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How Do Imidazole Groups Catalyze the Cleavage of RNA in Enzyme Models and in Enzymes? Evidence from "Negative Catalysis"

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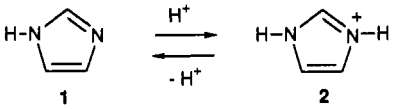
The imidazole ring in the side chain of the amino acid histidine plays an important role in many enzymes. Because its pK_a is close to 7, imidazole (1) is the strongest base that can exist at least partially unprotonated in biological solution, while in its protonated form the imidazolium ion (2) is the strongest acid that can exist at least partially protonated. Thus imidazole acts as the base catalyst in many enzymatic reactions, while imidazolium ion acts as the acid catalyst. In a particularly important example, the two imidazole rings of His-12 and His-119 in the enzyme[1] ribonuclease A act as the acid and base catalysts in the hydrolytic cleavage of ribonucleic acid (RNA).



1 Imidazole (Im) 2 Imidazolium ion (ImH^+)

RNA cleavage by the enzyme or by various other catalysts, as discussed below, occurs in two stages. In the first stage the phosphodiester linkage is converted to a cyclic phosphodiester, with cleavage of the chain. This ester interchange is catalyzed in the enzyme by the basic imidazole unit of His-12 and the acidic imidazolium unit of His-119. The ammonium ion on the side chain of Lys-41 is also important for catalysis. In biochemistry textbooks the mechanism commonly shown is as in Scheme I. The base catalyst is proposed to remove the proton from the attacking sugar hydroxyl group, and the acidic catalyst is proposed to protonate the leaving-group oxygen, converting it to the hydroxyl group of the product. In the second stage this process is essentially reversed: water, instead of a ribose hydroxyl group, adds back to open the phosphate ester ring. If this water enters in the same way that a ribose hydroxyl would, the two catalytic groups would just function as shown in Scheme I.

We decided to study the cleavage of RNA by imidazole and imidazolium groups as free species, not as part of an enzyme. There were several questions. (1) Would imidazole buffer catalyze RNA cleavage? (2) If so, what mechanism would it use? (3) Would this mechanism give us insight into the enzyme mechanism? (4) Would the preferred mechanism tell us how to design an effective enzyme mimic? We found that we saw such catalysis and were able to learn its mechanism. This indeed gave us new insight into the enzymatic process and led us to propose an enzyme mechanism different from that of Scheme I. Finally, the mechanistic information we obtained let us design a new mimic of the enzyme with improved properties.

Is RNA Cleaved by Simple Imidazole Buffers?

Using a new assay procedure for RNA cleavage,[2] we were able to observe catalysis by imidazole buffer.[3] After correcting away the part of the cleavage produced just by solvent or by the pH of the solution (by ex-

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(1) For reviews, see: (a) Blackburn, R.; Moore, S. *The Enzymes*; Academic Press: New York, 1982; Vol. 15, Chapter 12, pp 317–433. (b) Richards, F. M.; Wycoff, H. W. *The Enzymes*; Academic Press: New York, 1971; Vol. 4, Chapter 24, pp 647–806.

(2) Corcoran, R.; Labelle, M.; Czarnik, A. W.; Breslow, R. *Anal. Biochem.* **1985**, *144*, 563–568.

(3) Breslow, R.; Labelle, M. *J. Am. Chem. Soc.* **1986**, *108*, 2655–2659.

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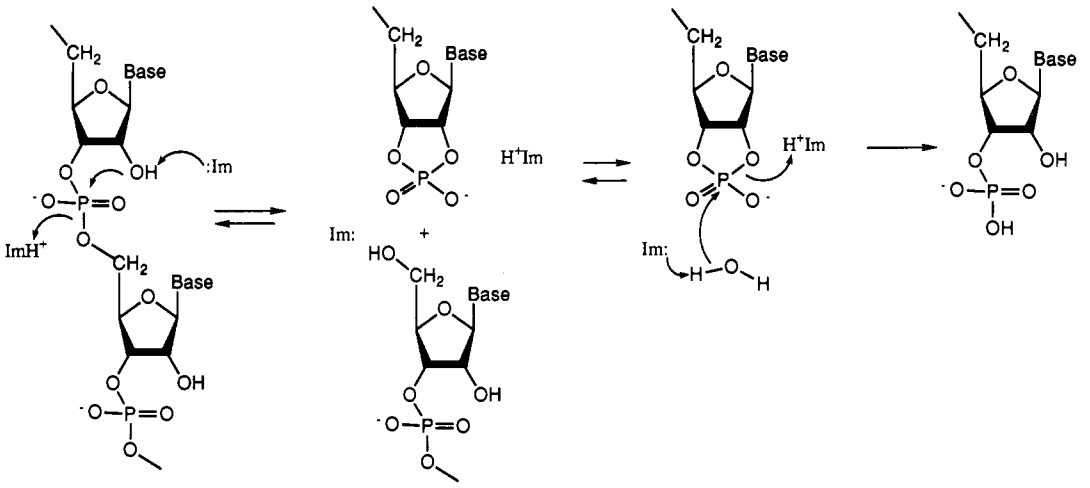
Scheme I
"Standard" Mechanism for Ribonuclease Action[a]



[a] A newer proposal is shown in Scheme VII.

trapolating the rate to zero buffer concentration), we saw a striking result. The rate had a maximum when both Im and ImH^+ were present, so both species were involved. However, the rate increased with only the first power of the buffer concentration. Thus the two buffer species were not acting at the same time in a single rate-determining step (or doubling the buffer concentration would have doubled the concentration of each species, leading to a 4-fold increase in the rate). We were able to show that there was no complexing that might have hidden a higher order kinetic dependence,[3] so this indicates that the two buffer species catalyze different steps of the cleavage process.

