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The Role of Metals in the Hydrolytic Cleavage of DNA and RNA

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The nucleic acids RNA and DNA consist of nucleoside building blocks joined by phosphodiester linkages. Phosphodiesters are generally inert to hydrolytic cleavage under physiological conditions because their negative charge disfavors nucleophilic attack. However, the hydrolytic scission of phosphodiester linkages is an important and common biological process, and can occur rapidly in the presence of appropriate catalysts such as ribozymes and nuclease enzymes. Metals play an important role in this process. Several possible modes of action can be invoked for metal-promoted phosphate ester hydrolysis, including Lewis acid catalysis, Brønsted base catalysis by metal-bound hydroxides, nucleophilic catalysis by metal-bound hydroxides, Brønsted acid catalysis by metal-bound water, and electrostatic stabilization of transition states by positively charged metal ions. Here we critically discuss the roles of metals in the hydrolytic cleavage of nucleic acids and related model substrates.

Key Words: *DNA, RNA, hydrolysis, chemical nucleases, synthetic ribozymes, metal ions*

INTRODUCTION

Phosphodiesters provide an effective scaffold for the genetic code in part because they are essentially inert to hydrolysis at pH 7, in

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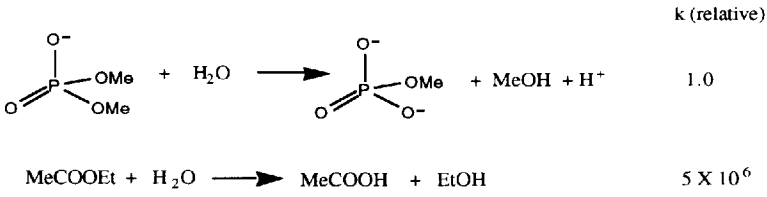
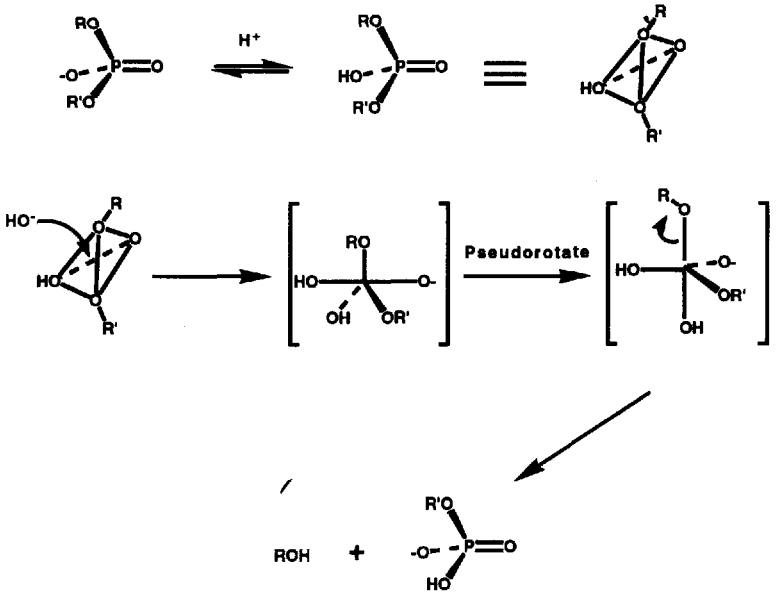


SCHEME 1

the absence of appropriate catalysts.[1] This kinetic property allows DNA to remain intact under normal cellular conditions, and arises because nucleophilic attack on the anionic phosphodiester groups is unfavorable. In contrast, uncharged carboxylic acid esters are hydrolyzed with relative ease. The hydrolysis of dimethyl phosphate and ethyl acetate is depicted in Scheme 1, along with relative rate constants for these reactions.[1]

The chemical steps of DNA hydrolysis include: (a) activation of the phosphate toward nucleophilic attack, (b) generation of a hydroxide nucleophile by deprotonation of water, (c) delivery of the nucleophile to the substrate, and (d) protonation of the leaving group. This hydrolysis reaction involves nucleophilic substitution at phosphorus. It is believed to occur by attack of hydroxide on a tetrahedral face of the substrate, giving a trigonal bipyramid.[2,3] Whether or not this exists as a true intermediate may vary from case to case. Departure of the leaving group from an apical position then occurs to form the products, as shown in Scheme 2.

