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Chemistry

On the mechanism of action of ribonucleases: Dinucleotide cleavage catalyzed by imidazole and Zn^{2+}

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ABSTRACT **Cyclization/cleavage of the 2-(*p*-nitrophenyl) phosphate ester of propylene glycol is catalyzed by imidazole and, much more effectively, by Zn^{2+} with imidazole. In the latter case, the mechanism involves simultaneous Lewis acid/base catalysis. Similar Zn^{2+} and imidazole catalysis of cyclization/cleavage is seen with the dinucleotide 3′,5′-UpU (uridylyluridine). Again, the zinc system is much more effective than is catalysis by imidazole alone, and in this case simultaneous Lewis acid/base catalysis substitutes for the sequential proton acid/base catalysis seen with polynucleotides or dinucleotides and imidazole buffer catalysts. A mechanism is proposed for catalysis of RNA cleavage by the enzyme ribonuclease A, and the relationship of that mechanism to the action of the enzyme model systems is discussed.**

The hydrolysis of RNA by ribonuclease A (bovine pancreatic ribonuclease) involves a two-step process (1). In the first step, the RNA chain is cleaved by a phosphate ester exchange, in which the 2′-OH group of ribose attacks the phosphate ester linkage and the 5′ oxygen of the next nucleotide is ejected. In the second step the resulting 2′,3′-cyclic phosphate is hydrolyzed, forming a 3′-phosphate monoester and regenerating the 2′-OH group. The principal catalytic groups for these reactions are the imidazole (Im) rings of His-12 and His-119; the ammonium ion of Lys-41 is also implicated. The pH vs. rate profiles indicate that in both the cyclization and the subsequent hydrolysis step an acid–base bifunctional mechanism is involved. One of the Ims acts as a base and the other, as the protonated imidazolium cation, acts as an acid. The details of the mechanism have not yet been clarified (but see Scheme **II** and the discussion below).

We have described a study of the cleavage of polyuridylic acid [poly(U)], a homogeneous RNA, by imidazole buffers (2). We saw that both buffer components were involved, but in a sequential mechanism, in which one step was catalyzed by one of the acid/base buffer species and the second step was catalyzed by the other. A pentacoordinate phosphorane was the intermediate between the two steps. In the original work (2), we could not tell whether the sequence was base, then acid, or the reverse. However, recently (3) we have found that the same type of sequential mechanism is observed for Im buffer catalyzed cleavage of the simplest RNA, the dimeric dinucleotide 3′,5′-UpU (uridylyluridine) **1**. In this case, we were able to show that the sequence was the less obvious one: first the acidic imidazolium ion (ImH^+) catalyzes conversion of the substrate **1** to the phosphorane intermediate, and then the basic Im catalyzes the decomposition of this intermediate to uridine 2′,3′-cyclic phosphate **2** and uridine. This sequence is not the one predicted (4) from stereoelectronic considerations, but it was established (3) for UpU by comparing kinetic effects on its cleavage to those on its competing isomerization to 2′,5′-UpU **3** (and vice versa).

The mechanism required for this acid, then base, catalyzed cleavage is shown in Scheme **I**. (The sequential bifunctional cleavage of 3′,5′-UpU **1** by Im buffer is shown. The first catalyst is imidazolium cation, the second is Im. An alternative path leads to the isomer **3**.) In the first step, the phosphate ester is activated by protonation, then Im promotes the cyclization step (the sequential reaction with a proton, then Im, is kinetically equivalent to reaction with ImH^+), then Im catalyzes the decomposition of the intermediate by removing the proton again and (probably) delivering it to the leaving group. We have pointed out (3) that a related mechanism is also likely for the enzyme ribonuclease A, in contrast to the mechanisms usually invoked for that enzyme. Our proposed mechanism (3) for the cleavage of RNA by ribonuclease A and related enzymes is shown in Scheme **II**. [Our proposed mechanism of catalytic hydrolysis of RNA by ribonuclease A is shown. The top sequence is the cleavage reaction; the bottom sequence shows hydrolysis of the cyclic phosphate intermediate. The first two steps of the top scheme (and last two steps of the bottom scheme) might be simultaneous if the hydrogen bond lengths simply change in response to changing electron demand. The critical point is that the ImH^+ of His-119 protonates the phosphate anion in the first step.] It is based on our study and on what is already known about the enzyme, such as the evidence (1) that imidazolium cations are involved in the binding of phosphate anion substrates, that His-119 moves to the phosphate group when substrate is bound, and that Lys-41 binds only to the phosphorane anion intermediate.

