All are indicative of substantial transition state bond cleavage in the rate-limiting step, and these results are most consistent with a concerted mechanism. The $^{18}k_{nonbridge}$ isotope effects of 1.0006 ± 0.0002, 1.0045 ± 0.0003, and 1.0057 ± 0.0009 with **1**, **2**, and **4** indicate that the transition state has a slight associative character. The kinetic $^{18}k_{nonbridge}$ isotope effects for dissociation of pNPP from **1** and **2** of 1.0135 ± 0.0004 and 1.0167 ± 0.0003 and the equilibrium one for **4** of 1.0081 ± 0.0006 are consistent with covalent character of the Co−O bond.

Introduction

Because of the central biological role of phosphate esters this class of compounds has been the object of considerable study. A number of enzymes catalyze the hydrolysis of phosphate esters at rates which are remarkable given the stability of the substrates in aqueous solution. Most of these enzymes require divalent metal ions, which can function both to coordinate the phosphate substrate and also to deliver a coordinated nucleophile.[1] Sargeson has studied several model systems in which a Co(III) complex serves both to coordinate *p*-nitrophenyl phosphate (pNPP) and to displace *p*-nitrophenolate ion via attack by an adjacent coordinated nucleophile. We have chosen three such systems, *cis*-[Co(en)$_2$(OH)pNPP]; (**1**; en = ethylenediamine),[2] [Co(NH$_3$)$_5$pNPP]$^+$ (**2**),[3] and a Co(cyclen) complex (cyclen = 1,4,7,10-tetraazacyclododecane) of pNPP (**4**) for a study to determine heavy-atom isotope effects in order to learn further details about the mechanisms of both the nucleophilic reaction and the cobalt−ligand dissociation, and to determine the transition-state structures. Isotope effect data have previously been measured for the uncatalyzed hydrolysis reactions

in solution[4a] and for several enzymatic reactions of pNPP,[4b] as well as for reactions of diesters and triesters where *p*-nitrophenol or its anion is the leaving group.[4c,d] These data provide a useful framework to interpret the results reported here with the Co-(III) complexes.

The positions of isotope effect measurement in the substrate are identified graphically in Figure 1. In phosphoryl transfer reactions the primary ^{18}O isotope effect in the bridging oxygen atom, $^{18}k_{bridge}$, gives an indication of the extent of bond cleavage to the leaving group. The secondary isotope effect in the nonbridge oxygen atoms, $^{18}k_{nonbridge}$, reveals the degree to which the transition state is dissociative (metaphosphate-like), or associative, resembling a pentavalent phosphorane. When the
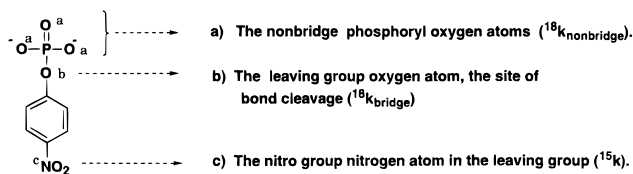
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Figure 1. The *p*-nitrophenyl phosphate substrate showing the positions where isotope effects were measured.

a) The nonbridge phosphoryl oxygen atoms ($^{18}k_{nonbridge}$).

b) The leaving group oxygen atom, the site of bond cleavage ($^{18}k_{bridge}$)

c) The nitro group nitrogen atom in the leaving group (^{15}k).

Figure 2. Diagrammatic representation of the preparation of the mixture used to measure ^{18}O isotope effects in pNPP (in this case $^{18}k_{bridge}$) using the remote label method, where the nitrogen atom in the nitro group serves as the remote label.

leaving group is *p*-nitrophenol or its anion, the ^{15}N isotope effect is sensitive to the amount of negative charge delocalized into the aromatic ring, thus giving a measure of the charge developed on the leaving group in the transition state. Together these isotope effects can give a detailed picture of the transition-state structure. In addition, since isotope effects will only be expressed on steps which are rate-limiting, they can give information about the kinetic mechanism of a reaction.

Experimental Section

Materials. Cyclen sulfate was obtained from Aldrich and recrystallized as the hydrochloride from 20% HCl after the sulfate was precipitated as barium sulfate. Alkaline phosphatase from *E. coli*, type III, from Sigma was used as received. Isotopically substituted versions of pNPP needed for isotope effect measurements were synthesized as previously described.[4]

Spectra. ^{31}P NMR were obtained in D_2O on a 500-MHz Bruker spectrometer, using 85% phosphoric acid as an external standard. In D_2O, pD was determined by converting the pH meter reading using the relationship pD = pH + 0.4. Proton spectra were recorded on a 200-MHz Bruker spectrometer using sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d_4* (TSP) as a standard. Visible spectra were recorded on a Cary 18 spectrophotometer.

Compound Synthesis. [Co(cyclen)Cl$_2$]Cl was synthesized by literature methods.[5] [Co(cyclen)PO$_4$] was synthesized following Hay's method,[6] starting with the [Co(cyclen)Cl$_2$]Cl instead of the bromide.

cis-[Co(en)$_2$(OH$_2$)pNPP]ClO$_4$·H$_2$O was synthesized with isotopically substituted Na$_2$pNPP by heating a solution that was 10 mM in [Co(en)$_2$Cl$_2$]Cl[7] and the desired Na$_2$pNPP, adjusted to pH 5.3, at 60 °C for 3 h. The solution was then chromatographed on Sephadex SP C25 that had been washed with 1 mM sulfuric acid, pH 3, and was eluted with 0.2 M NaClO$_4$. The second band contained the desired product. After concentration by rotary evaporation the pH was adjusted to 4 with dilute ammonia, and pink crystals formed after cooling on ice. Yield was 25%. This compound was also synthesized by Sargeson's method.[2]

[Co(NH$_3$)$_5$HpNPP](ClO$_4$)$_2$ was synthesized and purified in a similar fashion using [Co(NH$_3$)$_5$H$_2$O(NO$_3$)$_3$][8] as the starting material. However, the heating period was extended to 6 h.

Analysis of Cyclen Complexes. After converting the cyclen complex to the chloride by boiling in concentrated HCl, the compound was analyzed for cobalt by measuring the absorbance at 560 nm, using an extinction coefficient at 185 M^{-1}·cm^{-1}. Phosphate was analyzed by Carter's method.[9]

p-Nitrophenyl phosphate was quantitated by converting it to *p*-nitrophenol using alkaline phosphatase at pH 8.5 and then calculating the amount of *p*-nitrophenol from the absorbance at 400 nm in basic solution.

Reaction Kinetics. First-order rate constants for the hydrolysis of pNPP by the bis-ethylenediamine complex (pH 9) or the pentaammine complex (1 M NaOH) were obtained by plotting log(A_{400} at infinite

time minus A_{400} at time *t*) vs time, at a complex concentration of 0.1 mM. Initial rates for the hydrolysis of 0.5 mM pNPP by the cyclen complex were determined by varying the cobalt concentration from 0.0125 to 2.5 mM at pH 7.0.

