# *THE MECHANISM OF ACTION OF RIBONUCLEASE*

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*Abstract.*—The possible mechanisms of action of bovine pancreatic ribonuclease are discussed in the light of the detailed knowledge of the geometry of the active site that has been derived from studies of inhibitor binding by X-ray diffraction and nuclear magnetic resonance. When combined with a knowledge of the mechanism of phosphate ester hydrolysis, this information imposes severe geometric constraints on possible mechanisms of action of the enzyme. Two types of mechanism can be distinguished, the linear and the pseudorotation. The linear mechanism includes a catalytic role for both histidine residues at the active site and does not involve pseudorotation of the intermediate. In contrast, in the pseudorotation mechanism one histidine residue performs all the catalytic functions, while the other serves only to bind the phosphate anion; this necessarily involves pseudorotation of the intermediate and specific protonation of the leaving group by the enzyme.

The mode of binding of the product of the reaction, cytidine-3′-monophosphate, has been elucidated by X-ray diffraction and nuclear magnetic resonance. If the substrate binds in an analogous way, only the linear mechanism is possible. This mechanism is described in detail.

*Introduction.*—Bovine pancreatic ribonuclease A (RNase) is among the most extensively studied of enzymes (for reviews, see refs. 1–5). It catalyzes the hydrolysis of the 3′,5′-phosphodiester linkage of RNA at the 5′-ester bond in a two-step reaction. The first step is a transphosphorylation to give an oligonucleotide terminating in a pyrimidine 2′,3′-cyclic phosphate. The second is the hydrolysis of the cyclic phosphate to give a terminal 3′-phosphate.[6, 7] Numerous chemical studies have suggested that histidine 12, histidine 119, and lysine 41 are involved in the active site of the enzyme.[8, 9] This has been borne out by the crystal structure of RNase A[10] and of RNase S,[11] and by nuclear magnetic resonance (NMR) investigations.[12] However, the detailed enzymatic mechanism has remained undetermined, even though several proposals have been made.[13–16]

In this paper, we shall present a mechanism which is an extension of that proposed by Rabin and co-workers.[13] Current knowledge of the mechanism of hydrolysis of phosphate esters,[17, 18] together with the results of studies of RNase by NMR[19] and X-ray diffraction,[20, 21] enables us to describe this mechanism in a detail not previously attainable for this enzyme.

The mechanisms proposed by Witzel[14] and Rabin[13] are shown schematically in Figure 1. (In this figure and in the text, —OR represents a 5′-linked nucleoside.) Two aspects of Witzel's mechanism are difficult to reconcile with the present knowledge of the enzyme. Results of studies by NMR spectroscopy,[19] X-ray



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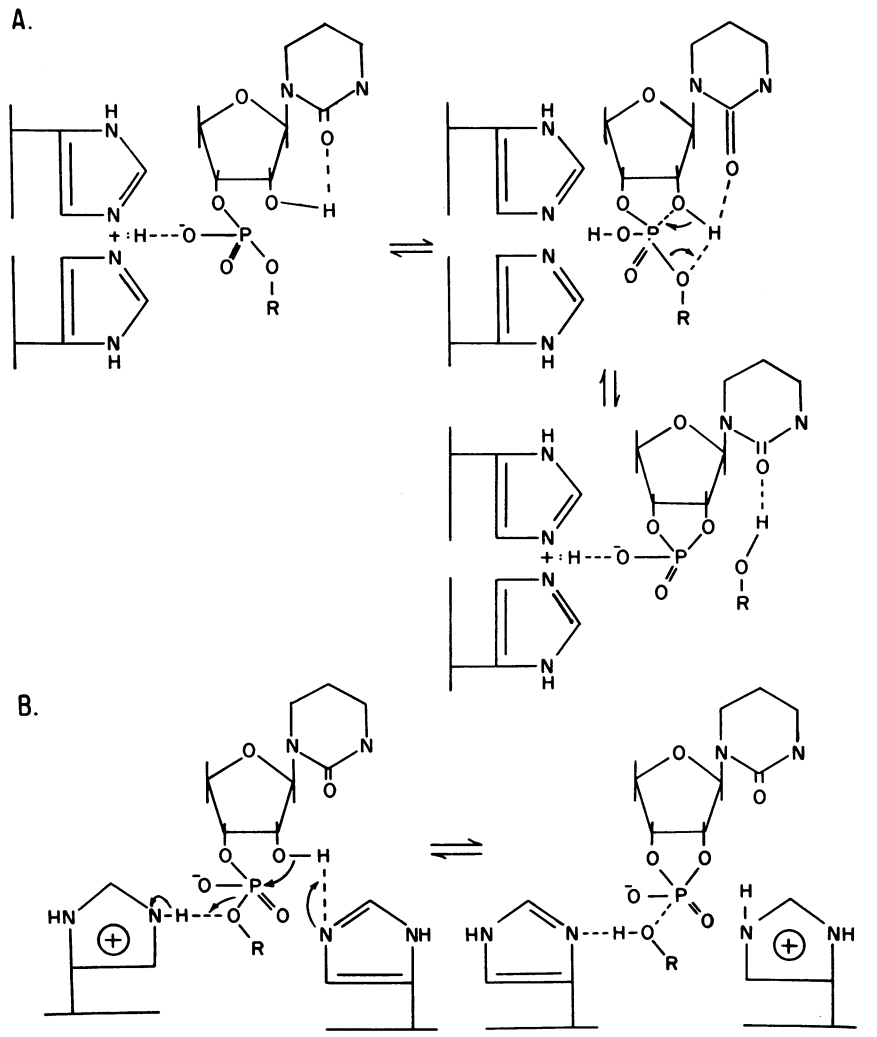


