$$k_3 = \frac{k_3' + \frac{[H^+]}{K_{A5}}k_3'' + \frac{[H^+]^2}{K_{A5}K_{B5}}k_3''' + \frac{[H^+]^3}{K_{A5}K_{B5}K_{C5}}k_3''''}{\left(1 + \frac{[H^+]}{K_{A5}} + \frac{[H^+]^2}{K_{A5}K_{B5}} + \frac{[H^+]^3}{K_{A5}K_{B5}K_{C5}}\right)}$$

$$k_{-3} = \frac{k_{-3}' + \frac{[H^+]}{K_{A6}}k_{-3}'' + \frac{[H^+]^2}{K_{A6}K_{B6}}k_{-3}''' + \frac{[H^+]^3}{K_{A6}K_{B6}K_{C6}}k_{-3}''''}{\left(1 + \frac{[H^+]}{K_{A6}} + \frac{[H^+]^2}{K_{A6}K_{B6}} + \frac{[H^+]^3}{K_{A6}K_{B6}K_{C6}}\right)}$$

$$k_4 = \frac{k_4' + \frac{[H^+]}{K_{A4}}k_4'' + \frac{[H^+]^2}{K_{A4}K_{B4}}k_4''' + \frac{[H^+]^3}{K_{A4}K_{B4}K_{C4}}k_4''''}{\left(1 + \frac{[H^+]}{K_{A4}} + \frac{[H^+]^2}{K_{A4}K_{B4}} + \frac{[H^+]^3}{K_{A4}K_{B4}K_{C4}}\right)}$$

$$k_{-4} = \frac{k_{-4}' + \frac{[H^+]}{K_{A5}}k_{-4}'' + \frac{[H^+]^2}{K_{A5}K_{B5}}k_{-4}''' + \frac{[H^+]^3}{K_{A5}K_{B5}K_{C5}}k_{-4}''''}{\left(1 + \frac{[H^+]}{K_{A5}} + \frac{[H^+]^2}{K_{A5}K_{B5}} + \frac{[H^+]^3}{K_{A5}K_{B5}K_{C5}}\right)}$$

$$k_s = \frac{k_s' + \frac{[H^+]}{K_{A3}}k_s'' + \frac{[H^+]^2}{K_{A3}K_{B3}}k_s''' + \frac{[H^+]^3}{K_{A3}K_{B3}K_{C3}}k_s''''}{\left(1 + \frac{[H^+]}{K_{A3}} + \frac{[H^+]^2}{K_{A3}K_{B3}} + \frac{[H^+]^3}{K_{A3}K_{B3}K_{C3}}\right)}$$

$$k_p = \frac{k_p' + \frac{[H^+]}{K_{A4}}k_p'' + \frac{[H^+]^2}{K_{A4}K_{B4}}k_p''' + \frac{[H^+]^3}{K_{A4}K_{B4}K_{C4}}k_p''''}{\left(1 + \frac{[H^+]}{K_{A4}} + \frac{[H^+]^2}{K_{A4}K_{B4}} + \frac{[H^+]^3}{K_{A4}K_{B4}K_{C4}}\right)}$$

$$k_5 = \frac{k_5' + \frac{[H^+]}{K_{A2}}k_5'' + \frac{[H^+]^2}{K_{A2}K_{B2}}k_5''' + \frac{[H^+]^3}{K_{A2}K_{B2}K_{C2}}k_5''''}{\left(1 + \frac{[H^+]}{K_{A2}} + \frac{[H^+]^2}{K_{A2}K_{B2}} + \frac{[H^+]^3}{K_{A2}K_{B2}K_{C2}}\right)}$$

$$k_{-5} = \frac{k_{-5}' + \frac{[H^+]}{K_{A3}}k_{-5}'' + \frac{[H^+]^2}{K_{A3}K_{B3}}k_{-5}''' + \frac{[H^+]^3}{K_{A3}K_{B3}K_{C3}}k_{-5}''''}{\left(1 + \frac{[H^+]}{K_{A3}} + \frac{[H^+]^2}{K_{A3}K_{B3}} + \frac{[H^+]^3}{K_{A3}K_{B3}K_{C3}}\right)}$$