Isotope Effect Measurements. The isotope effects in this study were measured by the competitive method, using an isotope ratio mass spectrometer. The ^{18}O isotope effects were measured by the remote label method.[10] In this technique substrate is synthesized with labels at two positions, one at the site of chemical interest and the other at a position which lends itself to facile isolation and isotopic measurement (the remote label). This double-labeled material is mixed with substrate containing only the natural abundance of ^{18}O in the position of interest, but depleted material in the remote label position (see Figure 2). The mixing ratio is such that the natural abundance of ^{15}N is restored in the remote label position. When this mixture is used in an experiment, the observed isotope effect is the product of that in the position of interest and that in the remote label position. The isotope effect in the latter position is determined with normal substrate containing only the natural abundance of isotopes in all positions, and the ratio of the two observed isotope effects is the desired one in the position of interest. With pNPP the nitrogen atom serves as a convenient remote label, as previously described.[4a] The observed isotope effects from these experiments were corrected for the ^{15}N effect and for incomplete levels of isotopic incorporation in the starting material.[4b]

Theory and Equations for Determination of Isotope Effects in Parallel Reactions. As shown in eq 1, the Co−pNPP complexes **1** and **2** undergo the parallel reactions of liberation of *p*-nitrophenol and of dissociation of pNPP, both of which are potentially isotopically sensitive. Following the widely adopted nomenclature of Northrop,[11] the isotope effects are denoted by a leading superscript ^xk, where *x* is 15 for ^{15}N isotope effects and 18 for ^{18}O effects. For ^{18}O isotope effects determined by the double-label method $x = 15, 18$ and $^{15,18}k$ is the isotope effect due to the presence of both heavy atoms in the substrate. In the equation below, k_1 denotes the rate constant for the light isotope and $^xk_1$ denotes the isotope effect on the rate constant k_1, which is the rate constant of the light isotopically labeled compound divided by that of the heavy. Thus the expression $k_1/^xk_1$ equals the rate constant for the heavy labeled compound.

$$A \xrightarrow{k_1} P = \text{free pNPP (from dissociation of pNPP from Co)}$$
$$A \xrightarrow{k_2} Q = \text{p-nitrophenol (from cleavage of Co-bound pNPP)} \tag{1}$$
$$A = \mathbf{1} \text{ or } \mathbf{2}$$

$$A_x \xrightarrow{k_1/^xk_1} P_x$$
$$A_x \xrightarrow{k_2/^xk_2} Q_x$$

The experimental quantities that need to be determined are the following: $R_p = Q_x/Q =$ the $^{15}N/^{14}N$ ratio in liberated *p*-nitrophenol at a known fraction of reaction *f*. $R_s = (Q_{x\infty} - Q_x)/(Q_\infty - Q) =$ the $^{15}N/^{14}N$ ratio in unreacted Co−pNPP at a known fraction of reaction *f*.

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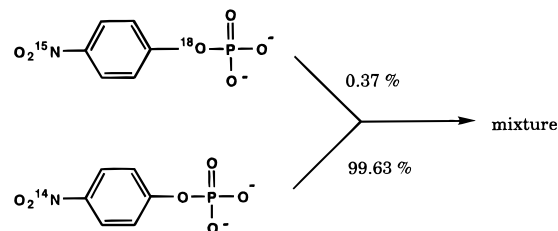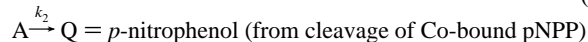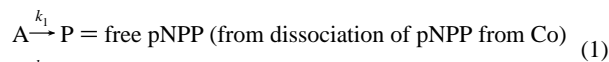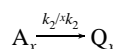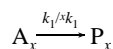(9) Carter, S. G.; Karl, D. W. *J. Biochem. Biophys. Methods* **1982**, *7*,

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$R_\infty = Q_{x\infty}/Q_\infty =$ the $^{15}N/^{14}N$ ratio in *p*-nitrophenol after complete reaction of Co−pNPP. $R_{p\infty} = P_{x\infty}/P_\infty =$ the $^{15}N/^{14}N$ ratio in pNPP dissociated from the Co complex.

The integrated equations describing the concentrations of the various molecules at a fraction of reaction *f*, or at infinite time, are as follows:

$$A = A_o e^{-(k_1+k_2)t}; \quad A_x = A_{xo}e^{-k't}$$

where

$$k' = \frac{k_1}{{}^x k_1} + \frac{k_2}{{}^x k_2}$$

$$Q = \frac{k_2 A_o(1 - e^{-(k_1+k_2)t})}{k_1 + k_2}; \quad Q_x = \frac{k_2 A_{xo}(1 - e^{-k't})}{{}^x k_2 k'}$$

$$P = \frac{k_1 A_o(1 - e^{-(k_1+k_2)t})}{k_1 + k_2}; \quad P_x = \frac{k_1 A_{xo}(1 - e^{-k't})}{{}^x k_1 k'}$$

The experimentally determined isotope ratios can be used to determine an isotope effect starting with the following two equations which yield an apparent isotope effect: ${}^x k_{app} = \ln(1 - f)/\ln(1 - fR_p/R_\infty) = \ln(1 - f)/\ln[(1 - f)(R_s - R_\infty)]$

$${}^x k_{app} = \frac{{}^x k_2(1 + k_1/k_2)}{1 + \dfrac{k_1 {}^x k_2}{k_2 {}^x k_1}} = \frac{{}^x k_2(k_1 + k_2)}{k_2 + k_1({}^x k_2/{}^x k_1)}$$

When the ratio k_1/k_2 is known, the above isotope effect can be used along with the ratio of ${}^x k_2/{}^x k_1$ to determine ${}^x k_1$ and ${}^x k_2$. The ratio of isotope effects ${}^x k_2/{}^x k_1$ is equal to $R_{p\infty}/R_\infty$.

The molecular nitrogen used for isotopic analysis was obtained by combustion of samples as previously described.[4] The nitrogen isotopic ratio in the starting material was determined from nitrogen obtained from combustion of samples of *p*-nitrophenyl phosphate substrate and, as a control, by completely hydrolyzing samples of the substrate and combustion of the nitrophenol produced and isolated by the same method used in the isotope effect experiments. The isotopic ratios obtained from both methods were the same within experimental error, showing that no isotopic fractionation occurs during the procedures used to recover *p*-nitrophenol.