FIG. 1.—Mechanisms proposed for RNase by Witzel[14] (*A*) and Findlay *et al.*[13] (*B*). These mechanisms are schematic, and the spatial relationship of the residues is not specified. They are written so that the forward direction (with R a 5′-linked nucleoside) represents the first step of the reaction, while the reverse direction (with R = H) represents the second step. In mechanism *B*, there are binding sites for the bases on either side of the phosphodiester group; in mechanism *A*, these explicitly do not exist.

diffraction,[20, 21] and ultraviolet spectroscopy[22] show clearly that, contrary to Witzel's suggestion,[14] the pyrimidine ring does bind to the enzyme. Furthermore, the model for the structure of the complex of cytidine 3′-monophosphate (the product of the action of RNase on cytidyl dinucleotides) with RNase derived from NMR[19] and X-ray crystallography[20, 21] suggests that the 2-carbonyl group of the pyrimidine ring is unlikely to be available to hydrogen-bond to the ribose 2′—OH. Finally, the normal shape of the titration curves of histidines 12 and 119 makes the existence of a hydrogen bond between them most un-

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likely,[23] even in the free enzyme. Since the phosphate group of bound mononucleotide inhibitors appears to be between these two histidine residues,[19, 20] such a hydrogen bond seems to be ruled out in the presence of substrate or inhibitor.

The basic features of the mechanism proposed by Rabin[13] are consistent with the results of both NMR and X-ray crystallography, and indeed the NMR results make it possible to identify each histidine with its proposed role in the reaction.[12, 24] Histidine 12 must be the residue which accepts a proton from the 2′—OH group, while histidine 119 would form a hydrogen bond to the phosphate ester oxygen in the first step, and to the water molecule in the second step of the reaction. In a criticism of this mechanism, Scheraga and Rupley[2] pointed out that, since the two histidines have different protonation states in the two steps of the reaction, the pH dependence of $1/K_m$ for the two steps should be different, unless the histidines both have the same pK. There is evidence that the pH dependence of $1/K_m$ is identical for uridine 2′,3′-cyclic phosphate and uridyluridine.[14] However, the pK values of histidines 12 and 119 differ by only 0.4 pH unit, and it is unlikely that this would produce a detectable difference in the pH dependence of the kinetic parameters. A further criticism of Rabin's mechanism is that it involves a transition state protonation of the leaving group (—OR) by histidine 119. Since $—O^-$ is a much better hydrogen-bond acceptor than —OR, it would be expected to be the preferred site of interaction for histidine 119 in the intermediate or transition state. Deavin, Mathias, and Rabin[25] have stated that an enzyme-substrate complex in which the histidine was bound to the $—O^-$ would be an abortive one; we shall suggest below that this may not be so.

Hammes[16] has proposed a modification of Rabin's[13] mechanism in which the protonation states of the histidine residues are the same in both steps of the reaction. He proposes that one histidine performs all the catalytic functions—accepting a proton from the 2′—OH *and* protonating the leaving group in the first step, and also hydrogen-bonding to the water molecule in the second step. The other histidine would simply bind the phosphate group.