$$k_6 = \frac{k_6' + \frac{[H^+]}{K_{A1}}k_6'' + \frac{[H^+]^2}{K_{A1}K_{B1}}k_6''' + \frac{[H^+]^3}{K_{A1}K_{B1}K_{C1}}k_6''''}{\left(1 + \frac{[H^+]}{K_1}\right)\left(1 + \frac{[H^+]}{K_{A1}} + \frac{[H^+]^2}{K_{A1}K_{B1}} + \frac{[H^+]^3}{K_{A1}K_{B1}K_{C1}}\right)}$$

$$k_{-6} = \frac{k_{-6}' + \frac{[H^+]}{K_{A2}}k_{-6}'' + \frac{[H^+]^2}{K_{A2}K_{B2}}k_{-6}''' + \frac{[H^+]^3}{K_{A2}K_{B2}K_{C2}}k_{-6}''''}{\left(1 + \frac{[H^+]}{K_{A2}} + \frac{[H^+]^2}{K_{A2}K_{B2}} + \frac{[H^+]^3}{K_{A2}K_{B2}K_{C2}}\right)}$$

Relaxation Spectra of Ribonuclease. V. The Interaction of Ribonuclease with Cytidylyl-3′:5′-cytidine[1]

James E. Erman and Gordon G. Hammes

Contribution from the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received July 14, 1966*

Abstract: The interaction of ribonuclease with a dinucleoside phosphate, cytidylyl-3′:5′-cytidine, has been studied with a stopped-flow, temperature-jump technique. At 15° in the pH range 6–7 two relaxation processes were observed. One relaxation time was independent of concentration and pH and is characteristic of an intramolecular isomerization of the dinucleoside phosphate–enzyme complex. The second relaxation time was dependent on the concentration and pH and is characteristic of the binding of the substrate to the enzyme. These results along with the results of previous studies have been incorporated into a schematic formal mechanism for the hydrolysis of cytidylyl-3′:5′-cytidine to cytidine 3′-phosphate and cytidine.

The ribonuclease-catalyzed hydrolysis of ribonucleic acid proceeds *via* a pyrimidine cyclic phosphate intermediate.[2] This catalytic hydrolysis of ribonucleic acid has been studied to some extent,[3,4] but since the system becomes quite inhomogeneous as ribonucleic acid is hydrolyzed, the data are difficult to interpret. Dinucleoside phosphates have been used to investigate the steady-state kinetics of the formation of the pyrimidine cyclic phosphate intermediate.[5] The second half of the reaction, the hydrolysis of the pyrimidine cyclic phosphate, has been investigated by steady-state techniques with cytidine 2′:3′-cyclic phosphate[6] and uridine 2′:3′-cyclic phosphate[7] as substrates.

The equilibrium temperature-jump method has been used to study the dynamic behavior of ribonuclease[8] and its interaction with cytidine 3′-phosphate, the product of hydrolysis of cytidine 2′:3′-cyclic phosphate, and with various competitive inhibitors of ribonuclease.[9] Since

(1) This work was supported by a grant from the National Institutes of Health (GM 13292).
(2) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).
(3) L. Vandendriessche, *Arch. Biochem. Biophys.*, **65**, 347 (1956).
(4) H. Edelhock and J. Coleman, *J. Biol. Chem.*, **219**, 351 (1956).
(5) H. Witzel and E. A. Barnard, *Biochem. Biophys. Res. Commun.*, 7, 294 (1962).
(6) D. G. Herries, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, **85**, 127 (1962).
(7) C. S. Cheung and H. J. Abrash, *Biochemistry*, **3**, 1883 (1964).
(8) T. C. French and G. G. Hammes, *J. Am. Chem. Soc.*, **87**, 4669 (1965).
(9) R. E. Cathou and G. G. Hammes, *J. Am. Chem. Soc.*, **87**, 4674 (1965).

the equilibrium between cytidine 2′:3′-cyclic phosphate and cytidine 3′-phosphate lies very much in favor of cytidine 3′-phosphate[10] and because of the very fast rate of conversion of cytidine 2′:3′-cyclic phosphate to cytidine 3′-phosphate, the interaction of pyrimidine cyclic phosphates and dinucleoside phosphates with ribonuclease could not be studied with the equilibrium temperature-jump method. In an accompaning paper[11] a stopped-flow temperature apparatus, which permits application of a temperature-jump perturbation 16 msec after mixing of reactants, was used to study the interaction of ribonuclease with cytidine 2′:3′-cyclic phosphate. In this paper, the results of a similar study on the interaction of cytidylyl-3′:5′-cytidine with ribonuclease are reported.