(a) Kinetic isotope effect experiments. For **1**, 2.5 mM solutions of cis-[Co(en)₂(OH₂)pNPP]ClO₄·H₂O were used at pH 9, in a 0.2 M Tris−perchlorate buffer, with NaClO₄ added so that that ionic strength was 1.0 M. Fifty milligrams of [Co(en)₂(OH₂)pNPP]ClO₄·H₂O were dissolved in 30 mL of water at 25 °C. Reaction was initiated by the addition of a solution of 10 mL of 0.8 M Tris−perchlorate, pH 9.0, with added sodium perchlorate. Reaction was stopped after about 50% completion by acidifying to pH 2 with HCl. This solution was extracted with ether (3 × 40 mL), and the combined ether portions were washed with 2 M NaOH (3 × 40 mL). The alkaline aqueous solution, containing the *p*-nitrophenol product, was acidified with HCl and extracted with ether (3 × 40 mL). These ether extracts were dried over MgSO₄, the ether was removed by rotary evaporation, and the *p*-nitrophenol was sublimed under vacuum prior to combustion for isotopic analysis. The aqueous solution from the first extraction, which contained the unreacted **1** (as well as dissociated pNPP), was titrated to pH 9 with 1 N NaOH and allowed to react for at least 10 half-lives. This solution was then acidified and the *p*-nitrophenol released from the residual **1** was isolated as described above. The complete hydrolysis experiments were performed using 30 mg of **1** in 15 mL of water, adding 5 mL of the Tris−perchlorate/sodium perchlorate solution, and allowing reaction to proceed for 3.5 h (> 10 half-lives). These samples were then acidified and the *p*-nitrophenol was isolated as described above. The total conversion experiments were set up in an identical manner, and after 3.5 h alkaline phosphatase was added to hydrolyze released pNPP prior to isolation of *p*-nitrophenol. Several experiments to look directly at the pNPP which dissociated from **1** were also

performed. These used 160 mg of **1** in 48 mL of water, which was mixed with 16 mL of 0.8 M Tris−perchlorate, pH 9, with added sodium perchlorate. After 3.5 h the samples were acidified and extracted with ether. The pH of the aqueous solution was adjusted back to 9 with NaOH, and alkaline phosphatase was added. After 12 h the reaction were acidified and the *p*-nitrophenol was isolated as described.

For **2**, 5.3 mM solutions of [Co(NH₃)₅HpNPP](ClO₄)₂ were used in 1 M NaOH, which partially ionizes an ammonia ligand forming the attacking amide. A solution of the pentaammine in water (112 mg, 200 μmol, in 32 mL) at 25 °C was mixed with 8 mL of 5 M NaOH to initiate reaction. The reaction was stopped after about 1 half-life by addition of 8 mL of 6 M HCl. The solution was extracted with ether (3 × 40 mL), and the combined ether extracts were dried over MgSO₄ and concentrated to dryness by rotary evaporation. The *p*-nitrophenol thus isolated was sublimed under vacuum prior to combustion for isotopic analysis. The remaining aqueous solution, containing unreacted **2** and dissociated pNPP, was made 1 N in NaOH using 5 N NaOH. After 10 half-lives (160 min) the solution was acidified, filtered to remove brown cobalt precipitates, and extracted with ether as before. The aqueous solution, still containing dissociated pNPP, was adjusted to pH 9 with 1 M NaOH and made 1 mM in Zn and Mg using ZnCl₂ and MgCl₂, and alkaline phosphatase was added. After 12 h the solution was acidified to pH 4 with HCl and extracted with ether as described. For isolation of *p*-nitrophenol from total reaction of the Co complex, reactions were performed with 100 μmol of **2** in 16 mL of water, and 4 mL of 5 M NaOH. These were allowed to react for 3.5 h before acidification and isolation of *p*-nitrophenol.

For the kinetic isotope experiments with **4**, the [Co(cyclen)Cl₂]⁺ and pNPP solutions were made separately, equilibrated at pH 7, 25 °C, and then mixed to give a solution 20 mM in cobalt and 5 mM in pNPP. Since buffers have been reported to catalyze the dimerization of the complex, the reactions were performed in unbuffered solution. Reactions were stopped by acidification to pH 4 with HCl, and *p*-nitrophenol was isolated as previously described.

(b) Trapping [Co(cyclen)]₂pNPP and Equilibrium Isotope Effect Experiments. The [Co(cyclen)Cl₂]Cl and Na₂pNPP solutions were separately adjusted to pH 6.0 and cooled to 15 °C. After mixing, the solution was equilibrated for 15 min at 15 °C and then chromatographed at 4 °C on Sephadex SP C-25, which had been equilibrated at pH 3. Identical results were obtained if the solutions were preincubated for 20 or 30 min. The column was washed with water, which removed any hydrolyzed or unbound pNPP, and then eluted with 0.2 M NaCl. The water wash was adjusted to pH 8.5, and the amount of hydrolyzed pNPP was determined from absorbance at 400 nm. The amount of unbound pNPP was quantitiated by adjusting the pH of the water wash to 9 and adding 5 units of alkaline phosphatase. The cobalt-bound pNPP in the 0.2 M NaCl eluant was converted to *p*-nitrophenol by raising the pH of the solution to 7. If necessary, the pH was adjusted to 8.5 and alkaline phosphatase was added to ensure completeness of reaction. The solutions used for the equilibrium isotope effect experiments were 9 mM in both the cobalt complex and pNPP.

Results

Characterization of Co−pNPP Complexes. When *cis*-[Co-(en)₂(OH₂)pNPP]⁺ which was synthesized by direct reaction of the cobalt precursor with Na₂pNPP and the complex that was synthesized by Sargeson's method[2] were compared, it was found that they had the same visible spectra (λ_{max} 507 nm) and ^{31}P NMR spectra and that k_{obs} values for formation of *p*-nitrophenol at pH 9 were in close agreement (7.5×10^{-4} vs 9.0×10^{-4} s^{-1}).

[Co(NH₃)₅pNPP]⁺ synthesized by direct reaction also had the same visible spectrum (λ_{max} at 517 nm) and similar k_{obs} (1.0×10^{-3} vs 8.2×10^{-4} s^{-1} in 1 M NaOH) as reported for this complex synthesized by a different route.[3]

Isotope Effect Experiments with 1 and 2. Both the *cis*-[Co(en)₂(OH)pNPP] and the [Co(NH₃)₅pNPP]⁺ complexes undergo release of pNPP from the Co complex by Co−O bond rupture in competition with nucleophilic attack at phosphorus to release *p*-nitrophenolate. Under the reaction conditions

Table 1. Isotope Effects for Reactions of Co(III)-Coordinated pNPP

reaction	^{15}k	$^{18}k_{bridge}$	$^{18}k_{nonbridge}$
Kinetic Isotope Effects for Nucleophilic Reactions Releasing *p*-Nitrophenol			
Co(en)$_2$pNPP	1.0021 ± 0.0002	1.0213 ± 0.0012	1.0006 ± 0.0002
Co(NH$_3$)$_5$pNPP	1.0012 ± 0.0001	1.0098 ± 0.0010	1.0045 ± 0.0003
[Co(cyclen)]$_2$pNPP	1.0016 ± 0.0002	1.0207 ± 0.0006	0.9976 ± 0.0003^a
			1.0057 ± 0.0009^b
Kinetic Isotope Effects for Dissociation of pNPP			
Co(en)$_2$pNPP	0.9998 ± 0.0002	1.0063 ± 0.0012	1.0135 ± 0.0004
Co(NH$_3$)$_5$pNPP	0.9998 ± 0.0001	1.0011 ± 0.0010	1.0167 ± 0.0003
Equilibrium Isotope Effects for Dissociation of pNPP			
[Co(cyclen)]$_2$pNPP	1.0004 ± 0.0004	0.9995 ± 0.0011	1.0081 ± 0.0006

[a] Isotope effect uncorrected for the equilibrium effect on formation of the Co complex. [b] Isotope effect after correction for the equilibrium effect on formation of the Co complex.