In Scheme **I**, for the chemical model system, a proton is first added to the phosphate to activate it; that proton must be removed again to promote cleavage to the 2′,3′-cyclic phosphate anion product. It seemed likely that this proton could be replaced by a coordinating metal ion and that the metal could cooperate with Im in an RNA cleavage mechanism of Scheme **III**. (The mechanism of cyclization/cleavage of 3′,5′-UpU **1** by Zn^{2+} and Im is shown.) We have now found that this is indeed the case.

In a preliminary study, we examined the cyclization of the *p*-nitrophenylphosphate ester **4** of propylene glycol, which has been used (5) as a model for some aspects of the RNA cyclization/cleavage reaction and whose kinetics are conveniently followed by UV light. We find that the cyclization of **4** is catalyzed by Im, with a first-order dependence, but shows no catalysis by imidazolium ion. Thus, it uses the simple mechanism of Scheme **IV**. However, the cleavage of **4** does show bifunctional catalysis by Zn^{2+} and Im, and a study (see below) of this bifunctional process indicates its mechanism as that of Scheme **V**. The differences in catalyses of substrates **1** and **4** are sensible in terms of our overall picture of the mechanisms involved and their dependence on the structure of the substrate.

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Abbreviation: Im, imidazole.

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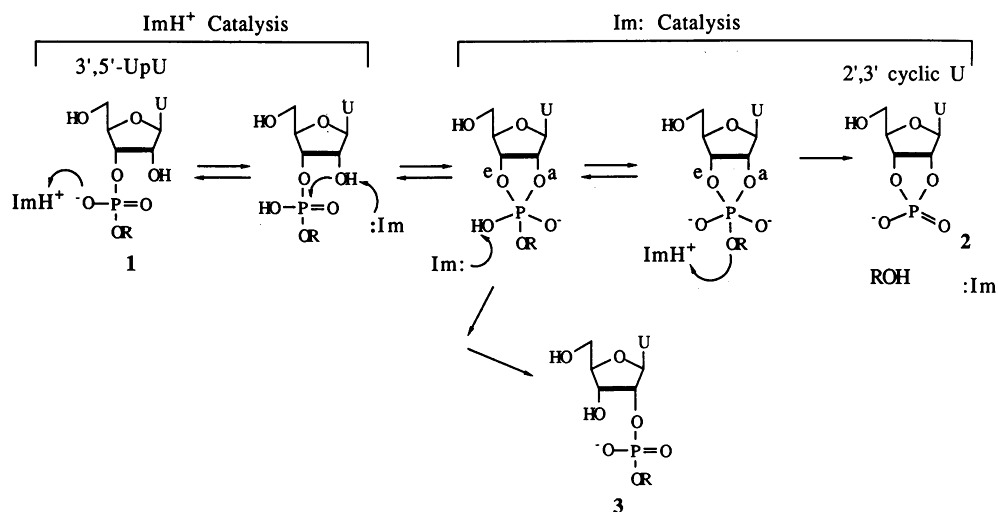
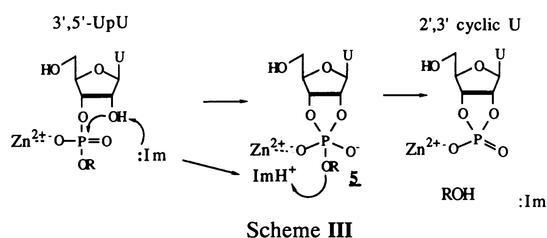
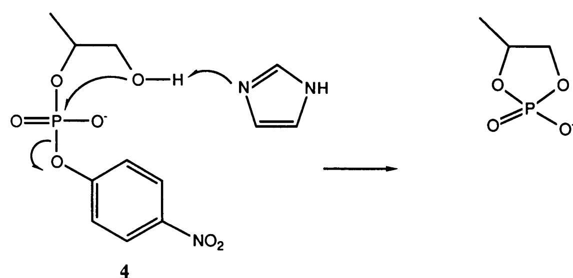