Experimental Section

The experimental methods and ribonuclease samples were the same as previously described.[11] Cytidylyl-3′:5′-cytidine was obtained from Gallard-Schlesinger Chemical Manufacturing Corp. lot no. B2669 and B4530. The purity was checked by paper chromatography using a 2-propanol–water–ammonia solvent system.[2] Concentration of the dinucleoside phosphate was determined spectrophotometrically using a molar extinction coefficient of 26,100 at 279 mμ in 0.1 *N* HCl. The Michaelis constants for cytidylyl-3′:5′-cytidine were determined spectrophotometrically by the procedure of Witzel and Barnard.[6]

Results and Treatment of Data

The experimental results for the ribonuclease–dinucleoside phosphate system are very similar to those found with ribonuclease–cytidine 2′:3′-cyclic phosphate. At 15°, two relaxation processes are observed. One, designated τ_7, is independent of substrate concentration and pH in the pH region where it was observed, pH 6.0 to 7.0. The other relaxation time is designated by τ_8 and is dependent upon the concentration of substrate. The relaxation process is characteristic of the binding of the dinucleoside phosphate to ribonuclease and was observed at pH 6.0 and 6.5. Attempts to extend these measurements to pH 5.5 and 7.0 were unsuccessful.

The simplest mechanism consistent with these two relaxation times and the isomerization of the native enzyme is

$$\begin{array}{c} E' \\ k_1 \uparrow\downarrow k_{-1} \\ E \end{array} + S \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} ES_1' \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} ES_2' \qquad (1)$$

An expression for $1/\tau_1$ is given in the accompanying paper;[11] $1/\tau_8$ is given by

$$1/\tau_8 = k_8([\bar{E}] + [\bar{S}]) + k_{-8} \qquad (2)$$

and $1/\tau_7$, at high substrate concentrations, is given by

$$1/\tau_7 = k_7 + k_{-7} \qquad (3)$$

$[\bar{E}]$ and $[\bar{S}]$ are the equilibrium concentrations of the free enzyme species, E, and the substrate, respectively. (This assumes the decomposition of ES_2 is slow relative to the time scale of the measurements.) The expressions for the relaxation times were derived with the assumption that $1/\tau_8$ is much greater than $1/\tau_7$ and that both $1/\tau_8$ and $1/\tau_7$ are much greater than $1/\tau_1$, which is in agreement with the experimental results. Plots of $1/\tau_8$ *vs.* $([\bar{E}] + [\bar{S}])$ are linear and permit the determination of k_8 and k_{-8} (see Figure 1). The results are collected in Table I along with the values of $1/\tau_7$ and the Michaelis constants, K_S'.

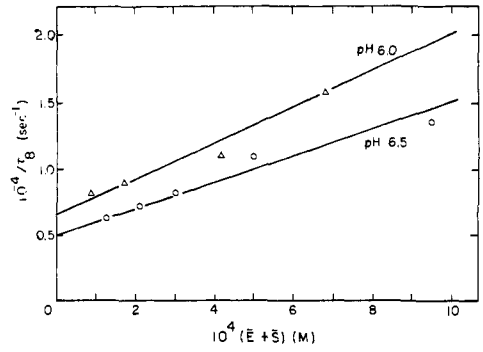

Figure 1. Variation of $1/\tau_8$ with ($[\bar{E}] + [\bar{S}]$) at 15°.

Table I. Summary of Ribonuclease–Cytidylyl-3′:5′-cytidine Data at 15°

pH	$1/\tau_7$, 10^{-3} sec^{-1}	k_8, 10^{-7} M^{-1} sec^{-1}	k_{-8}, 10^{-3} sec^{-1}	K_S', 10^4 M
6.0	8.6 ± 1.5	14 ± 4	7 ± 2	3 ± 1.5
6.5	8.9 ± 1	10 ± 3	5 ± 2	6 ± 3
7.0	8.9 ± 1	...	...	...