*Linear Mechanism.*—The mechanism which we propose is shown in Figure 2. The positions of histidine 12, histidine 119, and lysine 41 relative to each other and to the phosphate group of the substrate are derived from studies of RNase inhibitor complexes by NMR[12, 19] and by X-ray diffraction[20, 21]. The geometrical restrictions about the substrate are derived from a mechanism for phosphate ester hydrolysis,[17] which proposes that the hydrolysis proceeds through a pentacovalent intermediate with the geometry of a trigonal bipyramid. This mechanism consists of four postulates: (1) if a five-membered ring is present, ring strain is minimized if it spans one apical and one equatorial position;[26, 27] (2) more electronegative groups preferentially occupy apical positions[17] (by analogy to the alkylfluorophosphoranes[28]); (3) pseudorotation[29] of the intermediate may occur; and (4) groups must enter and leave the intermediate from apical positions (for a review, see ref. 18).

The combination of these spatial restrictions about the phosphorus atom and those imposed by the enzyme allows us to specify the geometry of the reaction rather precisely. Evidence from studies of the specificity of the enzyme[30] indi-

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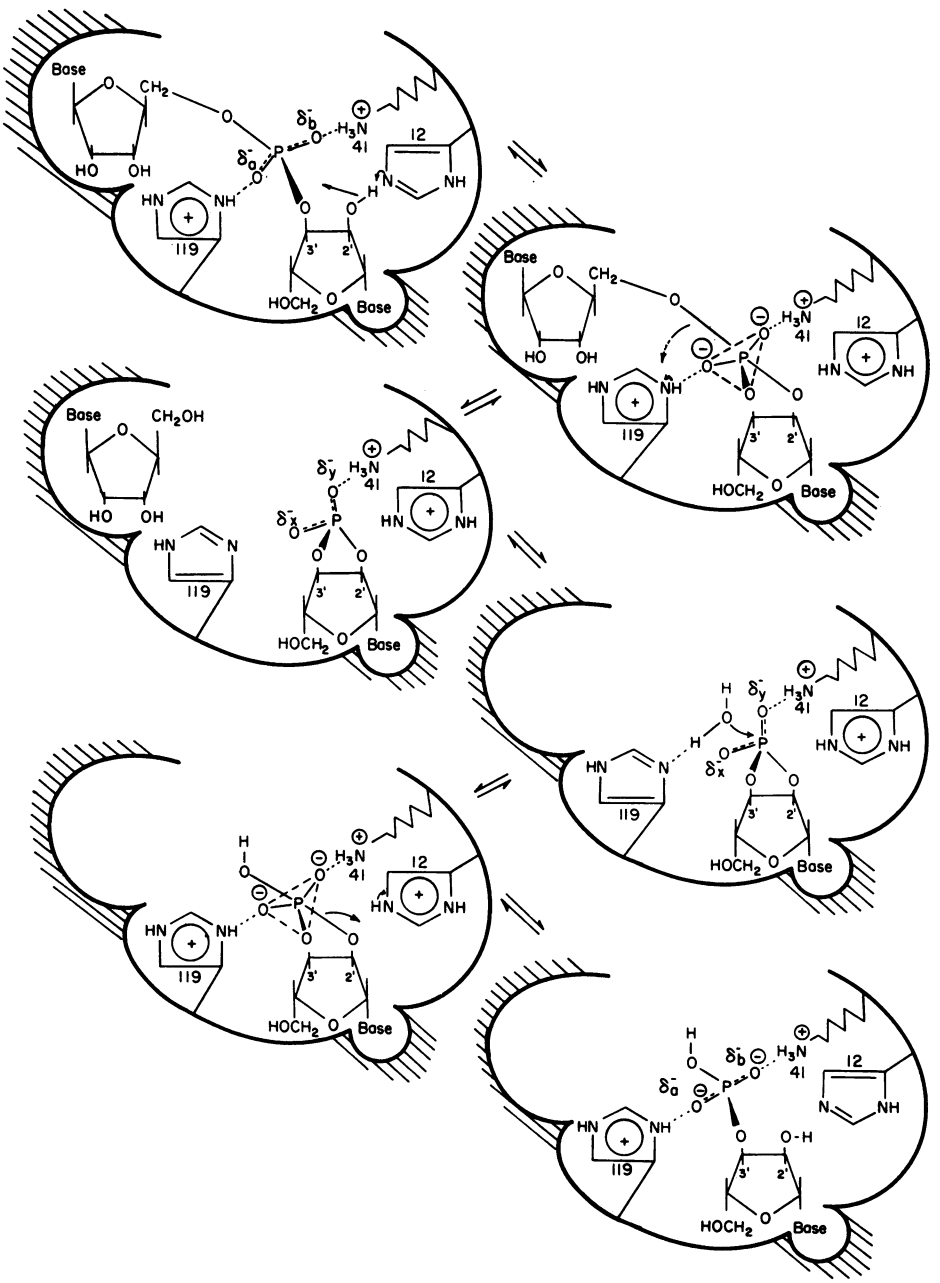