Reactions of phosphate esters frequently involve pentacoordinated intermediates, phosphoranes.[4] In our case, if the cleavage of the phosphate ester went through such an intermediate phosphorane (cf. **6**), then one buffer component could catalyze the formation of the intermediate while the other catalyst promoted the cleavage of the intermediate to form the cyclic phosphate product. With such a sequential mechanism (see Scheme III), a rate maximum can be seen when both species are present: when either the first or the second catalyst is at low concentration, the corresponding step becomes slow and rate determining, and the overall process is rapid only when both steps are catalyzed. If instead the two catalysts, Im and ImH^+, were cleaving RNA by parallel independent paths, no such rate maximum would be seen in the presence of both components; the rate would just get faster and faster as the buffer had increasing amounts of the better catalyst.

The first step might be catalyzed by either Im or ImH^+, with the other component catalyzing the second step. Two methods were used to decide between these possibilities. They both depend on the fact that another reaction accompanies the cleavage of RNA, an isomerization reaction.

To see this, we examined the cleavage of the simplest fragment of RNA, a dinucleotide. The first study[5] used UpU (**3**), the phosphate diester linking two uridine units. Later we saw[6] that similar results were observed with ApA, the dinucleotide with two adenosine groups. The starting material was 3′,5″-UpU, in which the phosphate group went from the 3 hydroxyl of one uridine to the 5 hydroxyl of the other. This is the kind of linkage seen in normal RNA. However, we found that cleavage of this compound, to form the 2′,3′-cyclic phosphate (**4**) of uridine along with a free uridine molecule (**7**), was accompanied by rearrangement.[5] Some 2′,5″-UpU (**5**) was also formed, by migration of the phosphate from the 3 hydroxyl to the neighboring 2 hydroxyl in the starting material.

Again this process was catalyzed by the imidazole buffer. However, in contrast to the cleavage reaction, the migration showed catalysis only by ImH^+, and not by Im. Such a migration could occur if the intermediate phosphorane was at a branch point, able both to fragment to the cleavage product *and* to reopen by ejecting the 3 hydroxyl, leaving the phosphate group attached to the 2 hydroxyl. The geometry of phosphoranes is such that a geometric change, called "pseudorotation", would have to occur before the 3 hydroxyl group could be ejected.[4]

If indeed the cleavage and isomerization reactions involved branching from the same phosphorane intermediate, this would prove that the ImH^+ was the catalyst for forming the intermediate; it was the only catalyst observed for the isomerization reaction. Observations with other buffers were consistent with this. With the imidazole buffer, the cleavage reaction dominated at all buffer ratios examined, and isomerization was only a minor reaction. However, with acetate buffer, the buffer-catalyzed reaction favored isomerization over cleavage.[5] Acetate ion is a weaker base than Im, so it does not send the intermediate as effectively along the cleavage pathway.

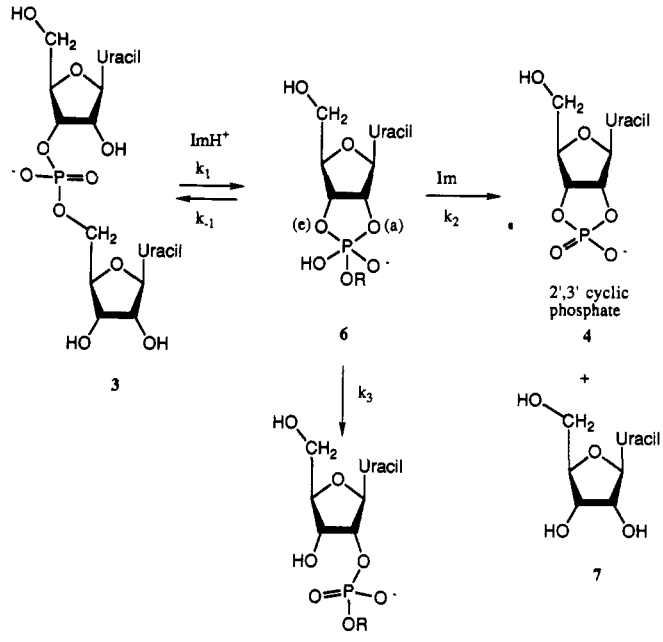
These observations were consistent with the idea that we were dealing with a two-step sequence (Scheme II) in which the ImH^+ was the first catalyst and Im the second catalyst, but they did not prove it. Definitive proof came with the observation of "negative catalysis".

How Does "Negative Catalysis" Establish the Mechanism?

As mentioned above, we found that the isomerization of 3′,5″ dinucleotides (**3**) to the 2′,5″ isomers (**5**), by

(4) Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1*, 70–78.
(5) Anslyn, E.; Breslow, R. *J. Am. Chem. Soc.* **1989**, *111*, 4473–4482.
(6) Breslow, R.; Huang, D.-L. *J. Am. Chem. Soc.* **1990**, *112*, 9621–9623.