Scheme I

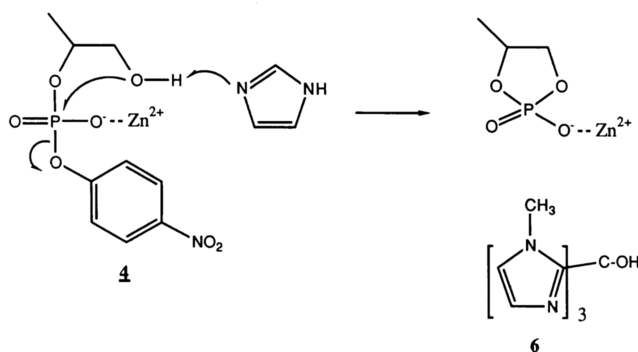
Scheme II



Scheme III



Scheme IV



Scheme V

METHODS

Uridine, 2′,5′-UpU, and 3′,5′-UpU were purchased from Sigma and used as received. Im was recrystallized from benzene. *p*-Nitrophenyl 2-hydroxypropyl phosphate (**4**) was prepared according to the reported procedure (5). All stock solutions, buffers, and eluents were prepared from deionized water.

The rate of cleavage of **1** to **2** was followed by HPLC analysis, as described elsewhere (2, 6). The same precautions to avoid foreign ribonuclease, and the same assay procedures, were used except for the following points: (*i*) The reaction buffers used for pH 7.0–7.5 were 10 mM Hepes and for pH 6.5–6.8 were 10 mM Pipes. (*ii*) The standard plots required for the assay were made from solutions that were 12 μM potassium *p*-nitrobenzenesulfonate (PPNBS, the internal standard) with various concentrations of both uridine and 2′,5′-UpU. Solutions of **1** were made up containing 3.2 mM **1** and 1 mM $ZnCl_2$ with 128 μM PPNBS and appropriate concentrations of imidazole in buffer. As before (2), the reactions were performed in sealed capillary tubes, and HPLC was used to determine the products uridine and 2′,5′-UpU.

The Im-catalyzed cleavage of **4** at 30°C was followed at 401 nm UV using a Beckman DU8 or DU8B spectrophotometer with temperature control. Im buffers were used with concentrations from 0.66 to 2.36 M, and (for the pH study) with 10–80% Im hydrochloride. For the temperature study, the molar extinction coefficient of the product *p*-nitrophenol was determined over the temperature range used. Cleavage of **4** with Zn^{2+} and Im was followed in a similar fashion at 400 nm and 37°C, but with 10 mM Hepes buffer (pH 7.00) except in

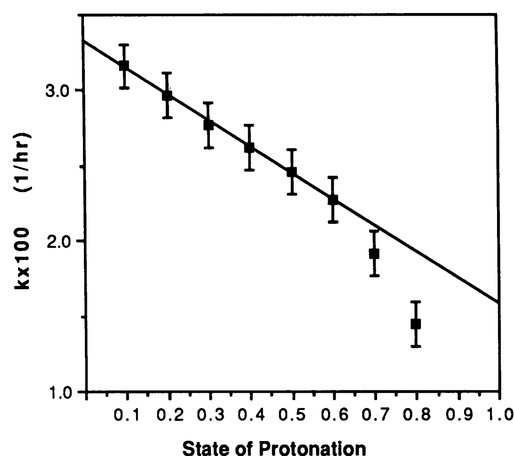


FIG. 1. The rate of cyclization/cleavage of **4** catalyzed by Im buffer vs. the buffer ratio.

the experiments exploring the pH dependence. The Im concentrations needed with Zn^{2+} cocatalyst were in the millimolar region.

RESULTS AND DISCUSSION

The cyclization/cleavage of poly(U) (2) and of UpU (3) is catalyzed by concentrated Im buffers in a reaction that is first order in buffer but that shows an optimum rate when both the basic Im and the acidic ImH^+ are present (a bell-shaped curve). As we have discussed (2, 3), this indicates sequential bifunctional catalysis. By contrast, we find that the cyclization/cleavage of **4** shows simple first-order dependence on Im, with no ImH^+ catalysis (cf. Fig. 1). From a temperature study, we find $\Delta H^{\ddagger} = 12.7 \pm 2.6$ kcal/mol (1 cal = 4.184 J) and $\Delta S^{\ddagger} = -43.5 \pm 8.9$ e.u. The base-catalyzed cyclization of **4** has been studied (5, 7).