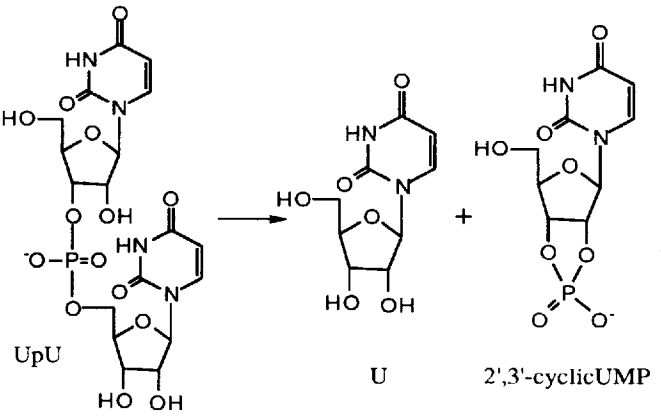
Phosphodiesterase enzymes are examples of catalysts that dramatically accelerate phosphate ester hydrolysis. With DNA substrates they typically produce 5′-phosphates and 3′-OH groups.[4] Many DNA hydrolysis enzymes are known, and the class known as restriction enzymes is particularly important for molecular biology because of their sequence-specific cleavage properties. However, there is only one report of DNA hydrolysis by a *small-molecule* catalyst under mild conditions.[5] This lack of success by synthetic catalysts may reflect the difficulty of achieving the appropriate juxtaposition of all the required catalytic elements within the relatively limited architecture of a small molecule. These catalytic elements may include: a general base to deprotonate water, a mechanism for delivering hydroxide to the target, a Lewis or



SCHEME 2

Brønsted acid to activate the phosphate toward nucleophilic attack, and a means of protonating the leaving group.

Although RNA and DNA have many common properties, the hydrolysis of RNA differs dramatically from that of DNA: RNA is cleaved one million times faster than DNA.[1] This relative rate of 10^6 is similar to the rate enhancement for the hydrolysis of ethyl acetate vs. dimethyl phosphate, even though RNA possesses negatively charged phosphodiester groups. As might be expected, this significant difference in rate reflects a change in mechanism. The 2'-OH group of RNA provides a hydrolysis pathway that is unavailable to DNA. In fact, the reaction that is conventionally referred to as RNA *hydrolysis* is actually a two-step process, the first step being a *transesterification* reaction. We choose to refer to this reaction as the *hydrolytic cleavage of RNA*, to distinguish it from oxidative pathways for strand scission.[6] Transesterification occurs when the 2'-hydroxyl group of RNA acts as an intramolecular nucleophile, replacing the intermolecular nucleophile (hydroxide)

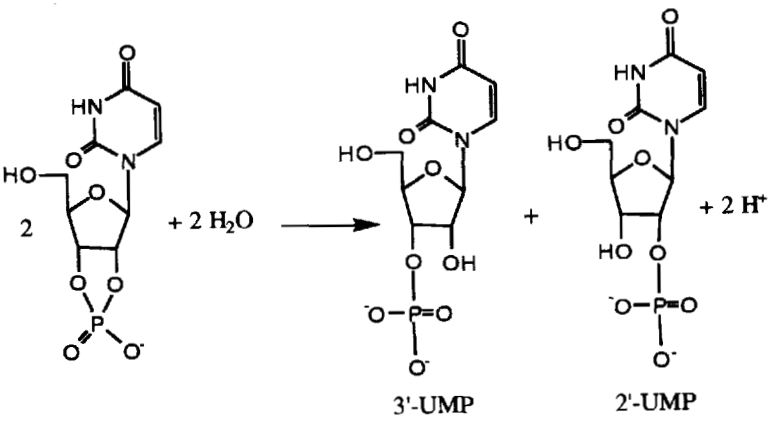


SCHEME 3

required for DNA hydrolysis. For example, Scheme 3 shows how the RNA dimer UpU undergoes hydrolytic cleavage to form uridine and 2′,3′-cyclic uridine monophosphate (cUMP).