Scheme II
Pathway by Which Imidazole Buffer Catalyzes the Cleavage of Dinucleotides Such as 3',5''-UpU While Simultaneously Catalyzing Their Isomerization to the 2',5''-UpU



migration of the phosphate linkage, is catalyzed by ImH^+ but not by Im. The situation is actually more unusual than that. With a given concentration of ImH^+, the rate of isomerization is *slowed* when Im is added (corrected as usual for pH effects).[6]

How is this possible? Normally there is no such thing as negative catalysis. If a "catalyst" would introduce a new pathway that is slower than the path without the catalyst, that slow path is simply not used. Of course added materials can inhibit other catalytic pathways, as with materials that poison hydrogenation catalysts. However, nothing like that is going on here.

With imidazole buffers, the cleavage reaction is much faster than the isomerization. Because the cleavage reaction shows cooperative sequential catalysis by both Im and ImH^+, the overall rate of disappearance of starting material is *increased* when Im is added to a given concentration of ImH^+.[6] Thus Im is a normal catalyst with respect to the rate of reaction of starting material, and also with respect to the rate of formation of cleavage products. The negative effect is seen only in the rate of isomerization.

This observation proves that the pathway involved is that shown in Scheme II. A common intermediate branches off to either cleavage or isomerization, and the major path shows positive catalysis throughout. The first step is catalyzed by ImH^+, so the rate of formation of *both* products shows catalysis by ImH^+. Then the cleavage of the intermediate is catalyzed by Im, but the isomerization is not. Adding Im decreases the steady-state concentration of the intermediate as more is diverted to cleavage product rather than isomerizing or returning to starting material. With a decreased concentration of the intermediate, the rate of formation of the isomer is less.

How do we know that the two paths branch off from an intermediate, rather than just branching off from the starting material? The observations were made early, when the reaction was run to only a few percent conversion of the starting material, so increasing the Im concentration would not have a significant effect on starting material concentration. By contrast, the intermediate **6** is always present at very low concentration as the result of a balance between its rate of formation and its rate of reaction. Speeding up one pathway will decrease the concentration of the intermediate and thus slow down the other pathway *provided that they branch off from the same intermediate.* Thus the curious observation of "negative catalysis" is really the strongest evidence for Scheme II.

Equations 1 and 2, the kinetic equations for this mechanism, make this clear.[6] (Some of the terms in the equations are there to account for steps that are not fully buffer catalyzed. For simplicity these steps are not shown in Scheme II. See ref 7 for an easy way to derive such equations.) For isomerization, Im appears only in the denominator, so increasing its concentration lowers the rate.

$$\text{rate constant of cleavage of UpU} = \frac{k_1k_2[ImH^+][Im] + k'_w}{k_{-1}[ImH^+] + k_2[Im] + k_3 + k_w} + k'[Im] + k''[ImH^+] \quad (1)$$

$$\text{rate constant of isomerization of UpU} = \frac{k_1k_3[ImH^+] + k''_w}{k_{-1}[ImH^+] + k_2[Im] + k_3 + k_w} \quad (2)$$

How Do These Catalysts Cleave RNA?

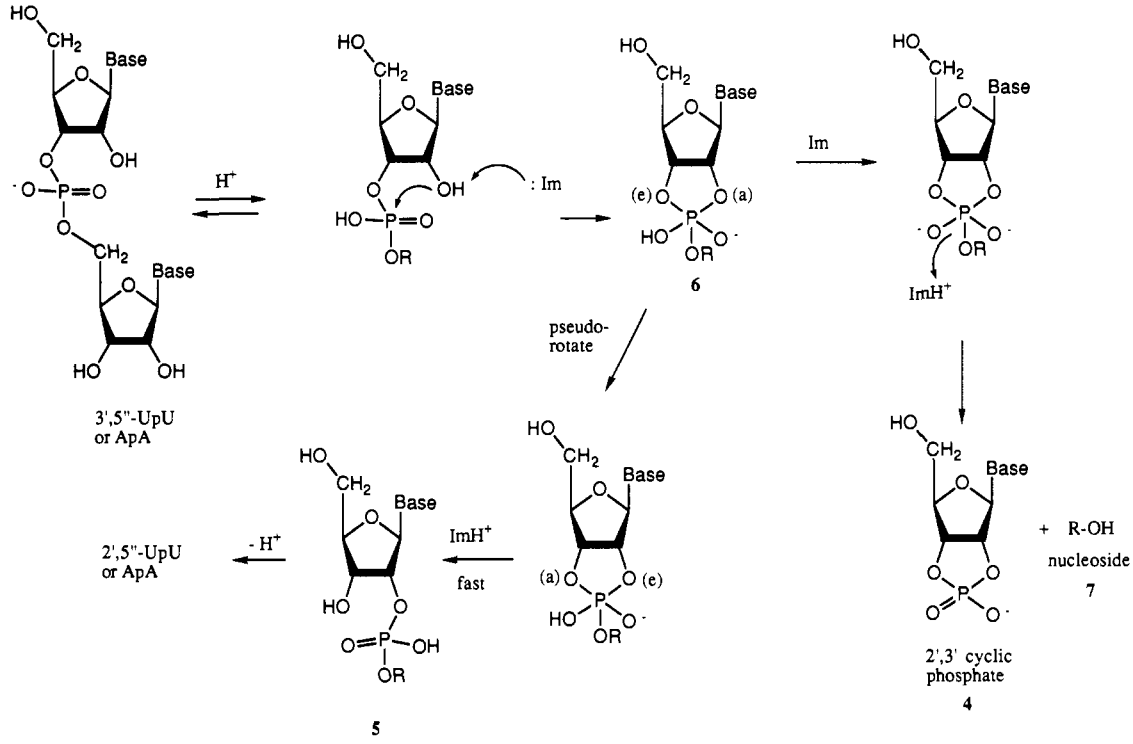
In the cleavage sequence (Scheme III), ImH^+ operates in the first step to convert the substrate to the phosphorane intermediate. Detailed mechanistic analysis[5] shows that the intermediate must be **6**, a phosphorane *mono*anion. Because this carries a proton on what used to be a phosphate oxyanion, the ImH^+ must be putting the proton on the substrate, activating the phosphoric acid species for addition of the neighboring hydroxyl group. This can add with assistance from an Im catalyst that removes its proton. (Reversible protonation of the substrate followed by general-base-catalyzed addition of the hydroxyl group is a sequence called specific acid/general base catalysis. Kinetically it behaves like catalysis by ImH^+, even though the two parts of ImH^+ acted in different steps.[8])