Table 2. Compositions of Initial Mixtures and Isolated Products from Chromatography of the Co(cyclen):pNPP Complex[a]

initial solution[b]		water wash[c]	isolated complex[d]		ratio:
CoN$_4$	pNPP	free pNPP	CoN$_4$Pi	bound pNPP	Co:pNPP
64.7	16.2	1.4	23.0	11.0	2.1
63.3	30.7	4.6	39.0	19.1	2.0
59.0	62.3	33.6	54.0	22.5	2.4
15.9	379.0		14.4	7.6	1.9

[a] All quantities is the table are in μmol. The missing amounts of pNPP and Co remain bound to the column after elution with water followed by 0.2 M NaCl. [b] Initial composition of solution as loaded on the cation-exchange column. [c] Quantity of uncomplexed pNPP, eluted by water wash. [d] Relative amounts of Co and pNPP in the Co(cyclen):pNPP complex eluted with 0.2 M NaCl.

Figure 3. Initial rates for release of *p*-nitrophenol from 0.5 mM pNPP as a function of Co(cyclen) concentration at pH 7.0.

employed the released pNPP does not undergo measurable hydrolysis during the reaction times employed. In the case of *cis*-[Co(en)$_2$(OH)pNPP] 89% of the complex underwent cleavage to release nitrophenol, with the remainder of the pNPP being released into solution. The [Co(NH$_3$)$_5$pNPP]$^+$ complex undergoes both processes at similar rates, with nitrophenol release comprising 48% of the reaction. These product ratios are very similar to the 86% and 47% reported by Sargeson.[2,3] Since release of pNPP from the complex will be sensitive to isotopic substitution in the nonbridge oxygen position (and possibly other positions as well), equations were derived for the calculation of the isotope effects on the two simultaneously occurring processes (see Experimental Section). The isotope effects at each of the three positions in the substrate (as shown in Figure 1) for *p*-nitrophenol cleavage and for Co–O rupture are listed in Table 1. The standard errors are calculated from at least six independent measurements of each isotope effect.

Isotope Effect Experiments with 4. The kinetic isotope effects for the nucleophilic reaction and the equilibrium effects for dissociation of pNPP from the complex are shown in Table 1. The observed effects for the nucleophilic reactions are the product of the kinetic effects on the nucleophilic reaction and the equilibrium ones for *formation* of the complex. The latter will be the reciprocal of the effects for dissociation shown in Table 1. Since ^{15}k and $^{18}k_{bridge}$ are unity within experimental error, only $^{18}k_{nonbridge}$ has been corrected for formation of the complex; both the observed and corrected effects are shown.

Reaction of [Co(cyclen)Cl$_2$]$^+$ + pNPP (pH < 6.0). A complex between [Co(cyclen)Cl$_2$]$^+$ and pNPP was formed at pH <6, which underwent only very slow hydrolysis (<5% in 30 min). The complex formed rapidly, within the time required to record an NMR spectrum. The ^{31}P NMR spectrum of a solution made with a cobalt to pNPP ratio of 3.4:1 showed one large phosphorus peak at 7.7 ppm and a small amount of unbound pNPP at −1.1 ppm. The proton NMR in the aromatic region showed two doublets at 8.18 and 7.24 ppm. If the Co to pNPP ratio was changed to 1:6.3, a similar ^{31}P spectrum was seen, except that the unbound pNPP signal was much larger.
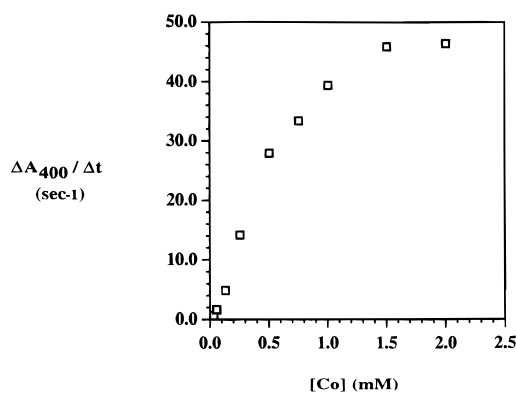
The complex could be isolated by putting this solution on a cation-exchange column. One purple band containing pNPP was eluted with 0.2 M NaCl. The band chromatographed faster than [Co(NH$_3$)$_5$H$_2$O]$^{3+}$ and had Co:pNPP in a 2:1 ratio, even when a large excess of pNPP was used (Table 2). Other cobalt-containing species remained bound to the column and were difficult to remove, even using 2 M NaCl.

The 2:1 Co(cyclen):pNPP species, after chromatography, was not completely active, with a small amount of it unable to liberate *p*-nitrophenol. (This could be released by adjusting the

pH to 8.5 and adding alkaline phosphatase, and thus presumably represents dissociated pNPP.) The purple band had λ_{max} at 520 nm at pH 5.

[Co(cyclen)Cl$_2$]$^+$ + pNPP (pD = 7.4). If the 3.4 Co:1 pNPP, pD 5.5 solution was adjusted to pD 7.4, the ^{31}P NMR spectrum showed transient signals at 13.3 and 8.5 ppm. As the reaction proceeded, signals at 31.2 and 38.1 ppm appeared. After 2 h, these were the only signals present, with the peak at 31.2 ppm predominating.

If the reaction mixture was then adjusted to pD 3.4, the pH used for the cation exchange column, a peak at 19.2 ppm developed, and the peak at 31.2 ppm decreased relative to the peak at 38.2 ppm.

Initial rate data for this complex showed a nonlinear dependence on cobalt concentration (Figure 3). At low Co:pNPP ratios, the reaction was very slow and did not go to completion.

{[Co(cyclen)]$_2$PO$_4$}. After completion of the reaction, a complex containing Co^{3+} and inorganic phosphate in a 2:1 ratio could be isolated from the reaction mixture by chromatography on Sephadex SP C25. This complex had a visible absorption maxima at 531 nm and a single ^{31}P NMR peak at 19.2 ppm at pD 3.4. The NMR signal shifted to 21.3 ppm at pD 5.4. [Co(cyclen)PO$_4$], prepared by the literature method, after chromatography on Sephadex SP C25 showed a ^{31}P NMR spectrum with a peak at 21.1 ppm at pD 5.45. The peak shifted to 19.1 ppm at pD 2.9. The complex had a visible spectra with λ_{max} at 532 nm at pH 3.5. Analysis of this complex also showed a 2 Co:1 phosphate ratio.