The cleavage of UpU has a mechanism (Scheme **I**) in which the phosphate diester is first activated for attack by protonation, before internal nucleophilic attack can occur. The more reactive phosphate diester of **4**, with an electron-withdrawing substituent, does not require such prior contrathermodynamic protonation. Furthermore, the leaving group of **4**, *p*-nitrophenoxide ion, should not require catalytic protonation.

Although the cleavage of **4** did not require acid catalysis, and Zn^{2+} can be thought of as a superproton, we find that Zn^{2+} is catalytically quite effective in the cleavage of **4**. The data of Table 1 indicate that 0.5 mM Zn^{2+} alone accelerates the cyclization of **4** at pH 7.00 by a factor of 150, while with addition of the optimum amount of Im (12 mM) (Fig. 2) the acceleration is 850-fold. An even better acceleration, 1500-fold, is seen with 0.5 mM Zn^{2+} and 9 mM 2-methylimidazole.

Table 1. Pseudo-first-order rate constants ($k \times 10^2$ hr^{-1}) and relative rate constants (k_{rel}) for the cyclization/cleavage of *p*-nitrophenyl ester **4** in H_2O at pH 7.0 and 37°C

Catalyst	$k \times 10^2$ hr^{-1}	k_{rel}
None	0.0115 ± 0.0014	1.00
$ZnCl_2$ (0.5 mM)	1.71 ± 0.12	150
Im (1.0 mM)	0.014	1.2
Im (12 mM) + Zn^{2+} (0.5 mM)	9.72 ± 0.58	850
2-MeIm (8 mM) + Zn^{2+} (0.5 mM)	17.4 ± 1.1	1513
Im (1 mM) + Zn^{2+} (0.5 mM)	2.65 ± 0.13	230
Im (1 mM) + Zn^{2+}/phen (0.5 mM)	1.61 ± 0.08	140
Im (1 mM) + Zn^{2+} (1 mM)	5.26 ± 0.13	457
2-TIC (1 mM) + Zn^{2+} (1 mM)	0.051	4.5

2-MeIm, 2-methylimidazole; Zn^{2+}/phen, equimolar $ZnCl_2$ and *o*-phenanthroline; TIC, tris-2-imidazolylcarbinol **6** (8).

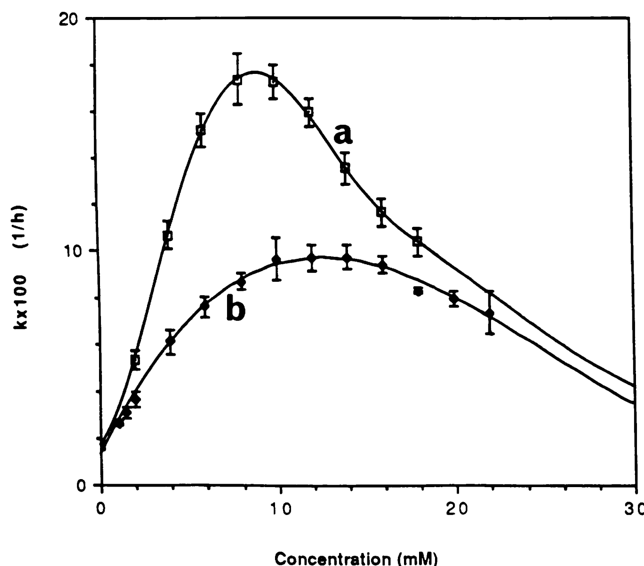


FIG. 2. The pseudo-first-order rate constant for cyclization/cleavage of **4** at pH 7.00 and 37°C with 0.5 mM $ZnCl_2$ and various concentrations of 2-methylimidazole (a) and Im (b).

One might wonder whether this Zn^{2+}/Im catalyzed reaction involves Im as a separate catalytic entity or simply as a zinc ligand. Indeed, Atwood and Haake (8) have reported that Zn^{2+} and Im catalyze the cleavage of catechol cyclic phosphate, and they propose that the active catalyst is simply a zinc–Im complex. We believe that this can be excluded, at least for our case.