Metal ions have long been known to promote the hydrolytic cleavage of RNA by this mechanism.[7,8] Ribonuclease A[9] and the hammerhead ribozymes[10] also cleave RNA by the transesterification pathway. Transesterification merely converts one phosphodiester into another, but this has two profound effects. First, the RNA strand is broken. Second, the resulting 2′,3′-cyclic phosphate contains a strained, 5-membered ring. Compounds of this type were shown by Westheimer to undergo hydrolysis ca. 10^6 times faster than their acyclic counterparts.[1] This rate acceleration can be attributed in part to the relief of ring strain, and in part to stereoelectronic effects.[11,12] As is illustrated in Scheme 4, this hydrolysis reaction can lead to both 2′- and 3′-monophosphates, although enzyme-catalyzed hydrolysis usually produces only the 3′-isomer.

Thus, due to the presence of the 2′-OH, both hydrolytic cleavage and "true hydrolysis" of RNA can take place much faster than the hydrolysis of DNA. There are, however, some alternate pathways for hydrolysis and cleavage that should be mentioned. For example, the catalytic core of ribozymes from *Tetrahymena thermophila* pre-ribosomal RNA employs an external guanosine res-



SCHEME 4

idue as the nucleophile for RNA cleavage rather than water or an internal 2′-OH.[2,13] Further, when the enzyme ribonuclease H (RNase H) cleaves the RNA strand of DNA–RNA duplexes, it produces 5′-phosphates and 3′-OH groups at each cleavage site.[14]

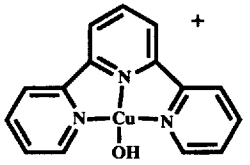
HOW DO CATALYSTS PROMOTE HYDROLYTIC CLEAVAGE OF NUCLEIC ACIDS?

There are several roles that a catalyst might play in promoting hydrolytic cleavage of DNA or RNA. *Brønsted base catalysis* can be employed to generate hydroxide and alkoxide nucleophiles, with either organic bases or metal hydroxides serving as the catalytic agent. Another important step is *activation of the phosphodiester toward nucleophilic attack*, which can be accomplished in several ways. Both *Brønsted* and *Lewis acid catalysis* will be discussed in this context, along with *electrostatic catalysis*. *Protonation of the leaving group* must also take place, and must involve a *Brønsted acid* mechanism; ammonium, imidazolium, and metal aquo species are all potential catalysts for this step.[15]

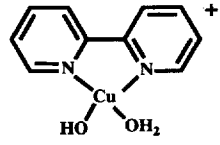
In the discussion below, examples of organic catalytic groups are provided for comparison with their inorganic counterparts. We also contrast the cleavage of DNA or RNA (both usually regarded

as unactivated substrates) with the cleavage of activated, *p*-nitrophenyl phosphates (e.g., bis(*p*-nitrophenyl)phosphate—BPNPP). This is relevant because the extensive body of literature on the hydrolysis of *p*-nitrophenyl phosphates is often used to model DNA and RNA hydrolysis.[16] However, there are important differences in the chemistry of these substrates. For example, the excellent leaving group *p*-nitrophenolate is a stable anion at pH 7, in contrast to the unstabilized alkoxide leaving groups found in DNA and RNA. Therefore, protonation of departing *p*-nitrophenolate is unnecessary, while protonation of the nucleic acid leaving groups is required at pH 7. Thus, while "activated" phosphates are usually hydrolyzed much faster than their unactivated counterparts, this difference most likely reflects a change in mechanism.[17] This complicates or invalidates conclusions about biological reactions that are based on the abundant data available for activated phosphates.