At pH 6.0, 15°, τ_1 became longer as cytidylyl-3′:5′-cytidine was added to the enzyme. The behavior of τ_1 as a function of substrate concentration was intermediate between that of cytidine 2′:3′-cyclic phosphate and cytidine 3′-phosphate.[11] A plateau region was not observed at high substrate concentrations as with cytidine 2′:3′-cyclic phosphate and the effect disappeared between 57 and 73% saturation of the enzyme. At 57% saturation of the enzyme, τ_1 is 40% longer than the value found in the absence of substrate.

An effort was made to find an additional relaxation process related to the interaction of the dinucleoside phosphate with ribonuclease, which was analogous to τ_4 in the cytidine 3′-phosphate–ribonuclease interaction.[7] No such effect was observed even though at pH 7.0 substrate concentrations up to 9×10^{-3} *M* were used.

Discussion

Relaxation studies of the interaction of various small molecules with ribonuclease show one feature which is common to all compounds which have a phosphate group. This feature is the intramolecular conversion characterized by τ_3, τ_5, and τ_7 for cytidine 3′-phosphate,[9] cytidine 2′:3′-cyclic phosphate,[11] and cytidylyl-3′:5′-cytidine, respectively, and for intramolecular conversions of ribonuclease complexes with cytidine 2′-phosphate and pyrophosphate.[9] The similarity of the relaxation effects is seen in the level plateau in the low pH region, the sharp rise at high pH values for cytidine 3′-phosphate and cytidine 2′-phosphate, and the comparable effects of D_2O on the relaxation time τ_3 and τ_5.[6,11]

(10) J. T. Bahr, R. E. Cathou, and G. G. Hammes, *J. Biol. Chem.*, 240, 3372 (1965).
(11) J. E. Erman and G. G. Hammes, *J. Am. Chem. Soc.*, 88, 5607 (1966).

The similarity of the relaxation effects and its presence for the interactions of all the compounds mentioned above strongly suggest that when the molecule containing the phosphate grouping binds to the enzyme, the presence of the phosphate induces an isomerization of the initial enzyme–phosphate compound complex. Analogous relaxation processes are not observed for the interaction of ribonuclease with sulfate ion and cytidine.[9] This isomerization may be a conformational change which brings the catalytic site of the enzyme and the substrate into the proper spatial arrangement. The occurrence of a conformational change is supported by the ultraviolet difference spectrum observed when nucleotides bind to ribonuclease[12,13] and by the results of optical rotatory dispersion studies,[14] which suggest that the environment of the tyrosine residues in ribonuclease is altered when a nucleotide is bound to the enzyme. This conformational change need not be involved in the catalytic process: an inactive complex may be formed or both of the species involved in the isomerization may react further, *i.e.*, two catalytic pathways may exist. The ubiquitous nature of this relaxation effect makes it seem likely that the conformational change is a necessary part of the binding process and is therefore necessary for the catalysis. However, at the present time there is no way of distinguishing between these three possibilities.

Enough information has not been obtained to give as detailed a description of the conversion of cytidylyl-3′:5′-cytidine to cytidine 2′:3′-cyclic phosphate and cytidine as that given for the hydrolysis of cytidine 2′:3′-cyclic phosphate to cytidine 3′-phosphate.[11] However, a schematic diagram for the over-all conversion of cytidylyl-3′:5′-cytidine to cytidine 3′-phosphate and cytidine is shown in Figure 2. The observed relaxation times[9,11] and steady-state parameters[6,15] have been associated with specific steps in the reaction sequence. This assumes the relaxation times are not significantly kinetically coupled and that the turnover numbers can be roughly identified with the rate-controlling steps. The diagram does not take into account the different possible states of ionization of each species but represents only the number and sequence of the species which have been distinguished by relaxation and steady-state techniques. A minimum of seven states of the enzyme–substrate complex are required to be consistent with the relaxation spectrum. Additional states could exist which have not been detected by relaxation techniques, but no fewer states are possible. In the mechanism depicted, cytidine is released from the dinucleoside phosphate after the rate-determining step in the first half of the reaction. This is reasonable if it is assumed that rupture of the 5′-oxygen–phosphorus linkage and formation of the cyclic phosphate occur in the rate-determining step. A process which could be associated with the binding of cytidine was not detected with the temperature-jump method; this is not surprising as the binding of cytidine to ribonuclease is quite weak, as shown by the value of the inhibition constant, 1.4×10^{-2} M at pH 6.5, 25° (with cytidine 2′:3′-cyclic phosphate as substrate).[8] All enzyme–substrate complexes are assumed to be obligatory intermediates in the absence of information to the contrary.