Now the Im catalyzes the conversion of this intermediate to the cleavage products **4** and **7**. Imidazole is a base and must remove the proton from intermediate **6**. That proton was added to make the ring closure easier, but now it must be removed to promote fragmentation in the forward direction. Then it seems likely that the proton that was removed, to form ImH^+, is used to protonate the leaving oxygen as it departs. The full mechanism is shown in Scheme III.

The proposed protonation of the leaving oxygen in the last step of the cleavage has no direct evidence. The overall sequence of the last two steps—removing a proton, then adding it elsewhere to promote

(7) Breslow, R. *J. Chem. Educ.* **1990**, *67*, 228–229.
(8) Chemical experience indicates that proton transfer from an imidazolium ion to a phosphate anion in a general-acid mechanism could not be rate limiting, so the alternative specific acid/general base mechanism must be involved.

Scheme III
Detailed Mechanism of the Reactions of Scheme II



cleavage—is related to the action of imidazole in many enzymes, such as chymotrypsin. We have pointed out that if the Im is converted to an ImH^+ in one step and the ImH^+ is then used in a later step, the ImH^+ will be kinetically invisible.[7] (It is interesting that the evidence requires that the intermediate cleaves in the forward direction by losing a proton to form a phosphorane dianion, but when the intermediate cleaves back to starting material, it does so *without* losing that proton first. The reasons for this subtlety have been discussed;[5] they do not necessarily apply to the enzymatic reaction.)

We have also examined[9] the cleavage of simple dinucleotides, and of models for them, with imidazole buffer and various metal ions such as Zn^{2+}. Whereas ribonuclease does not normally use metal ions in this way, it can act as a catalyst with added metal ions,[10] and other RNA-cleaving enzymes do use Zn^{2+}.

Does the Enzyme Ribonuclease Use Its Imidazole Groups in the Same Way?

With the simple buffer it was unlikely that both Im and ImH^+ could be tied down next to the substrate at the same time, and *sequential* bifunctional catalysis was observed. In the enzyme both catalytic groups are fixed in place, so they can be used at the same time to promote *simultaneous* bifunctional catalysis. However, the enzyme might still use a simultaneous version of the *mechanism* that was preferred in the simple chemical system. That seems to be the case.

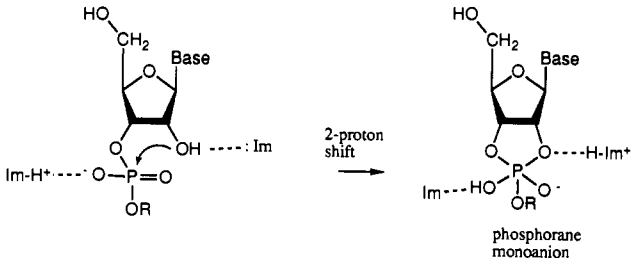
The essential point from our model studies has to do with the role of the ImH^+ group, the acid catalyst. We found that this first protonated the phosphate oxyanion of the substrate, to promote attack of the 2 hydroxyl group so as to form a phosphorane monoanion. Only later was a proton donated to the leaving oxygen by an ImH^+. In previous proposed mechanisms for the enzyme, the prior protonation of the phosphate by ImH^+ was not invoked, but there is good reason to believe that it occurs. In the X-ray structure of ribonuclease A with a bound substrate or substrate analogue,[11] the Im ring of His-12 is seen hydrogen bonded to the 2 hydroxyl of the ribose which must attack the phosphate, as expected if the imidazole is to remove that proton during attack. However, the ImH^+ ring of His-119 is seen hydrogen bonded to the anionic oxygen of the phosphate group. This is where it should be if it is to protonate that group, as we propose. There is also evidence of a strong hydrogen bond of this type from titration studies[12] on the enzyme.

Two protons move *simultaneously* in the enzyme-catalyzed reactions. Matta and Voh looked at the hydrolysis of cytidine 2',3'-cyclic phosphate by ribonuclease A,[13] corresponding to the second stage of enzymatic RNA hydrolysis. As we have mentioned above, it is reasonable to believe that the attack on the 2',3'-cyclic phosphate by water, in the forward hydrolysis direction, follows the same mechanism as would the attack by a ribose hydroxyl group to reverse the first step and re-form the dinucleotide. If two protons are moving in the *reverse* reaction, they must also be moving in the forward reaction (by the principle of microscopic reversibility). The evidence for simultaneous motion of two protons (Scheme IV) comes from use of the *proton inventory* method.