One would expect that binding of a strong donor ligand such as Im to Zn^{2+} would decrease its Lewis acidity and, therefore, its catalytic effectiveness if it is acting as an acid. Consistent with this, we find that with Zn^{2+} (1.0 mM) and the strong chelating tris-imidazole ligand **6** (1.0 mM) (9) the cleavage rate of **4** drops to only 1% of that with 1.0 mM Im instead of **6** (Table 1). Other donor ligands also decrease the catalytic effectiveness of Zn^{2+} in the reaction of **4**; for instance, with 0.5 mM Zn^{2+} and 1.0 mM Im (Table 1), the addition of 0.5 mM *o*-phenanthroline drops the catalytic rate

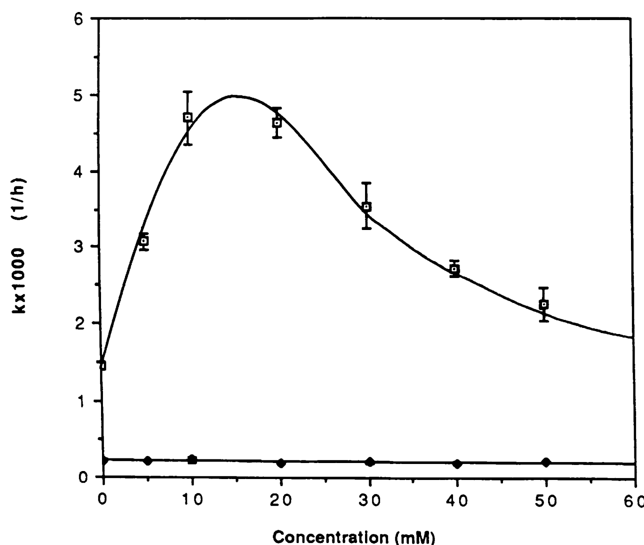


FIG. 3. The pseudo-first-order rate constant for cyclization/cleavage of 3′,5′-UpU **1** (3.2 mM) in 10 mM Hepes buffer (pH 7.00) at 80°C with 1.0 mM $ZnCl_2$ and various concentrations of Im. The top curve is for the cleavage process, forming uridine and uridine-2′,3′-cyclic phosphate, while the lower straight line is for isomerization of 3′,5′-UpU to 2′,5′-UpU; the isomerization shows no catalysis by Zn^{2+} or Im under these conditions.

Table 2. Pseudo-first-order rate constants ($k \times 10^3$ hr^{-1}) and relative rate constants (k_{rel}) for the cyclization/cleavage of **1** in 10 mM Hepes buffer (pH 7.00) at 80°C

Catalyst	$k \times 10^3$ hr^{-1}	k_{rel}
None	0.046 ± 0.004	1.00
Im (10 mM)	0.10 ± 0.02	2.3
$ZnCl_2$ (1 mM)	1.46 ± 0.04	32
Im (10 mM) + $ZnCl_2$ (1 mM)	4.70 ± 0.35	103

by 40%. Thus, the extra catalytic effect of Im with Zn^{2+} must be in spite of its binding to the metal and because of an additional catalytic role. As expected, an excess of Im completely saturates the Zn^{2+} and decreases the rate (Fig. 2). Consistent with this idea, 2-methylimidazole is a more effective catalyst with Zn^{2+} (Fig. 2); the hindrance from the extra methyl group should favor dissociation rather than metal binding.

The Zn^{2+} promoted cleavage of RNA has been described (10). We find that 3′,5′-UpU **1** is also cleaved by a combination of Zn^{2+} and Im/ImH^+ buffer. In contrast to the result without Zn^{2+}, this reaction uses only the basic Im component of the buffer. Catalysis is much more effective than with Im buffer alone, and much lower concentrations are used; also in contrast to the case with Im buffer only, the Zn^{2+}/Im system does not catalyze detectable competing isomerization of the substrate to **3**.