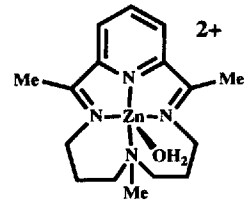
Brønsted Base Catalysis. A Brønsted base can act to generate the required nucleophile by deprotonating water (for hydrolysis) or an alcohol (for transesterification). In both chemical and enzymatic cleavage of nucleic acids, imidazole (Im) plays prominent roles because its pK_a of ca. 7 is ideal for performing both acid and base catalysis at pH 7. Metal hydroxides can act as inorganic analogues of Im. Coordination of water to metal ions can dramatically lower its pK_a, and metal hydroxides are the predominant species at pH 7 for a variety of zinc and copper complexes. It is established that metal hydroxides can act either as nucleophiles or as base catalysts. For example, in the first report of hydrolytic cleavage



Cu(II)terpyridine



Cu(II)bipyridine



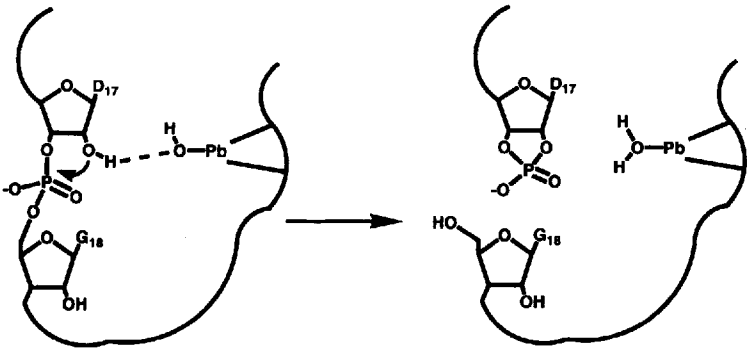
Zn(II)MeCR

SCHEME 5

of RNA by well-defined metal complexes, using the Cu(II) and Zn(II) complexes shown in Scheme 5, we found that the products contained 2′,3′-cyclic phosphates (2′,3′-cNMP's).[18] Formation of these cyclic phosphates requires deprotonation of the 2′-OH, indicating that base catalysis has occurred. If these reagents were effective nucleophilic catalysts, one might expect to observe direct hydrolysis of RNA to a terminal phosphate and ROH, as well as DNA hydrolysis. However, the intermediate cyclic phosphate was found, and DNA was inert to these inorganic reagents under conditions where the RNA was completely degraded hydrolytically.

Several reports have described the sequence-specific cleavage of tRNA molecules by metal ions. For example, when Pb^{2+} ion was employed as a heavy atom in the crystallographic analysis of yeast phenylalanine transfer RNA ($tRNA^{Phe}$), a break in the 76-nucleotide-long RNA phosphodiester chain was observed between nucleotides 17 and 18.[19] The binding site of the Pb^{2+} which promoted the cleavage was apparently between residues 59 and 60—*not* at the cleavage site. In addition, the cleavage was observed at pH 7.4 but not at pH 5.0 (the reported pK_a of lead-bound water was ~7). Thus, the mechanism shown in Scheme 6 was proposed, whereby a lead-bound hydroxide acts as a Brønsted base and deprotonates the 2′-OH on nucleotide 17.

Further kinetic studies[20] on the same system indicated a first-order dependence on Pb^{2+} and a bell-shaped pH dependence with