Discussion

Characterization of Complexes. Monomeric and dimeric cobalt complexes containing ammine ligands are well-known. The monomers can be distinguished from the dimers by the size of the rate constant for the hydrolysis reaction. Dimers

Scheme 1
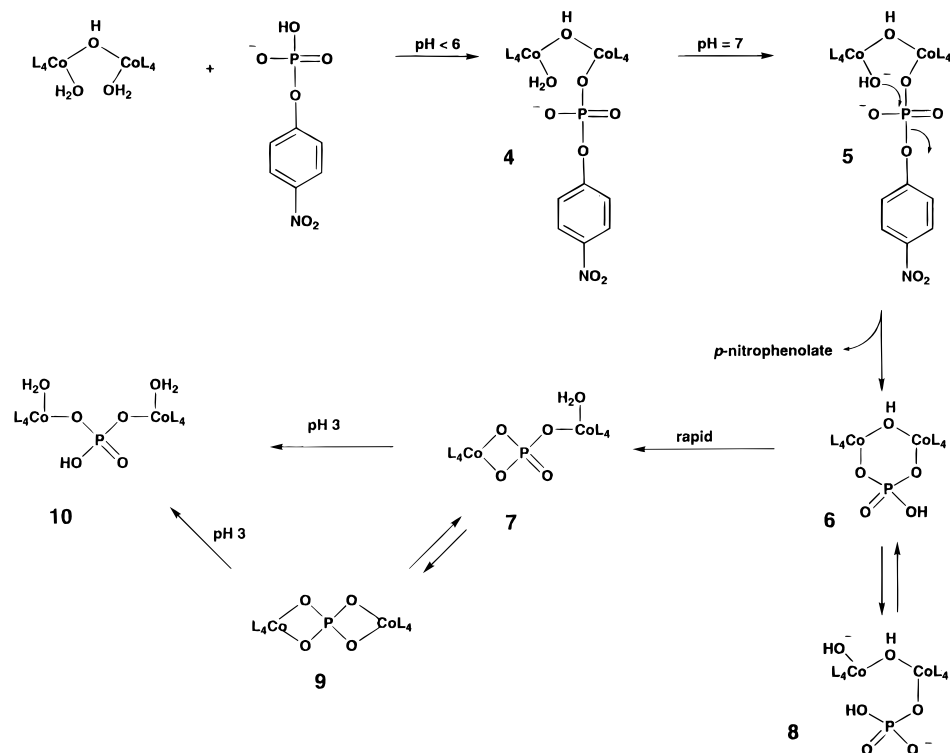
also show distinctive ^{31}P NMR signals. The dimeric cobalt complex {[(NH$_3$)$_5$Co]$_2$pNPP}(ClO$_4$)$_4$[12] has been synthesized and contains pNPP that is coordinated to both cobalt ions. Under basic conditions, one NH$_3$ deprotonates and then attacks the phosphorus, giving *p*-nitrophenolate and a phosphoramidate that is chelated through N and O to one cobalt and is also bonded to the other Co(NH$_3$)$_5$ moiety. The rate constant for formation of *p*-nitrophenolate is approximately 100 times greater than the corresponding rate constant for the monomeric complex.[12] Another compound that has been synthesized,[13] {[Co(en)$_2$(μ-pNPP)]$_2$}$^{2+}$, contains two pNPP ligands that both bridge the two cobalts. Reaction involves rapid displacement of one end of a bridged pNPP by hydroxide and then attack of this coordinated hydroxide on the remaining bridging pNPP. The rate constant for the fast release of *p*-nitrophenolate in the dimer reaction is 26 times larger than k_{obs} for the monomer reaction.[13]

The rate constants for the isotopically substituted complexes that were synthesized by direct heating of the cobalt precursor with the isotopically substituted pNPP are in agreement with the rate constants expected for the monomeric compounds. ^{31}P NMR and visible spectra are also consistent with an assignment of the ethylenediamine and pentaammine complexes used here as monomeric species.

The analytical data for the cyclen complex, however, indicate that it has a dimeric structure with a 2 Co:1 pNPP ratio. The formation and fate of this complex are described in Scheme 1. The initial species at pH <6 has a ^{31}P NMR signal at 7.7 ppm that is characteristic of a pNPP ligand that has replaced one water molecule in the cobalt coordination sphere and is monodentate.[13] Unlike the dimeric ethylenediamine complex, with its 2 Co:2 pNPP ratio, the NMR spectrum is not consistent with a structure in which the 2 cobalt ions in the complex are bonded to each other through a bridging pNPP. A bridging pNPP would have an NMR signal around 13 ppm which is not

seen for solutions of the cyclen complex at pH <6. The cobalt atoms in the cyclen complex are most probably bonded to each other through a bridging hydroxide (**4**).

When the pH is raised to 7, the rate of the reaction increases as the water molecule on the cobalt not bound to pNPP becomes deprotonated (**5**) and attacks the pNPP, releasing *p*-nitrophenolate and forming a bond to the phosphorus atom. Although no NMR signal is found for this bridging phosphate that has one bond to each cobalt (**6**), an NMR signal at 31 ppm is observed during the cyclen reaction that is close in position to the 33-ppm signal seen during the reaction of the dimeric ethylenediamine complex which was assigned as a triply bonded phosphate ion in a cobalt dimer.[13] Thus, we propose that species **6** rapidly reacts to form the triply bonded phosphate complex **7**. Although the ethylenediamine system does give a somewhat stable doubly bonded bridging phosphate species, with an NMR signal at 20.97 ppm, the extra stability of this doubly bonded species in the ethylenediamine system may be due to the presence of the extra pNPP ligand occupying one coordination position. A small NMR signal at 13.3 ppm in the cyclen system indicates that the doubly bridged phosphate species may also break down to give a small amount of the transient monodentate phosphate species **8**.

An NMR signal at 38.1 ppm is also observed during the cyclen reaction that can be assigned to a phosphate ion that bridges two cobalts, with all four O atoms coordinated by cobalt, **9**. A dimeric Co−tris(aminopropyl)amine (Co−trpn) complex with quadruply bridged inorganic phosphate has been characterized by X-ray[14] and shows a ^{31}P NMR signal at 40.5 ppm.[13]

As reaction nears completion, the solution contains only a mixture of triply and quadruply bonded phosphate in a cobalt dimer. If the pH is lowered, a new signal at 19.3 ppm was observed. This is consistent with the loss of one or two Co−

(12) Henry, P.; Sargeson, A. M. *Inorg. Chem.* **1990**, *29*, 92−97.
(13) Jones, D. R.; Lindoy, L. F.; Sargeson, A. M. *J. Am. Chem. Soc.* **1984**, *106*, 7807−7819.