In D_2O all rapidly exchangeable protons in the enzyme or substrate will be deuteriums. If those protons

(9) Breslow, R.; Huang, D.-L.; Anslyn, E. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1746–1750.
(10) Eichhorn, G. L.; Clark, P.; Tarien, E. *J. Biol. Chem.* **1969**, *244*, 937–942.
(11) Campbell, C. L.; Petsko, G. A. *Biochemistry* **1987**, *26*, 8579–8584 and references cited therein.
(12) Reference 1b, pp 759–769.
(13) Matta, M. S.; Vo, D. T. *J. Am. Chem. Soc.* **1986**, *108*, 5316.

Scheme IV
Simultaneous Two-Proton Shift along Two Hydrogen Bonds[a]



[a] The protons are vibrating in indeterminate positions, and their motion may not be concerted, but the two positions of minimum energy, about which they vibrate, move at the same time.

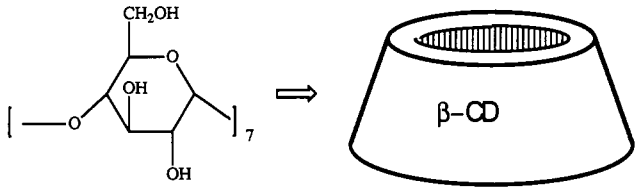
are moving in the rate-determining step, one can expect to see a slowing of the reaction by the isotope effect. This is observed with the ribonuclease reaction. However, when the solvent is a mixture of H_2O and D_2O, the situation is more interesting. If a *single* proton is moving, all the rates will lie on a straight line between the H_2O and the D_2O extremes; if two protons are moving, the line will be curved. The change from D to H is a form of catalysis, and the shape of the line indicates whether the reaction is first order or second order (or higher) in the catalyst. Matta and Vo saw[13] a curved line that could be fit by the theoretical line for *two* protons moving, each with an isotope effect near 2.0.

On the basis of all these facts, including in particular our finding that phosphate oxyanion protonation is involved in the nonenzymatic reaction, we proposed[5,9] the enzymatic mechanism shown in Scheme V. Further support for this mechanism came from some molecular modeling calculations by Karplus et al.[14] They simulated the hydrolysis of RNA by ribonuclease A using either our mechanism with the intermediate phosphorane anion or the alternative in which the proton is instead being placed directly on the leaving oxygen. They saw that indeed a phosphorane could be a stable intermediate on the path *provided it had been protonated so as to be a monoanion*, as we had proposed. They furthermore saw that this path, with prior protonation of the phosphate anion by the ImH^+ group, was actually the preferred one. Although such calculations cannot yet replace direct experimental evidence, they certainly support the idea that the enzyme uses a key feature of the mechanism that the simple model system preferred.

A Bifunctional Enzyme Mimic

Some years ago we initiated a study of β-cyclodextrin derivatives carrying two imidazole groups,[15] as mimics of ribonuclease. β-Cyclodextrin (**8**) is a doughnut-shaped molecule made up of seven glucose units in a ring.[16] It is water soluble and binds hydrophobic species into its cavity if they fit correctly. Thus it has often been used as a building block for the construction of artificial enzymes;[17] it furnishes the binding, and then additional groups can be used to furnish the catalysis.

With appropriate rigid reagents, it is possible to sulfonate the primary hydroxyl groups (on carbon 6) of two different sugar residues with geometric control. For instance, a biphenyldisulfonyl dichloride can sulfonate two primary hydroxyl groups of sugar units on opposite sides of the cyclodextrin ring.[18,19] We letter the glucose rings alphabetically; the result is the formation of a 6A,6D-functionalized cyclodextrin. With standard chemistry this was converted to the 6A,6D cyclodextrin bis(imidazole) **9**.



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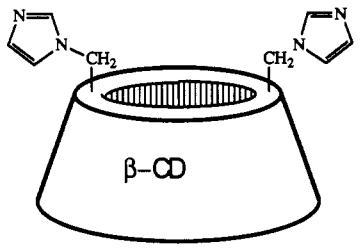