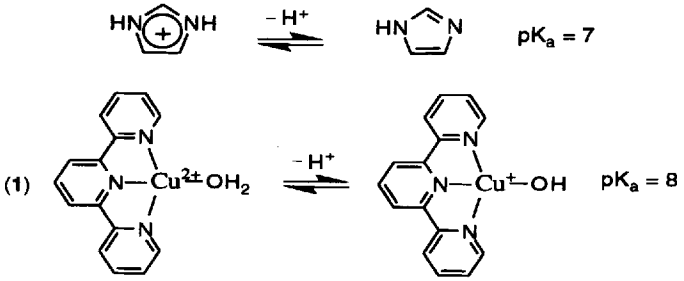
SCHEME 6

a maximum at pH 7. The first-order dependence on lead was attributed to competition between the added lead and the Mg^{2+} already present at the binding site. The upward slope of the pH profile was consistent with Brønsted base catalysis by a lead-bound hydroxide. The downward slope above pH 7 was attributed to a decrease in Pb^{2+} concentration caused by the formation of polyhydroxo species (see Scheme 8). While this mechanism has merit, it does not provide for activation of the phosphate toward nucleophilic attack, a step that is usually required for RNA transesterification at neutral pH. An alternative explanation of the kinetic results is that the Pb^{2+} bound between residues 59 and 60 acts as a base, but that free metal ions in solution provide the necessary acid catalysis.

Brønsted Acid Catalysis. A Brønsted acid can protonate a phosphodiester, neutralizing its negative charge and decreasing the electrostatic barrier to nucleophilic attack. This activity is usually associated with weak organic acids such as imidazolium (ImH^+, $pK_a = 7$). There are two imidazole residues in the active site of Ribonuclease A, a thoroughly studied enzyme that cleaves and hydrolyzes RNA. Recently, two groups have shown that polyamines and polylysines may also serve as Brønsted acid catalysts at moderate pH's. The monomeric subunits of these polymers are too basic to perform efficient acid catalysis in the range $6 < pH < 8$. However, incorporation of the amines into oligomers or polymers results in a range of pK_a's, since the basicity of each amino group is affected by how many ammonium groups are present in its immediate environment. Polyelectrolyte theory provides a description of these altered properties.[21]

Given that all examples cited above for Brønsted acid catalysis are organic acids, one might ask whether metals can participate in this reaction. It is common to invoke base catalysis by metal hydroxides. We suggest here that Brønsted acid catalysis by metal aquo species may also be an important reaction pathway. In fact, many metal aquo species have pK_a's near 7 and 8, and can be regarded as inorganic analogues of imidazolium. This is illustrated in Scheme 7 for **1**, $Cu(trpy)(H_2O)^{2+}$.

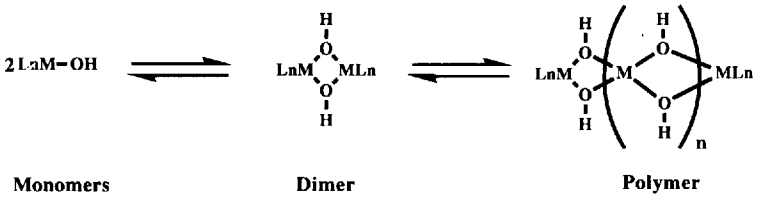
For compound **1**, both the acid and conjugate base forms are positively charged, so both will be electrostatically attracted to a



SCHEME 7

nucleic acid substrate. Since both acid and base catalysis are normally required for the hydrolytic cleavage of nucleic acids (*vide infra*), this provides a potential advantage for inorganic Brønsted acids over reagents such as Im. Whether inorganic Brønsted acids play an important role in nucleic acid cleavage is not yet established. However, the following observation is intriguing. When organic reagents such as Im were found to exhibit a bell-shaped pH-rate profile for hydrolytic cleavage of phosphate esters, this was interpreted to indicate a bifunctional, acid–base catalytic mechanism. Bifunctional catalysis can be either simultaneous (as with RNase A)[8] or sequential (as with free Im).[22] However, when a bell-shaped pH-rate profile was observed for *metal*-promoted RNA cleavage, it was attributed to a decrease in catalyst concentration at elevated pH.[20,23,24] Several mechanisms have been suggested to account for this change in catalyst concentration as a function of pH: (a) inhibition caused by the formation of stable nucleotide metal complexes at higher pH, (b) dimerization of metal aquo or hydroxy species to form inactive complexes, and (c) precipitation of metal hydroxides from solution at higher pH. It is certainly true that a threshold pH exists for many metal aquo complexes, above which oligomeric metal hydroxides may form and polymers may precipitate (Scheme 8).