(14) Connolly, J. A.; Banaszczyk, M.; Hynes, R. C.; Chin, J. *Inorg. Chem.* **1994**, *33*, 665−669.
(15) Chin, J.; Banaszczyk, M. *J. Am. Chem. Soc.* **1989**, *111*, 4103−4105.

phosphate bonds under acidic conditions, resulting in a doubly bonded phosphate that has one bond to each cobalt ion (**10**). Loss of chelate structure has routinely been observed under acidic conditions.[6]

The same ^{31}P NMR species (19.2 ppm at pH 3) is seen for the 2 Co:1 P_i (P_i = inorganic phosphate) complex isolated by chromatography from the cyclen reaction mixture. Presumably, with the chromatography under somewhat acidic conditions, enough time has elapsed for the phosphate to break the third or fourth bond to the cobalt ion. The spectrum of this species at pD 5 (21.3 ppm) is consistent with the spectra reported for the bidentate phosphate-bridged Co^{3+}-ethylenediamine dimers (20.97 ppm).[13] The [Co(cyclen)P_i] complex, although reported to be monomeric, after chromatography on Sephadex, also shows a similar pH-dependent NMR spectrum and analyzes for 2 Co per phosphate.

The kinetic data for the cyclen complex, which show a nonlinear dependence on cobalt concentration, are also consistent with a dimeric structure, and the high reactivity of the cyclen complex at pH 7 may well result from this dimeric structure. Thus the mechanism proceeds by releasing *p*-nitrophenol and forming a phosphate that is bidentate bridging two cobalt atoms, with a subsequent Co−O bond rapidly formed to break the hydroxo bridge and generate the triply bonded phosphate. For some of these triply bonded molecules, the fourth O atom also becomes coordinated, in a structure that must resemble the [Co-(trpn)]$_2$$P_i$ complex.

The rate of formation of the pNPP complex seems to be rapid, within the time required to record an NMR spectrum. Although Co^{3+} species are known as "substitution inert", the rapid formation of the pNPP complex is consistent with the observed rate of substitution of water by phosphate ion in the cyclen complex.[16] Thus, although some work on [Co(en)$_2$(OH)OH$_2$)]$^{2+}$ complexes indicate that the rate of substitution of the cobalt complex by several diesters may be partially limiting in the hydrolysis reaction,[17] the substitution rate with the cyclen complex seems to be fast enough that the rate-limiting step is the nucleophilic reaction.

Isotope Effects on the Dissociation Reactions. The bond between the Co atom and the coordinated nonbridge oxygen atom of the pNPP moiety is sensitive to isotopic substitution. In the dissociation reactions of the complexes **1** and **2** the nonbridge isotope effect is a primary one, and the nonbridge isotope effects in these cases, 1.35% and 1.67%, respectively, are kinetic ones for cleavage of the Co−O bond. The cyclen complex was formed reversibly in solution, and the isotope effect of 0.81% for the formation of this complex is an equilibrium effect. Contributions from reaction coordinate motion in the primary effects presumably account for their larger magnitudes, although differences between the Co−O bond resulting from differing Co ligands may also account for some of this difference. The most interesting aspect of these data is the fact that they indicate that these Co−O bonds have significant covalent character. This contrasts with the purely electrostatic interactions of metal ions like Mg^{2+} with phosphate groups, which do not cause significant isotope effects.[18] The IR spectra of Co^{3+} complexes have been interpreted as indicating covalent character in the coordination bonds.[19]

As expected, none of the dissociation reactions give rise to measurable ^{15}N isotope effects in the nitrophenol leaving group. However, there are measurable effects in the phenolic oxygen position with **1** and **2**; these are 0.63% for **1** and 0.11% for **2**. These secondary effects arise partly from differences in the bridging P−O bond as the phosphate is converted from the equivalent of a diester to a monoester.[20] Another possible contribution to this isotope effect is the partial loss of the Co−O−P−O torsional vibration in the transition state of the dissociation reaction. The smaller value with the pentaammine complex **2** suggests an earlier transition state for dissociation of the complex with less Co−O bond cleavage compared with that for the Co(en)$_2$ complex.

The isotope effects in Table 1 for dissociation of the Co-(cyclen)pNPP complex are equilibrium values, in contrast to those for complexes **1** and **2** which are kinetic effects. The ^{15}N and bridge oxygen isotope effects are unity within experimental error, the only significant isotope effect being the $^{18}k_{nonbridge}$ effect of 0.81%.

Nucleophilic Reactions Liberating Nitrophenol. Due to the covalent character of the Co−O bond in these complexes, pNPP exists as a species that probably more nearly resembles a diester than it does the solution structure of pNPP. This is an important distinction because of the different nature of the reaction pathways followed by phosphoryl transfer reactions of monoesters as compared with diesters. In aqueous solution monoesters hydrolyze via a concerted process where bond formation to the nucleophile and bond cleavage to the leaving group occur in the same step, with a highly dissociative transition state with bond cleavage far advanced and bond formation very small.[21] Diesters exhibit more associative transition states and greater nucleophilic participation in the transition state. Both linear free energy relationships and isotope effects indicate that diesters with good leaving groups (such as *p*-nitrophenol) undergo hydrolysis by concerted mechanisms.[4c,22] Phosphodiesters with less labile leaving groups may react via phosphorane intermediates, particularly under acidic conditions. Triesters have even more associative transition states. A useful diagnostic tool to determine the dissociative versus associative nature of phosphoryl transfers is the nonbridge ^{18}O isotope effect. Measurements with monoesters, diesters, and triesters with *p*-nitrophenol as the leaving group show the expected trend, and are slightly inverse (from near unity to 0.5% inverse) in the dissociative monoester reactions, slightly normal (near unity to 0.5% normal) for diesters, and larger and normal (0.63%) for the triester diethyl *p*-nitrophenyl phosphate. A larger nonbridge ^{18}O isotope effect of 2.5% found in the triester with the poorer leaving group *p*-carbamoylphenol is indicative of the expected later, more associative transition state.[4d]

Significant isotope effects in the *p*-nitrophenol leaving group (^{15}k and $^{18}k_{bridge}$) will be observed if bond cleavage occurs in the rate-limiting step, and the magnitudes are indicative of the extent of bond cleavage in the transition state. An ^{15}N effect should arise only if measurable P−O bond cleavage to nitrophenol occurs, as a result of the partial negative charge arising from bond cleavage which delocalizes into the aromatic ring. Interpretation of the primary ^{18}O isotope effect in terms of precise degrees of bond cleavage is complicated by contributions from reaction coordinate motion, but this isotope effect is smaller

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(17) Chin, J.; Zou, X.; Chin, J.; Banaszczyk, M. *J. Am. Chem. Soc.* **1988**, *110*, 223−225.