FIG. 2.—Proposed mechanism of action of RNase. The relative positions of the substrate and the amino acid side-chains deduced from X-ray crystallography and NMR spectroscopy are shown, as far as is possible in two dimensions. The shaded areas represent binding sites for the two nucleoside bases. The notation $\delta^-$ is used to indicate the uncertainty in the negative charge on the phosphate oxygens: $(\delta_a^- \sim \delta_b^- \sim 1/2)$ and $(0 < \delta_x^- < 1/2 < \delta_y^- < 1)$. In the last structure, the ionization states shown are those which result from the reaction; optimal binding of the product results when the phosphate group is doubly ionized and histidine 12 is protonated.

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cates that there is a binding site on the enzyme for the nucleoside on the 5′-side of the phosphate group, and the results of X-ray diffraction[20] suggest the spatial relationship between the two nucleoside binding sites shown in Figure 2. This binding configuration places the phosphate group near the side of the active site cleft occupied by histidine 119. We suggest that the dinucleotide binds to the enzyme by both bases and by an interaction between the phosphate group and histidine 119. There may also be an interaction between the phosphate and lysine 41, as shown in Figure 2. The histidine 12 accepts the proton from the 2′—OH, activating the 2′—oxygen for attack on the phosphorus to form the pentacovalent intermediate. The geometry of this intermediate is restricted by the relative orientations of the binding groups described above and by the requirement that the attacking 2′—oxygen must be apical. In the pentacovalent intermediate, each of the nonesterified phosphate oxygens has a full formal charge, strengthening the interaction with histidine 119 and lysine 41. The geometry is constrained in such a way that the —OR group is apical (see Fig. 2) and can thus leave directly to give the cyclic phosphate. It should be pointed out that the mechanism in Figure 2 is drawn out in detail for clarity—several of the steps shown may in fact be concerted. In addition the conformational isomerizations of the enzyme,[16] one of which probably involves a movement of lysine 41 toward the phosphate group,[19] are not shown explicitly.

It cannot be specified with absolute certainty whether the leaving $RO^-$ group receives its proton from histidine 119, histidine 12, or water. However, consideration of the position of the —OR group suggests that, of the two histidines, histidine 119 is the more likely proton donor. The pK value of histidine 119 is known to be sensitive to the exact position of the phosphate group,[19] and the slight movement of the phosphate on cyclization toward the opposite side of the active site cleft would be expected to lower its pK value. This, together with the reduction in the negative charge density at the nearby phosphate oxygen, would facilitate the loss of a proton to $RO^-$ or to water, and subsequent hydrogen bonding to a water molecule to initiate the second step. Some movement of histidine 119 might be necessary to accommodate the water molecule; from the X-ray model this appears to be feasible.[20] In contrast, the pK value of histidine 12 is insensitive to the position of the phosphate group,[19] and it would therefore be expected to remain protonated. The X-ray model[20] indicates that the proton on histidine 119 is positioned in such a way that it may be bound to the $—O^-$ and subsequently transferred directly to the leaving group. This proton transfer could be concerted with the departure of the —OR group and the movement of the phosphate. No distinction between the possible pathways of protonation of the leaving group can be made at this time; in Figure 2 we have shown the simplest direct pathway.

The second step of the reaction—the hydrolysis of the cyclic phosphate—basically follows the mechanism proposed by Rabin[13] (cf. Figs. 1*B* and 2). It is therefore the exact reverse of the first step only if histidine 119, in the first step, donates a proton directly to the leaving group in a concerted fashion. In the second step, the leaving group is specified not only by the geometry of the inter-

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mediate, but also by the ability of histidine 12 to act as a proton donor to the 2′—oxygen, while there is no corresponding group near the 3′—oxygen.

*Pseudorotation Mechanism.*—In addition to the pathway described above (shown schematically as *A* in Fig. 3), a second pathway (*B* in Fig. 3) involving pseudorotation of the pentacovalent intermediate is possible on purely chemical grounds. In pathway *A* of Figure 3, the leaving —OR group is in the apical position in the first-formed pentacovalent intermediate (IIa), and the reaction can thus proceed directly to product. In pathway *B*, the initial intermediate has the —OR group in an equatorial position (IIb). This intermediate must pseudorotate to IIIb to place —OR in an apical position so that it can leave to give the product.[32] The initial intermediate of pathway *B* has an —$O^-$ group in an apical position. According to the "rules" summarized above, this would