These suggested mechanisms do not rule out Brønsted acid catalysis by metal aquo species, but do make it more difficult to establish. We are studying a series of reactions including the cleavage of oligomeric RNA, ApA, activated substrates, and 2′,3′-cAMP under identical conditions (buffer, ionic strength, etc.) to

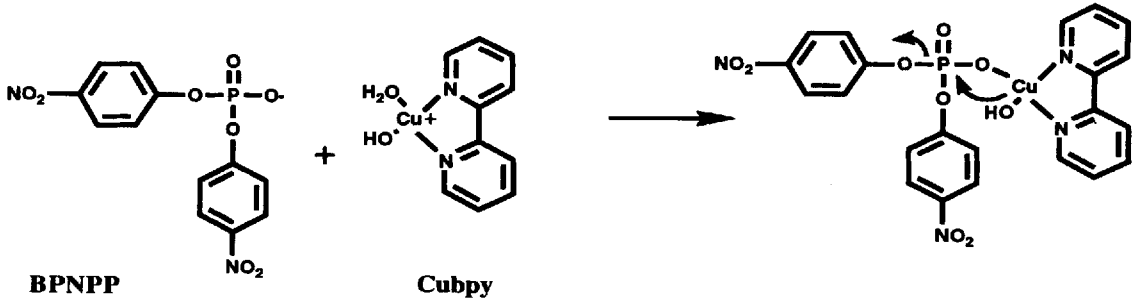


SCHEME 8

confirm or refute the occurrence of inorganic Brønsted acid catalysis in metal-promoted nucleic acid cleavage.

Lewis Acid Catalysis. Proton transfer is not the only mechanism for activating phosphates toward nucleophilic attack. Direct coordination by a metal can provide the required charge neutralization and transition state stabilization. For example, the active site of the enzyme DNase I contains an Im and a Ca^{2+} ion. The Im is believed to generate hydroxide by deprotonating water with assistance from glutamate 75, and the Ca^{2+} is believed to activate the phosphate toward nucleophilic attack by coordination.[4] A related mechanism has been established for metal-promoted hydrolysis of activated phosphate esters such as *p*-nitrophenyl phosphates through the elegant work of Sargeson and Buckingham,[25] and has been demonstrated in catalytic reactions by Morrow and Trogler.[26] One particular advantage of a directly bound metal ion is that it can deliver additional catalytic functional groups such as hydroxide to the substrate. This is illustrated in Scheme 9 for both Lewis acid/nucleophilic hydroxide and Lewis acid/basic hydroxide mechanisms.

A Controversy Surrounding Hydrolysis. As shown in Scheme 9, a metal complex or ion with two labile coordination sites can bind a phosphate and a hydroxide simultaneously, effectively delivering the hydroxide to the substrate. This hydroxide can act as either a base or a nucleophile. Morrow and Trogler reported[26] that (2,2′-bipyridyl)(aquo)(hydroxy)Cu(II), bpyCu(II), catalyzes the hydrolysis of BPNPP, but that **1**, its Cu(II) terpyridine analogue, is inactive for this reaction. Their very reasonable interpretation was



SCHEME 9

that the terpyridine complex could not coordinate both hydroxide and phosphodiester simultaneously, so that the metal-assisted nucleophilic attack on phosphate was an inaccessible mechanism for complex **1**. We later reported that **1** was active in promoting the hydrolytic cleavage of RNA.[18] In this case, the nucleophile is the 2′-OH, and no nucleophilic metal hydroxide is required. However, this reaction is similar to true hydrolysis in that activation of the phosphate, generation of the nucleophile (RO- or HO-), and protonation of the leaving group are all required. As part of a later study on RNA cleavage and hydrolysis,[23] Morrow concluded that **1** could promote RNA cleavage but *must* be incapable of promoting RNA hydrolysis, since it had failed to hydrolyze BPNPP. Deprotonation of a 2′-OH or water did not seem to us to be fundamentally different reaction steps, so we investigated this issue.

