ACCOUNTS OF CHEMICAL RESEARCH

VOLUME 1 NUMBER 11 NOVEMBER, 1968

Relaxation Spectrometry of Enzymatic Reactions

GORDON G. HAMMES

Department of Chemistry, Cornell University, Ithaca, New York

Received May 29, 1968

Understanding the mechanism of enzyme action is a goal of long standing in biochemistry. The major impetus for the study of enzymes derives, of course, from their physiological importance as catalysts of biochemical reactions. Apart from this, enzymes are innately fascinating because of the extreme efficiency with which they catalyze chemical reactions, an efficiency without parallel in ordinary chemistry. For example, consider the enzyme fumarase which catalyzes the hydration of fumaric acid to *l*-malic acid (and the reverse dehydration reaction). A fumarase molecule saturated with substrate catalyzes the hydration reaction with an apparent first-order rate constant of 2×10^3 sec^{-1} at 25°,[1] whereas the corresponding rate constant is approximately 2×10^{-8} sec^{-1} [2] in 1 *M* acid and 5×10^{-9} sec^{-1} in 1 *M* base.[3] Thus the enzymic reaction is over 10^{11} times more efficient than the simple acid- or base-catalyzed reaction.

In recent years, interest has turned from rather qualitative aspects of enzyme catalysis, such as the nature of the reactions catalyzed and the placement of enzymes in metabolic cycles, to a search for understanding the mechanism of enzyme action in terms of the molecular structure of the enzymes and substrates. Although the macromolecular nature of enzymes introduces considerable complexity, many enzymes have now been isolated and purified, and in a number of cases the entire amino acid sequence is known.[4] In a few cases three-dimensional structures are available from X-ray crystallographic studies.[5] Structural information is, of course, necessary for the understanding of enzyme mechanisms, but in addition kinetic data must be obtained in order to elucidate the dynamics of enzyme catalysis. The most common kinetic approach to enzymic mechanisms is through "steady-state" investigations. In such studies, the enzyme concentration is maintained *much* lower than the substrate concentrations; this is possible because of the extreme catalytic efficiencies of enzymes. Even at enzyme concentrations of 10^{-7} to 10^{-10} *M*, conversion of substrates to products occurs at a conveniently measurable rate. Under such conditions the steady-state approximation can be used on the time rate of change of all enzyme species, *i.e.*, free enzyme and enzyme–substrate complexes. Steady-state investigations provide general information about the over-all mechanism; for example, the sequence of reaction of substrates in multiple substrate reactions[6] and the minimal values of rate constants.[7] However, since enzyme–substrate species are not observed directly, no direct information about the nature or number of reaction intermediates can be obtained, although inferences about possible intermediates are sometimes made.

This shortcoming of steady-state kinetics can be avoided by working at sufficiently high enzyme concentrations so that the enzyme species themselves can be detected. However, at appropriate concentrations the reactions become very rapid (typical half-times are considerably less than 1 sec), so that special techniques must be employed to study them. One of the most useful techniques involves use of rapid mixing devices: the substrate and enzyme are mixed as rapidly as possible and the progress of the reaction is immediately followed, usually by a spectroscopic method. Although this technique is very useful,[8] solutions cannot be conveniently mixed in times much less than about 1 msec, so that reactions with characteristic time constants shorter than 1 msec cannot be studied in detail.

Recently a new group of experimental methods has become available; relaxation techniques, which permit reactions with time constants as short as 5×10^{-10} sec to be studied.[9] With these methods the reactants are

(1) C. Frieden and R. A. Alberty, *J. Biol. Chem.*, **212**, 859 (1955).
(2) L. T. Rozelle and R. A. Alberty, *J. Phys. Chem.*, **61**, 1637 (1957).
(3) L. E. Erickson and R. A. Alberty, *ibid.*, **63**, 705 (1959).
(4) R. V. Eck and M. O. Dayhoff, "Atlas of Protein Sequence and Structure," National Biomedical Research Foundation, Silver Spring, Md., 1966.
(5) D. R. Davies, *Ann. Rev. Biochem.*, **36**, 321 (1967).
(6) W. W. Cleland, *ibid.*, **36**, 73 (1967).
(7) L. Peller and R. A. Alberty, *Progr. Reaction Kinetics*, **1**, 235 (1961).
(8) B. Chance in "Technique of Organic Chemistry," Vol. VIII, Part II, S. L. Friess, E. S. Lewis, and A. Weissberger, Ed., Interscience Publishers, New York, N. Y., 1963, p 1314.
(9) M. Eigen and L. de Maeyer in ref 8, p 895.