9 substituted on A,D glucoses

10 substituted on A,C glucoses

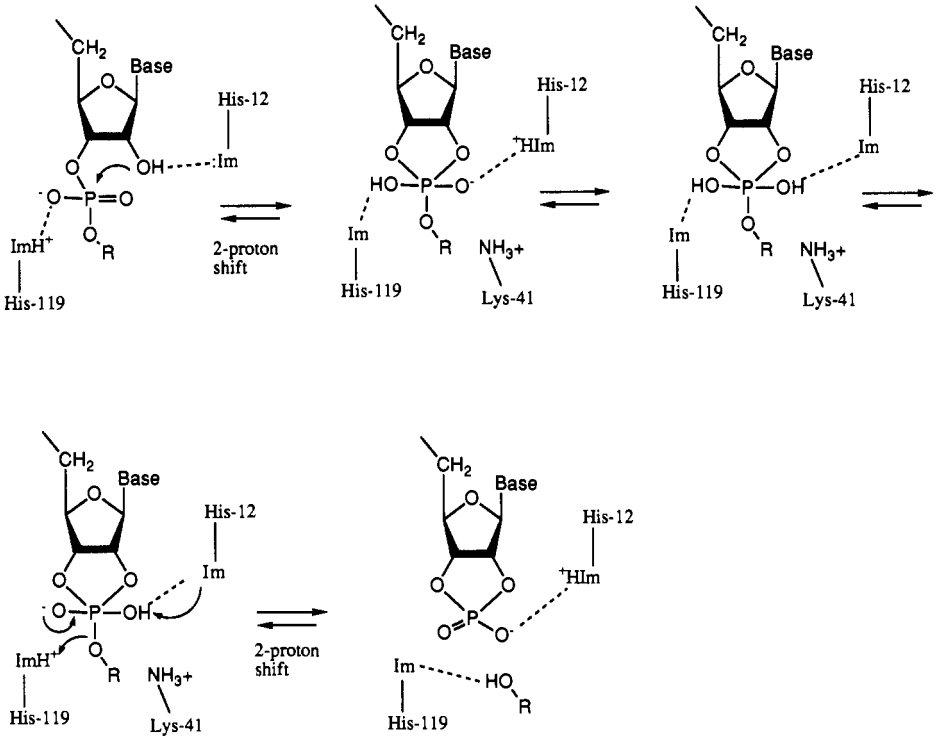
12 substituted on A,B glucoses

We selected a substrate that could bind into the cyclodextrin and that resembled an RNA derivative, at least formally. Molecular models indicated that the cyclic phosphate **11** could bind into the cavity so as to place the cyclic phosphate group in reach of the imidazole rings in **9**. This substrate is related to the 2′,3′-cyclic phosphate of a ribonucleotide, in the sense that it also has a five-membered phosphate group. Of course, there is a chemical difference between the two (**11** is more reactive), but RNA derivatives such as adenosine 2′,3′-cyclic phosphate do not fit the catalyst correctly and are not suitable substrates for this catalyst.[20]

The A,D isomer was constructed because it was believed that the enzyme used the mechanism of Scheme I, in which the Im delivers a water molecule to the cyclic phosphate group as the ImH^+ protonates the leaving group. The geometry of such a displacement (Scheme VI) would require the catalysts to be situated on opposite sides of the phosphate group, as in the A,D isomer. We found that **9** was indeed a reasonable catalyst for the hydrolysis of **11**, and the pH vs rate profile showed that it was operating as a bifunctional catalyst. That is, there was a rate maximum at pH 6.3, when the catalyst groups could be present as an equilibrium mixture of Im and ImH^+, with a lower rate at either lower pH (both ImH^+) or higher pH (both Im).

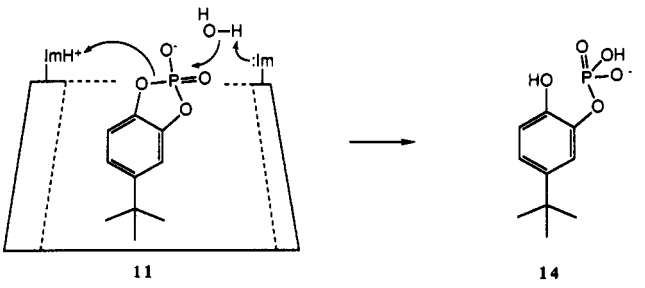
(14) Haydock, K.; Lim, C.; Brünger, A. T.; Karplus, M. *J. Am. Chem. Soc.* **1990**, *112*, 3826 and later work.
(15) Breslow, R.; Doherty, J.; Guillot, G.; Lipsey, C. *J. Am. Chem. Soc.* **1978**, *100*, 3227–3229.
(16) For an early review, see: Bender, M. L.; Komiyama, M. *Cyclodextrin Chemistry*; Springer-Verlag: New York, 1978.
(17) Cf.: (a) Breslow, R. *Science* **1982**, *218*, 532–537. (b) Tabushi, I. *Acc. Chem. Res.* **1982**, *15*, 66.
(18) Tabushi, I.; Yamamura, K.; Nabeshima, T. *J. Am. Chem. Soc.* **1984**, *106*, 5267.
(19) Anslyn, E.; Breslow, R. *J. Am. Chem. Soc.* **1989**, *111*, 5972–5973.
(20) Breslow, R.; Huang, D.-L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4080–4083.

Scheme V
Proposed Detailed Mechanism for the Hydrolysis of RNA by Ribonuclease[a]



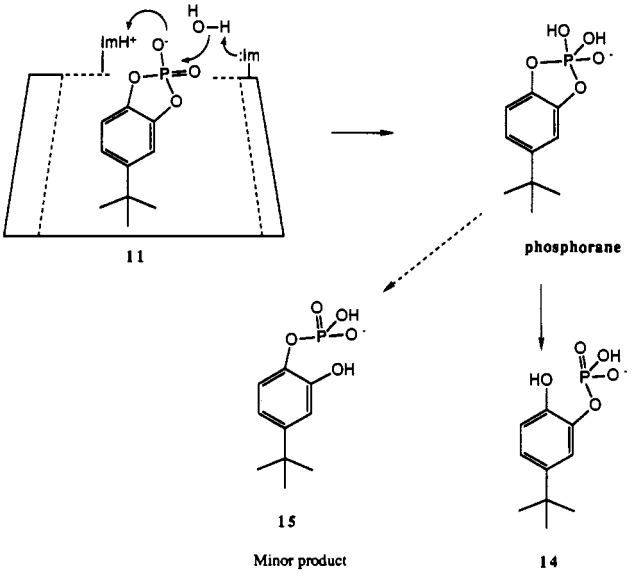
[a] The cyclization step is shown; for the hydrolysis of the cyclic phosphate, the same mechanism is run *backwards*, substituting H_2O for the alcohol ROH. Thus in the first step of the hydrolysis sequence there is again a two-proton shift as an Im delivers the water while an ImH^+ protonates the phosphate group. Lysine-41 is shown stabilizing the intermediate phosphorane anions, consistent with X-ray data. The switch in protonation state between the two imidazole groups in the middle steps is needed to achieve proper geometry. Note that after cyclization the enzyme ends up in a protonation state *reversed* from that of the starting enzyme, but is restored to its original state after hydrolysis. As we have pointed out (ref 5), this oscillation in enzyme states explains some otherwise curious observations.