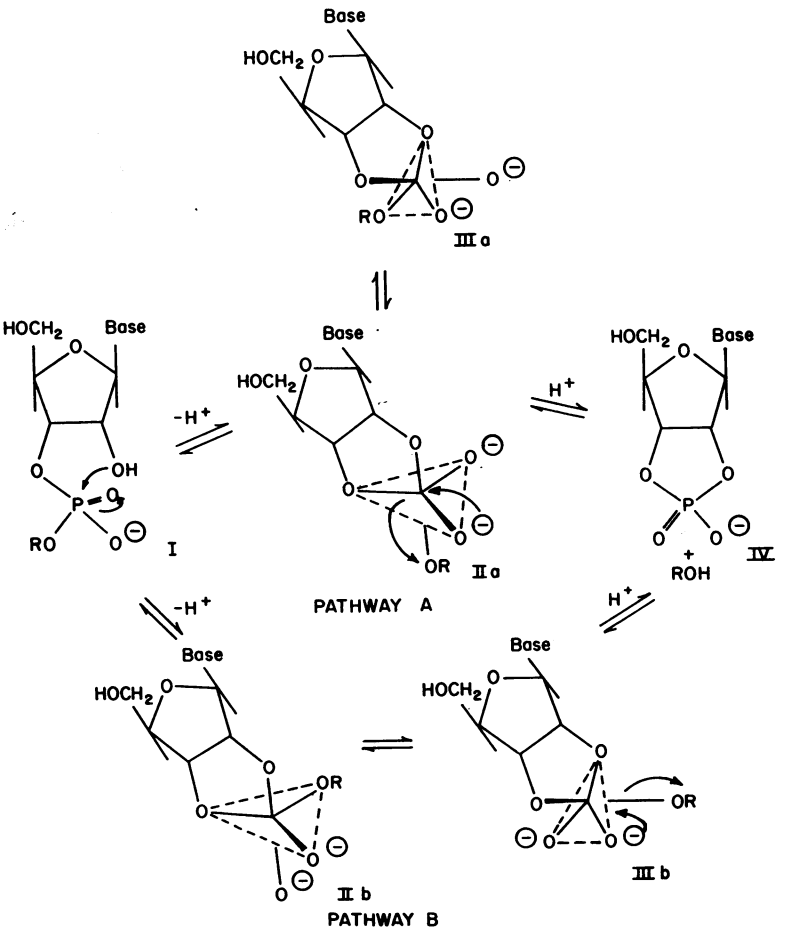


FIG. 3.—Possible mechanisms for the reaction catalyzed by RNase. The second step is the reverse of the reactions shown, with R = H. In the pentacovalent intermediates, the phosphorus atom in the center of the trigonal bipyramid is not specifically lettered.

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be strongly unfavored, since —O⁻ is more electropositive than —OR[31]. Protonation of the apical—O⁻ by a group on the enzyme could be invoked to favor the formation of intermediate IIb, and removal of the proton would then facilitate pseudorotation.[15] The mechanism proposed by Hammes,[16] in which the same histidine residue interacts first with the 2′—oxygen and then with the —OR group, would seem to require either a substantial movement of this histidine (which seems unlikely from the results of X-ray diffraction[20]) or a pseudorotation as shown in pathway *B*.[32]

Mechanism *B* may be considered only if specific protonation of the leaving group is invoked as a necessary part of the mechanism. This limitation arises because in the absence of specific protonation both the apical substituents, —OR and the 3′—oxygen of the ribose (Fig. 3, IIIb), should be equally good leaving groups. Thus one of the products would be a 2′,5′-linked dinucleotide. The work of Brown, Dekker, and Todd[6] shows that this compound (which is not a substrate for RNase) is not detectable to the limit of their measurements.

*Conclusion.*—NMR[12, 19] and X-ray diffraction[20, 21] studies of inhibitor-enzyme complexes show that the 2′—OH group of the inhibitor is close to histidine 12, while the phosphate group is close to histidine 119. The —OR-group binding site is fairly close to histidine 119 and on the opposite side of the cleft from histidine 12, as shown in Figure 2. This binding configuration is not compatible with any mechanism which requires that *both* the 2′—OH and the —OR leaving group lie close to the same histidine residue.[15, 16, 32] In contrast, the structural evidence is entirely compatible with the linear mechanism shown in Figure 2.

The unique specificity and catalytic activity of ribonuclease derive from two major factors. First, the binding of the substrate imposes geometric constraints which define the pathway of the reaction. Second, the function of the histidine residues in proton transfer is promoted by their location in close juxtaposition to the appropriate groups of the substrate. The mechanism proposed in this paper describes in detail the interactions that produce these effects.

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