The second-order and dissociation rate constants for the binding of cytidylyl-3′:5′-cytidine, cytidine 2′:3′-cyclic phosphate, and cytidine 3′-phosphate at pH 6.0, 15°, are collected in Table II. The values of all of the second-order rate constants are of the order of magnitude of 10^7 to 10^8 M^{-1} sec^{-1} which is approaching the value expected for a diffusion-controlled reaction.[16] For cytidine 2′:3′-cyclic phosphate the second-order rate constant is smaller and the dissociation rate constant is larger than for the other two species; these results should be compared with the Michaelis constants which indicate that cytidine 2′:3′-cyclic phosphate binds less strongly to ribonuclease than either of the other two species.

Table II. Second-Order and Dissociation Rate Constants for the Binding of Various Nucleotide Species to Ribonuclease at pH 6, 15°

Species	Rate constants: Second-order, 10^{-7} M^{-1} sec^{-1}	Rate constants: Dissociation, 10^{-3} sec^{-1}
Cytidylyl-3′:5′-cytidine	14 ± 4	7 ± 2
Cytidine 2′:3′-cylic phosphate[a]	2–5	10–20
Cytidine 3′-phosphate[b]	6 ± 2	4 ± 1

[a] Reference 11, pH 6.2. [b] Interpolated from data at 12.5° and 25°, ref 9.

The second-order rate constant for the dinucleoside phosphate binding to ribonuclease is about twice as large as that for cytidine 3′-phosphate. However, since it is not known which ionized form(s) of cytidine 3′-phosphate binds to ribonuclease, this difference may be misleading. At pH 6 about 50% of cytidine 3′-phosphate is in the monoanion form and about 50% in the dianion form. If only one ionized form binds to ribonuclease, either the monoanion or the dianion, then the actual rate constant would be about twice the observed value and comparable to that observed with cytidylyl-3′:5′-cytidine. However, it can be concluded that if the dianion binds at all, the second negative change does not have much effect on the rate of binding of the nucleotide.

The apparent equilibrium constant between cytidylyl-3′:5′-phosphate, cytidine 2′:3′-cyclic phosphate, and cytidine can be estimated from the steady-state parameters according to the equation

$$K_{eq}' = \frac{[C2':3'P][C]}{[CpC]} = \frac{K_S V_S' K_C}{V_P' K_S'} \qquad (4)$$

where [C2′:3′P], [C], and [CpC] are the concentrations of cytidine 2′:3′-cyclic phosphate, cytidine, and cytidylyl-3′:5′-cytidine, respectively, K_S, K_C, and K_S' are the Michaelis constants for the same three species, and V_S' and V_P' are the maximum velocities for the breakdown and formation of the dinucleoside. For the case under consideration the Michaelis constants are true equilibrium constants since the rates for the binding step and the isomerization between the en-

(12) J. P. Hummel, D. A. Ver Ploeg, and C. A. Nelson, *J. Biol. Chem.*, 236, 3168 (1961).
(13) G. G. Hammes and P. R. Schimmel, *J. Am. Chem. Soc.*, 87, 4665 (1965).
(14) R. E. Cathou, G. G. Hammes, and P. R. Schimmel, *Biochemistry*, 4, 2687 (1965).
(15) H. Witzel, *Prog. Nucleic Acid Res.*, 2, 221 (1963).
(16) R. A. Alberty and G. G. Hammes, *J. Phys. Chem.*, 62, 154 (1958).