Scheme VI
Direct Mechanism for the Bifunctional Cleavage of Substrate 11 by a Cyclodextrin Bis(imidazole)[a]



[a] This mechanism suggested that the two catalyst groups should be on opposite sides of the cavity.

Scheme VII
Preferred Mechanism for the Cleavage of 11[a]



[a] This mechanism is directly related to that for the enzyme in Scheme V and explains why the cyclodextrin with two imidazoles on neighboring glucose units is the best catalyst. The details of the conversion of the phosphorane to the product are not established, but may be related to those of Scheme V.

With another rigid disulfonyl reagent we could prepare[5] the 6A,6C cyclodextrin bis(imidazole) **10**. This was also a good catalyst for the hydrolysis of **11**, and in molecular models it was also possible for these Im and ImH^+ groups to assist in the mechanism of Scheme VI, even though they are less clearly on opposite sides of the cavity.

As a result of the studies described above, it seemed likely that these enzyme mimics had been constructed to perform the wrong mechanism. Our evidence indicated that we wanted the ImH^+ group to protonate the phosphate oxyanion in the first step, not the leaving group. This mechanism (Scheme VII) would require that the Im and the ImH^+ be near each other, not on opposite sides of the cavity. For this reason we used a disulfonyl reagent to functionalize the 6A,6B positions of β-cyclodextrin, and we prepared[19] 6A,6B cyclodextrin bis(imidazole) **12**. Models showed that this had a much better geometry to perform the process of Scheme VII, although the A,D and A,C isomers might still be able to use Scheme VII.

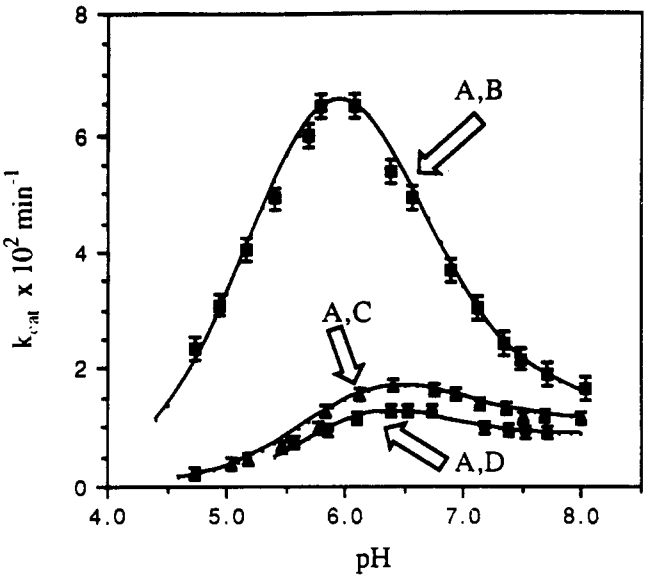
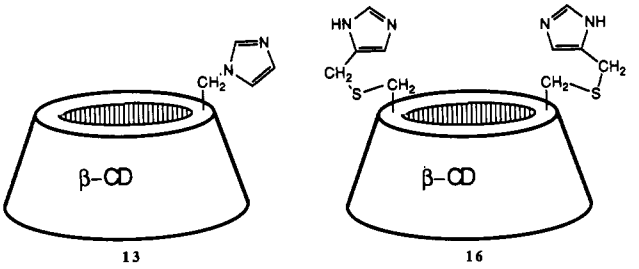


Figure 1. The k_{cat} at 25.0 °C for the hydrolysis of 11 fully complexed within the cavities of the A,B cyclodextrin bis(imidazole) 12 and the A,C and A,D isomers 10 and 9, respectively. Reprinted with permission from ref 19. Copyright 1989 American Chemical Society.

Gratifyingly, it turned out[19] that the 6A,6B isomer 12 was the best of the three catalysts for the hydrolysis of 11. As Figure 1 shows,[19] all of them have the bell-shaped pH vs rate profile that indicates bifunctional catalysis, but the highest rate is seen with the A,B isomer 12. This is consistent with the idea that this catalyst uses the mechanism of Scheme VII, not that of Scheme VI. Thus the mechanistic studies on simple imidazole buffer catalysis have guided the construction of the best catalyst in this case.

The dependence of catalytic effectiveness on the geometry of the catalyst suggests that in this case the bifunctional catalysis is *simultaneous*, not sequential. If the ImH^+ had protonated the phosphate group in the first step, and the Im had then acted in a second discrete step, we might have expected the intermediate protonated phosphate substrate to spin in the cavity so that the relative positions of the two catalytic groups would not matter. We found that indeed this system uses simultaneous bifunctional catalysis. The proton inventory method described above, with D_2O and H_2O mixtures, was applied here.[21] We saw that two protons are moving in the rate-determining step, and with isotope effects similar to those seen for the enzyme ribonuclease itself.