(18) Jones, J. P.; Weiss, P. M.; Cleland, W. W. *Biochemistry* **1991**, *30*, 3634−3639.
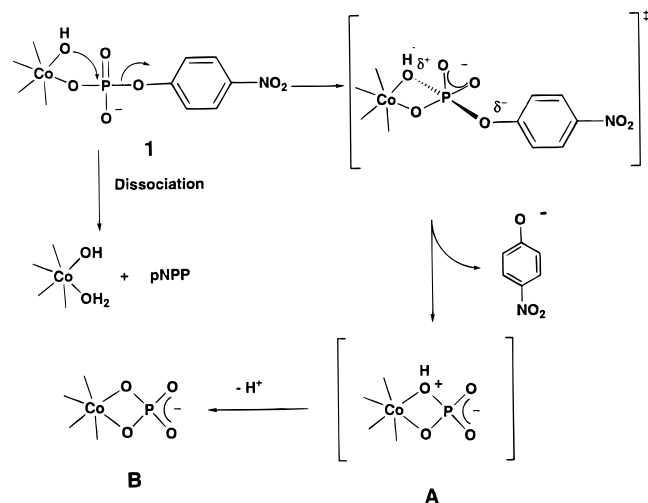
(19) Nakamoto, K. *Infrared and Raman Spectra of Inorganic and Coordination Compounds*; Wiley: New York, 1986; pp 193−205.

(20) Ray, W. J.; Burgner, J. W.; Deng, H.; Callender, R. *Biochemistry* **1993**, *32*, 12977−12983.

(21) Bourne, N.; Williams, A. *J. Am. Chem. Soc.* **1984**, *106*, 7591−7596. Skoog, M. T.; Jencks, W. P. *J. Am. Chem. Soc.* **1984**, *106*, 7597−7606. Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1989**, *111*, 7579−7586.

(22) Davis, A.; Hall, A. D.; Williams, A. *J. Am. Chem. Soc.* **1988**, *110*, 5105−5108.

Scheme 2

Scheme 3

Scheme 4

in the triester reaction (0.6%) than in monoester reactions (around 2%) as expected from the mechanisms. This effect varies considerably in diester reactions from 0.5% to 3%.[23]
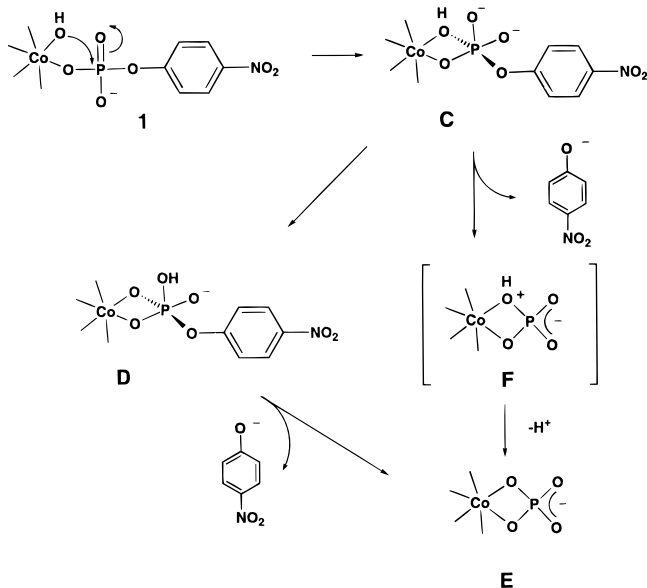
The isotope effects for the nucleophilic reactions of complex **1** will be considered first. The leaving group isotope effects of 0.21% for ^{15}k and 2.13% for $^{18}k_{bridge}$ are both large and indicate that this P–O bond is largely cleaved in the transition state of the rate-limiting step. The small normal value of 0.06% for $^{18}O_{nonbridge}$ indicates at most minimal associative character for the transition state.

Two limiting mechanistic possibilities for reaction of **1** exist. One possibility is that the complex reacts via nucleophilic attack simultaneous with leaving group departure in a concerted mechanism, followed by rapid deprotonation to give **B** (Scheme 2). The acidity of the proton on the Co-bound hydroxide will increase rapidly with bond formation between this oxygen atom and phosphorus, and proton loss may in fact be simultaneous with nucleophilic attack. In any event species **A** should have no appreciable lifetime at pH 9 where the reaction was studied. The isotope effects indicate a large degree of bond cleavage to the leaving group, and minimal associative character as measured by the isotope effect in the nonbridge oxygens.
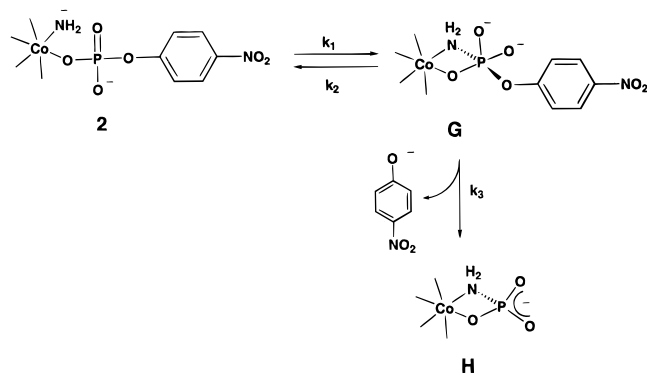
The alternative possibility is an associative mechanism via a phosphorane intermediate (Scheme 3). The species initially formed upon intramolecular attack of the Co-bound hydroxide (**C**) would not be a transition state as in Scheme 2, but would be a finite intermediate. This hypothetical phosphorane could partition either of two ways depending upon the relative rates of deprotonation and loss of *p*-nitrophenolate. Proton loss will be extremely rapid given the low pK_a of this proton, and the pH of the reaction. The isotope effect data require that loss of *p*-nitrophenolate be rate limiting, thus slower than proton loss from **C**. Thus the most feasible pathway is **C–D–E**. The formation of intermediate **D** should be rapid and irreversible under the reaction conditions employed. Intermediate **D** should partition completely forward by loss of *p*-nitrophenolate to give **E**, and in this event isotope effects on the bond-cleavage step **D–E** will not be expressed and only those on the formation of **D** would be observed. No ^{15}k effect should be seen since the bond to the leaving group is not broken, and only minimal $^{18}k_{bridge}$ effects are predicted. The experimentally observed magnitudes of both of these leaving group isotope effects thus make this mechanism very unlikely.

Further evidence against the stepwise mechanism comes from

computational evidence[24] suggesting the instability of a dianionic pentavalent phosphorane such as **C** in Scheme 3. Protonation of phosphate gives rise to an inverse ^{18}O isotope effect of 0.984%[25] and formation of a monoanionic phosphorane such as **D** should reveal itself by an inverse value for $^{18}k_{nonbridge}$, which is not observed.