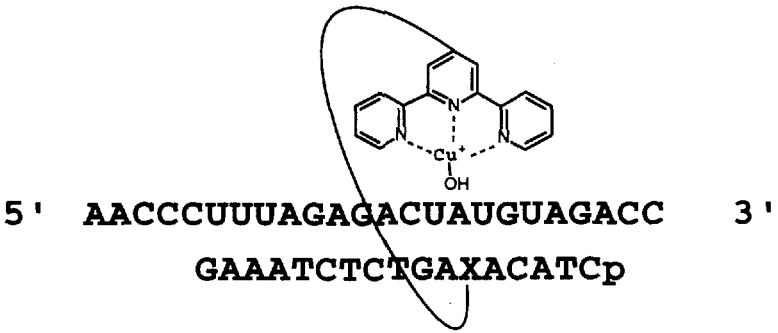
As shown in Scheme 4, hydrolysis is the step during which hydroxide attacks a 2′,3′-cyclic phosphate. We have recently found[17] that **1** *does* promote the hydrolysis of 2′,3′-cyclic adenosine monophosphate, cAMP, at pH = 7.4. A summary of these results is as follows: both aqueous bpyCu(II) and trpyCu(II) promote the hydrolytic cleavage (transesterification) of RNA. BpyCu(II) catalyzes the hydrolysis of "activated" esters such as bis(*p*-nitrophenyl)phosphate, but trpyCu(II) is inactive for this hydrolysis. However, trpyCu(II) does promote the hydrolysis of the "unactivated," biologically important substrate, cAMP. A minimal conclusion is that the mechanism of hydrolysis of activated phosphate esters is different from that of their unactivated counterparts. This would invalidate any simple extrapolation of results obtained with "activated" substrates to the biological arena. It is clear that the hydroxide of **1** can promote the hydrolysis of cAMP, although we have yet to determine whether the Cu–OH acts as a nucleophile or a base.

Electrostatic Catalysis. The active site of RNase A contains two histidine imidazoles and a lysine. A conventional mechanism of action invokes one imidazole acting as a base to deprotonate the 2′-OH, the second acting as an acid to protonate the leaving group, and the lysine acting to stabilize the negative charge that develops on the phosphate during the reaction.[9] In this mechanism, an electrostatic argument is invoked to explain the activation of the phos-

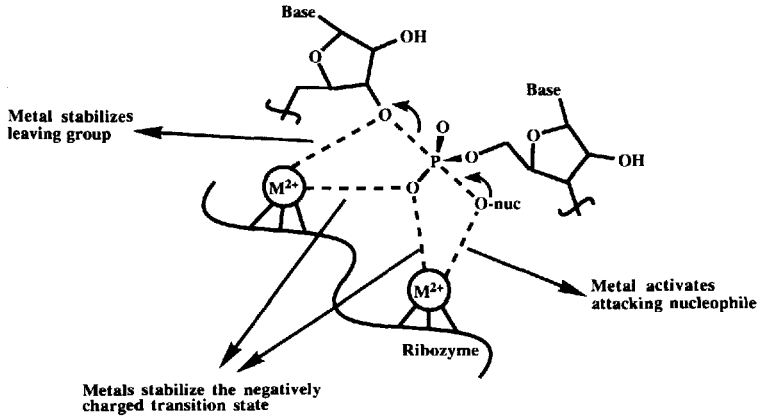
phate toward nucleophilic attack. It is important to note that this mechanism is disputed by Breslow, whose studies on the Im-catalyzed cleavage and hydrolysis of RNA indicate that initial Brønsted acid catalysis takes place.[27] In Breslow's mechanism, acid catalysis leads to formation of a phosphorane intermediate by protonation of the phosphate and deprotonation of the 2′-OH. In a subsequent step, which constitutes a cycle of Brønsted base catalysis by Im, the phosphorane is deprotonated and the leaving group is protonated. Of course, the possibility remains that, in the absence of a group capable of stabilizing the developing negative charge on the phosphate, a different mechanism must operate. Intermediate mechanisms, in which complete proton transfer does not take place, may also be important, since the required proton transfers are inconsistent with pK_a values.[28]