zyme–substrate complexes of the various species are much faster than for the rate-determination step. At pH 6.5, 25°, K_S is 3.38 × 10^{-3} M,[6] K_S' is approximately 2 × 10^{-3} M (estimated from the value at pH 7 and the pH dependence of the Michaelis constants for several dinucleoside phosphates).[5] An estimate of K_C can be made from the inhibition constant of cytidine which was found to be 1.4 × 10^{-2} M.[8] The Michaelis constant and inhibition constant for cytidine are certainly not the same because the latter constant measures the binding of cytidine to the cytidine 2′:3′-cyclic phosphate binding site while in the former case the binding of cytidine to the water site is being measured. Since the Michaelis constant for cytidine has not been measured the value for the inhibition constant is used as an estimate for the binding of cytidine to ribonuclease. The maximum velocity V_S' is 440 sec^{-1},[5] and if it is assumed that V_P' is about the same as the maximum velocity for the hydrolysis of cytidine 2′:3′-cyclic phosphate to cytidine 3′-phosphate, V_S, then V_P' is about 14 sec^{-1}.[6] The assumption that V_P' is equal to V_S means that the maximum velocity for the cleavage of the cyclic phosphate bond and incorporation of either cytidine or water is independent of whether cytidine or water is incorporated. This is not an unreasonable assumption. Findlay, *et al.*,[17] have studied the activity of ribonuclease in various organic solvents and found that the rate of reaction of various alcohols with cytidine 2′:3′-cyclic phosphate was the same order of magnitude as the rate of reaction with water. They also present evidence for a binding site for the molecule which reacts with cytidine 2′:3′-cyclic phosphate. These results suggest that the maximum velocity is relatively independent of the molecule which is incorporated into cytidine 2′:3′-cyclic phosphate once that molecule has been bound to the enzyme.

Using the experimental values and the estimates for the steady-state parameters, K_{eq}' is about 0.7 M and is independent of pH except at very low pH values (~pH 1). Since K_{eq}' is about unity, the over-all reaction, the conversion of the dinucleoside phosphate to cytidine 3′-phosphate and cytidine, is driven toward products primarily because of the unfavorable equilibrium between cytidine 2′:3′-cyclic phosphate and cytidine 3′-phosphate.[10] If the above numbers are qualitatively correct, a rough calculation indicates that if high concentrations of pyrimidine nucleotide 3′-phosphates (~0.1 M) are incubated with ribonuclease around pH 5 appreciable synthesis of oligonucleotides should occur.

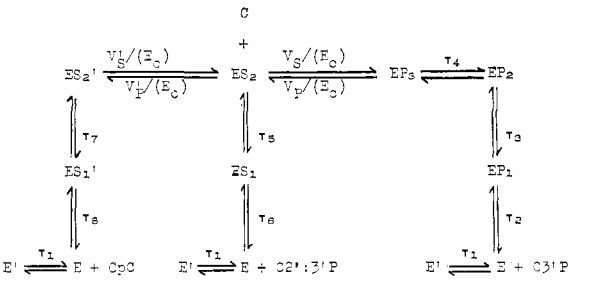

Figure 2. Schematic mechanism for the hydrolysis of cytidylyl-3′:5′-cytidine by ribonuclease. Symbols used: CpC, cytidylyl-3′:5′-cytidine; C2′:3′P, cytidine 2′:3′-cyclic phosphate; C3′P, cytidine 3′-phosphate; C, cytidine; E′ and E, isomers of native ribonuclease; ES_1' and ES_2', ribonuclease–cytidylyl-3′:5′-cytidine complexes; ES_1 and ES_2, ribonuclease–cytidine 2′:3′-cyclic phosphate complexes; EP_1, EP_2, and EP_3, ribonuclease–cytidine 3′-phosphate complexes. The observed relaxation times and steady-state parameters have been associated with the individual steps in the mechanism.

The low molecular weight of ribonuclease suggests that its structure is one of the simplest of the known enzymes, yet the relaxation studies show that the catalytic action of the enzyme is quite complex. The most direct path from cytidylyl-3′:5′-cytidine to cytidine 3′-phosphate and cytidine involves a minimum of six intermediate states (see Figure 2). This assumes the cyclic phosphate and ES_1 are not obligatory intermediates. The relationship between the structure and function of ribonuclease is still not known. The kinetic data suggest the existence of three ionizable groups at the active site; likely groups are His 12, His 119, and Asp 121.[18] In addition, Lys 41[19] and some tyrosine residues[12–14] are probably closely related to the active site. Unfortunately kinetic and chemical modification data are not sufficient to establish reliably the three-dimensional structure of ribonuclease. Although a knowledge of the three-dimensional structure will not reveal the dynamics of catalysis, a synthesis of kinetic and structural information should yield a molecular mechanism. We now await the elucidation of the three-dimensional structure of ribonuclease.

(17) D. Findlay, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, **85**, 134 (1962).

(18) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 2421 (1963).

(19) R. L. Heinrikson, *ibid.*, **241**, 1393 (1966).