The rates of cyclization/cleavage of **1** with 1.0 mM Zn^{2+} and various concentrations of Im at pH 7.00 are plotted in Fig. 3. The optimum rate is 100 times that with the Hepes buffer alone under these conditions (Table 2). At lower pH values the rate falls as expected for the decrease in the Im concentration (Table 3), establishing that catalysis indeed involves Im and a Zn^{2+}. The relationship is not simple, since, as Fig. 3 shows, the binding of Im to Zn^{2+} leads to a complex dependence of the rate on free Im, but the calculated rates for pH values below 7 agree, using the data of Fig. 3 (Table 3), with those observed if indeed Im but not imidazolium ion is a catalyst.

As the buffer pH is raised above 7 the rate of cleavage of **4** continues to increase, but that for cleavage of **1** decreases. At these higher pH values the observed rates no longer agree (Table 3) with those expected if we are simply changing the concentration of Im. However, we find that the decrease for **1** is due to precipitation of Zn^{2+} complexes at higher pH, including complexes with the nucleotides. The cleavage of **4** is also slowed rather than accelerated at higher pH, and to the same extent, if its reaction is run under the same conditions [using 1.0 mM Zn^{2+} and 10 M Im/ImH^+ buffer and with 1.0 mM UpU (or ApA) present]. Thus, we detect no kinetic involvement of ImH^+ in the cleavage reactions of **1** or **4**.

Our data for the Zn^{2+}/Im catalyzed cleavage of UpU are consistent with the mechanism of Scheme **III**. In contrast to Scheme I, coordination by Zn^{2+} replaces protonation in the first step. The added proton of Scheme I had to be removed by Im prior to fragmentation of the phosphorane anion

Table 3. Observed pseudo-first-order rate constants ($k_{obs} \times 10^3$ hr^{-1}) of cyclization/cleavage of **1** by 1 mM Zn^{2+} and 10 mM Im buffer at 80°C at various pH values vs. the values calculated ($k_{calc} \times 10^3$ hr^{-1})

pH	% ImH^+	$k_{obs} \times 10^3$ hr^{-1}	$k_{calc} \times 10^3$ hr^{-1}
7.48*	25	4.01 ± 0.24	4.80
7.27*	35	3.60 ± 0.18	4.69
7.00*	50	4.70 ± 0.35	4.37
6.74†	65	3.34 ± 0.31	3.81
6.52†	75	3.44 ± 0.35	3.30

*Hepes buffer (10 mM).
†Pipes buffer (10 mM).

intermediate to form the cyclic phosphate product, but zinc coordination probably persists throughout. The cyclic phosphate does not want a proton at the operating pH, but some metal coordination ought to be stabilizing. The decomposition of the intermediate **5** should be catalyzed by ImH^+ in the UpU case, since its leaving group needs protonation. However, since the ImH^+ is formed in the first step, it must be kinetically invisible even if it is involved in the second step of UpU cleavage and if that step is the slow one (3). This invisibility is also seen for chymotrypsin, in which a base catalyst in one step forms the acid catalyst needed for the next step (3). We have pointed out that this is the reason that the rate of chymotrypsin catalysis is not decreased at higher pH, even though ImH^+ is a catalyst for the second step (3).

It is interesting that we observed some isomerization of **1** to **3** with Im/ImH^+ catalysis (3), but not with the Zn^{2+}/Im catalyst system described here. Instead, isomerization goes on at the same slow rate as it does in the absence of these catalysts (Fig. 3). As we have described (3), isomerization and cleavage paths branch from the common phosphorane intermediate, but in the Zn^{2+} system a proton on that phosphorane (Scheme **I**) is replaced by Zn^{2+} (Scheme **III**). This makes the cleavage step easier, since a separate deprotonation step is not required. It probably makes the pseudorotation required for isomerization more difficult, especially if the Zn^{2+} is actually bridged between two oxygens of the phosphorane. Thus, isomerization does not compete with cleavage in the Zn^{2+} catalyzed pathway.

For both substrates **1** and **4** the Zn^{2+}/Im system is much more effective as a catalyst than is the Im/ImH^+ buffer catalyst without metal. Thus, it is an attractive candidate for the elaboration of multifunctional catalysts for RNA cleavage. The Im/ImH^+ system has also given us additional insights into the mechanism of RNA hydrolysis by ribonuclease A, which uses these two catalytic groups. The mechanism used in the Zn^{2+}/Im model system may furnish similar insights into the activity of nucleases that contain catalytic metal ions.

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