The nucleophilic reaction of complex **2** shows leaving group isotope effects of 0.12% for ^{15}k, 0.98% for $^{18}k_{bridge}$, and 0.46% for $^{18}k_{nonbridge}$. As a group these three isotope effects resemble the pattern seen in hydrolysis reactions of diesters of *p*-nitrophenol. These reactions are concerted and are characterized by a more associative transition state (small normal $^{18}k_{nonbridge}$) with less advanced bond cleavage to the leaving group (smaller ^{15}k and $^{18}k_{bridge}$) as compared with reactions of the monoester pNPP.

In the original study of this reaction by Sargeson and co-workers, two mechanisms were considered consistent with their results, namely a concerted displacement and a stepwise reaction via a phosphorane, analogous to those discussed above for complex **1**. The hypothetical stepwise mechanism is shown in Scheme 4 and differs in key respects from the analogous pathway for **1** shown in Scheme 3. The initially formed phosphorane species **G** will not be labile to deprotonation as is the corresponding intermediate **C** in Scheme 3. The pK_a of the bound ammonia in the complex **2** is thought to be about

(23) Cleland, W. W.; Hengge, A. C. *FASEB J.* **1995**, *9*, 1585−1594.

(24) Lim, C.; Karplus, M. *J. Am. Chem. Soc.* **1990**, *112*, 5872−5873.
(25) Knight, W. B.; Weiss, P. M.; Cleland, W. W. *J. Am. Chem. Soc.* **1986**, *108*, 2759−2761.

17.[3] The pK_a of the phosphoramidate monoanion $H_3N^+-PO_3^{2-}$ is close to that of ammonium ion[26] suggesting that a phosphoryl group has an inductive effect not too different from that of a proton. Thus the pK_a of the bridging NH_2 moiety in **G** should not be much different from 17. The very large difference in the pK_a values of the potential leaving groups makes the breakdown of the hypothetical phosphorane **G** much more likely to proceed by loss of p-nitrophenol (pK_a of 7.1) than to revert to **2** by ring opening. Whether the reversion of **G** to **2** has a finite rate at all under the alkaline conditions of reaction is questionable. Separately, on the basis of the kinetic behavior of **2** Sargeson and co-workers concluded that if the phosphorane pathway is followed then loss of nitrophenol from **G** must be fast, and must also be more rapid than its formation, that is $k_3 > k_2$, meaning that if such a stepwise mechanism is followed formation of the intermediate will be rate determining.[3] The formation of **G** should produce a minimal $^{18}k_{bridge}$ effect, no ^{15}k effect, and an inverse value for $^{18}k_{nonbridge}$ since for reasons previously discussed the phosphorane structure is likely to exist only as the monoanionic species with one of the nonbridge oxygen atoms protonated. The small normal value for $^{18}k_{nonbridge}$ and the large magnitudes of the values for $^{18}k_{bridge}$ and ^{15}k are inconsistent with this mechanism. The much more likely explanation of the isotope effect data is a concerted mechanism for the reaction of **2** analogous to that shown in Scheme 2 for **1**. Compared to the transition state for reaction of **1**, that for **2** exhibits considerably less bond breaking to the leaving group (about half) and has more associative character. The pNPP moiety in the transition state of the reaction of **2** more nearly resembles that typically seen in diesters of p-nitrophenol, whereas the leaving group with **1** more nearly resembles that in the typical reaction of pNPP in solution. Whether this is a result of differences in the Co−O pNPP bond in the two complexes or, more likely, is due to the difference in nucleophile cannot be answered definitively by these data.

The isotope effects observed for the hydrolysis of pNPP by Co(cyclen) will be a product of that for the equilibrium formation of the Co−pNPP complex and the kinetic effects on the hydrolysis. The isotope effects in the leaving group are very close to those measured for **1**. The same mechanistic arguments presented for **1** apply to **4** and thus this reaction is most likely a concerted one as well. The isotope effect for reaction of **4** is given below, where the superscript x represents the isotope effect on the following kinetic quantity. The partition ratio (or commitment factor) k_3/k_2 will affect expression of the kinetic isotope effect on the nucleophilic step. If this ratio is large the observed isotope effects will be $^{x}k_1$, the kinetic

(26) Chanley, J. D.; Feageson, E. *J. Am. Chem. Soc.* **1963**, *87*, 1181.

isotope effect for formation of the complex. By contrast, if the equilibrium formation of complex **4** is rapidly reversible, the observed effects will be the product of the equilibrium isotope effect on formation of **4** and the kinetic one on the hydrolysis, or $^{x}K_1{}^{x}k_3$. The isotope effects on the equilibrium formation of **4** given in Table 1 were independently determined as described in the experimental section. Chemical precedent as well as the observation of isotope effects for the hydrolysis reaction that are significantly different from the equilibrium values argue that the ratio k_3/k_2 is small. The observed isotope effects for hydrolysis thus corrected for the equilibrium values are each given in Table 1.

$$2\text{Co(cyclen)} + \text{pNPP} \underset{k_2}{\overset{k_1}{\rightleftharpoons}}$$

$$[\text{Co(cyclen)}]_2 - \text{pNPP} \overset{k_3}{\longrightarrow} p\text{-nitrophenol}$$

$$\text{observed isotope effect} = \frac{^{x}K_1{}^{x}k_3 + {}^{x}k_1(k_3/k_2)}{1 + (k_3/k_2)}$$
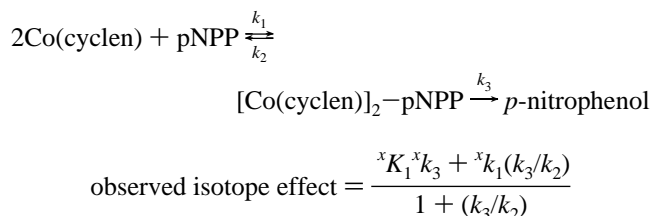
Conclusions. The significant values of the ^{15}k and $^{18}k_{bridge}$ isotope effects on the nucleophilic reactions of all three cobalt−pNPP complexes require that cleavage of the bond to the leaving group p-nitrophenol occur in the rate-limiting step. This is consistent with concerted mechanisms for these complexes, where nucleophilic attack and bond cleavage occur in the same step. Bond cleavage is far advanced in the transition states of the nucleophilic reactions of **1** and **4**, similar to what is observed in the uncatalyzed solution reaction of the pNPP dianion. With both of these complexes the nucleophile is a Co-bound hydroxide. With the pentaamine complex **2** the nucleophile is a Co-bound NH_2 of much higher pK_a, and bond cleavage is only about half as advanced in the transition state. The small normal values for $^{18}k_{nonbridge}$ in the nucleophilic reactions resemble values previously measured for diesters of p-nitrophenol, and indicate a small degree of associative character to the phosphoryl group in the transition state. The large $^{18}k_{nonbridge}$ isotope effects for dissociation of pNPP from Co in these complexes are consistent with the covalent character of this bond. This is in contrast to the purely electrostatic interaction of phosphate with magnesium which does not exhibit an ^{18}O isotope effect.