We might ask if metals can mimic lysine and provide electrostatic relief *without acting directly as Lewis acids*, i.e., with metal–phosphate coordination. Cowan[29] has recently shown this to be possible, and in so doing has forced a revision of certain common assumptions about the role of metal ions as enzyme cofactors. Working with the enzyme RNase H, which hydrolyzes the RNA strand of RNA–DNA hybrids, Cowan replaced the usual cofactor $Mg(H_2O)^{2+}{}_6$ with $Co(en)^{3+}{}_3$. These two metal cations have approximately the same size. The Co(III) complex is kinetically inert to ligand exchange, but Cowan and Jou found it to serve as an effective catalytic cofactor for the enzyme. This clearly demonstrates that direct coordination of the substrate to a metal is not required for phosphodiester cleavage to take place. The catalytic action may involve through-space electrostatic attraction between metal cation and substrate, or may be mediated by intervening hydrogen bonds.

Ribozyme Activity. Similar principles may govern the activity of ribozymes, the catalytic RNA molecules.[30] Ribozymes require metal cofactors, yet still seem to lack all the necessary functional groups with appropriate pK_a's to promote cleavage and hydrolysis of RNA. We have chosen to study this reaction by preparing functional mimics of ribozymes for which the identity, number and coordination environment of the reactive metal center can be varied. An



SCHEME 10



SCHEME 11

example is shown in Scheme 10.[31] We are also pursuing functional mimics of ribozymes which are wholly organic.[32]

A recent report by Steitz and Steitz proposed a general, two-metal ion mechanism for RNA splicing and cleavage.[33] The authors cite likely structural similarities between ribozymes and enzymes that perform RNA and DNA phosphoryl transfer reactions to yield 5′-phosphates and 3′-OH's. These enzymes include *E. coli* DNA polymerase I, alkaline phosphatase, RNase H, P1 nuclease and phospholipase C. General features of the Steitz and Steitz mech-

anism are shown in Scheme 11. The crystal structure of the 3′,5′-exonuclease domain of DNA polymerase I is in agreement with the Steitz and Steitz mechanism. Two metal ions form inner-sphere complexes with the substrate phosphate and the nucleophile which can be water or ROH from a nucleoside. They are poised to assist formation of the nucleophile and to stabilize the activated complex (or intermediate) for hydrolysis. The interaction between a cationic metal center and the leaving group oxygen may also assist the bond-breaking step. Steitz and Steitz proposed that chelation of a phosphodiester by a metal ion compresses one edge of the tetrahedral phosphate, resulting in a strained structure that is readily hydrolyzed. This new "two-metal" mechanism has many attractive features, including generality for enzymes and ribozymes. It does not, however, encompass Cowan's finding that the coordinatively saturated metal complexes can activate RNase H, and so may need further refinement.

CONCLUSIONS

It is clear that direct Lewis acid coordination of metals to phosphate is an important step in the cleavage of activated esters such as *p*-nitrophenyl phosphates. However, studies have shown these activated substrates to have limited or no relevance to biological phosphate ester cleavage.[34] It remains unclear when direct metal–phosphate binding is important for substrate cleavage by natural or synthetic nucleases, and it may be difficult to determine the importance of an intervening water molecule or other ligand from X-ray crystal structure analyses. Labile coordination sites on a metal may be more important for properly binding the metal to the active site than for actual substrate activation. We have described a number of catalytic mechanisms by which metals may promote RNA and DNA hydrolysis, including:

(1) Brønsted base catalysis by organic bases and metal hydroxides
(2) Nucleophilic catalysis by M–OH and M–OR groups
(3) Brønsted acid catalysis by organic acids and $M-OH_2$ species
(4) Lewis acid catalysis by metals
(5) Electrostatic catalysis by ammonium groups and metal cations

It remains to be determined when these various catalytic steps are operative, if Brønsted acid catalysis by $M-OH_2$ groups does occur, and how general the phenomenon of outer-sphere, electrostatic catalysis is.

Acknowledgments

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