The proton inventory method is not unambiguous. The rates must be corrected for isotope effects on pH and pK's, as we did, and even then the method does not indicate *which* two protons are moving. In the enzyme, for instance, there are many protons whose bonding could change in the rate-determining step. In our case we were able to check this point. We had seen[15] that the monofunctional β-cyclodextrin 6-imidazole 13 is also a catalyst for the hydrolysis of 11. The pH vs rate profile indicated that it acted as a base catalyst, with its Im group, not as an acid catalyst. The proton inventory method showed that in this case only *one* proton was moving in the rate-determining step.[21] Thus it is clear that in the bifunctional case the two protons detected by the proton inventory method are indeed the two shown.



13 16

In our earliest work[15] we had observed a striking geometric selectivity in the hydrolysis of 11 catalyzed by cyclodextrin bis(imidazoles). With the A,D isomer 9 we saw high selectivity for the formation of hydrolysis product 14, with only a little of 15 formed. By contrast, ordinary hydrolysis of 11 in solution, even with imidazole buffers, yields an essentially equal mixture of 14 and 15. With our best catalyst, the cyclodextrin A,B isomer 12, we observe[22] very high selectivity for the formation of 14. Only 2% or so of the other product, 15, is formed. This is similar to the high selectivity of the enzyme, which hydrolyzes the 2′,3′-cyclic phosphate of a nucleoside regioselectivity so as to produce the 3′-phosphate and liberate the 2′-hydroxyl group.

The geometric selectivity in our enzyme mimic is expected from the mechanism of Scheme VII. Models show that the delivery of water by the Im group can be in line with the P–O bond to O-1 of 4-*tert*-butylcatechol cyclic phosphate, not O-2. This means that initially both the attacking water oxygen and O-1 of the substrate are *apical*, while the other three oxygens of the phosphorane intermediate are *equatorial*. Only apical oxygen atoms can be leaving groups on a phosphorane.[4] Unless the system undergoes a pseudorotation to interconvert apical and equatorial groups, which it apparently does not do, O-1 must be the leaving group and lead to the formation of product 16. The small amount of 17 formed could result from a little pseudorotation, or from imperfect geometrical control of the approach of the water molecule.

Imperfect control can be built in by adding flexibility. We prepared the A,D isomer of compound 16, in which the two imidazole rings are linked by flexible chains.[23] We found that this was also a bifunctional catalyst, with a rate maximum when both Im and ImH^+ are present, but now hydrolysis of 11 leads to an almost equal mixture of 14 and 15. Thus the selectivity seen with our best catalyst 12 must reflect its well-defined geometry.

Even 12 is not a perfect enzyme mimic. It does cleave its substrate 11 with a specificity constant (a hybrid of rate and binding) that is only 230-fold less than that of the enzyme for its cyclic phosphate substrate,[19] but our substrate is intrinsically more reactive. However, it is easy to propose further improvements, such as the addition of a surrogate for the Lys-41 of the enzyme and somewhat better control of flexibility. These improvements are best guided by consideration of the preferred mechanism we have discovered for RNA cleavage. This mechanism has given us insight into the pathway used by the enzyme and has guided us to the

(21) Anslyn, E.; Breslow, R. *J. Am. Chem. Soc.* **1989**, *111*, 8931–8932.

(22) Liu, T., unpublished work.

(23) Breslow, R.; Bovy, P.; Lipsey Hersh, C. *J. Am. Chem. Soc.* **1980**, *102*, 2115.

synthesis of our best enzyme mimic to date.[24]

Conclusions

Imidazole buffer can catalyze the cleavage of RNA and of simple dinucleotides. The mechanism is bifunctional and sequential, with a phosphorane intermediate. The first catalyst is the imidazolium ion, ImH^+, whereas the forward decomposition of the intermediate phosphorane is catalyzed by imidazole, Im. Definitive evidence for this sequence comes from the observation of "negative catalysis" of a rearrangement reaction by Im. This is a normal positive catalyst with respect to the disappearance of starting material, but it affects the partitioning of a common intermediate such that the rate of formation of one of the products is slowed with increasing Im.

The mechanism of action of the enzyme ribonuclease A is suggested to be similar, except that the bifunctional mechanism is now *simultaneous*, not sequential. This idea is supported by X-ray structural evidence and by calculated simulations. A simultaneous process is also indicated from results of the proton inventory method.

Cyclodextrin bis(imidazoles) can catalyze the hydrolysis of a cyclic phosphate substrate by a bifunctional mechanism. Again proton inventory studies show that this is *simultaneous* as with the enzyme. The isotope effects and pH effects are very similar for the enzyme and the enzyme mimic. Geometric optimization of the enzyme mimic was guided by our mechanistic evidence. The catalyst shows excellent selectivity of product formation, consistent with the mechanism invoked.

I thank my co-workers, named in the references, for their intellectual and experimental contributions. This work has been supported over the years by the National Institutes of Health and the Office of Naval Research.

Registry No. 1, 288-32-4; ribonuclease, 9001-99-4.

(24) For a recent review of some of the material in this Account, see: Breslow, R.; Anslyn, E.; Huang, D.-L. *Tetrahedron* **1991**, *47*, 2365–2376.