mixed and the system is allowed to come to chemical equilibrium; some external parameter such as temperature or pressure is then rapidly changed and the rate of approach of the concentrations to their new equilibrium values at the new value of the external parameter is measured. By far the most useful of these methods is the temperature-jump method where the temperature is raised 5 to 10° in a few microseconds by discharging a large voltage through the solution. The rate of change of the concentrations as they approach their new equilibrium values at the higher temperature can be conveniently measured by spectroscopic techniques. This technique permits reactions with characteristic time constants greater than a few microseconds to be studied. Even faster reactions can be studied by the use of ultrasonic relaxation methods. If a reaction is essentially irreversible, equilibrium relaxation techniques are not usually applicable. However, a combination of rapid mixing and relaxation techniques can alleviate this problem. If, for example, the over-all reaction occurs in times longer than milliseconds, a temperature jump can be applied immediately after mixing. This perturbs the reactants from their steady-state concentrations and permits study of the fast steps in the mechanism occurring before the rate-determining step.[10] This combined rapid mixing–relaxation technique is especially useful for enzymatic reactions, many of which are essentially irreversible.

This article will be concerned with the application of fast reaction techniques, especially relaxation methods, to the elucidation of the elementary steps in enzymatic reactions. Only by investigating the entire time sequence of a reaction can meaningful mechanistic conclusions be reached. Firstly the results obtained with a particular enzyme, bovine pancreatic ribonuclease A, are considered in detail. This example illustrates the type of detailed mechanistic information which can be obtained, particularly with regard to the detection of reaction intermediates, and the interpretation of these intermediates in terms of mechanism. Secondly, some general aspects of the elementary steps occurring in enzymatic mechanisms and their relationship to appropriate model systems are discussed. Finally, some speculations concerning the macromolecular nature of enzymes and their unusually high catalytic efficiency are presented. More comprehensive reviews of the use of relaxation methods in biochemistry have been presented elsewhere.[11,12]

Ribonuclease

The enzyme which has been most extensively studied with relaxation methods is bovine pancreatic ribonuclease A (RNase A).[12–17] This enzyme is unusually

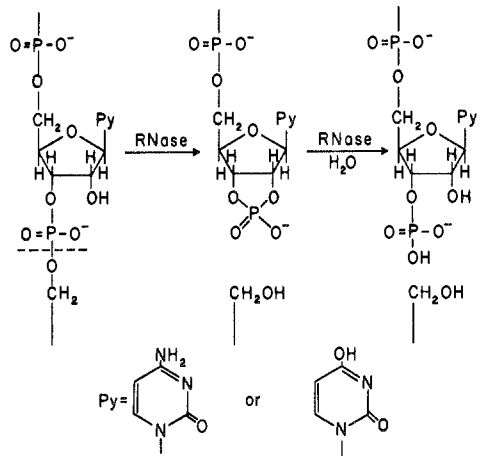


Figure 1. The breakdown of ribonucleic acid by pancreatic ribonuclease A. Both steps of this reaction are catalyzed by the enzyme.

small, having a molecular weight of 13,683; its amino acid sequence is known,[18–20] and even the tertiary structure has been elucidated by X-ray structural studies.[21,22] Thus correlations between structural and kinetic studies are possible. RNase A catalyzes the breakdown of ribonucleic acid in two distinct steps, as shown in Figure 1: first, an oxygen–phosphorus bond is cleaved, if the nucleoside attached through a C3′–O–P has a pyrimidine base (cytosine or uracil), and a 2′,3′ cyclic phosphate derivative is formed; secondly, the cyclic phosphate derivative is hydrolyzed to give a terminal pyrimidine 3′-monophosphate. As the ribonucleic acid is degraded, the system becomes nonhomogeneous with respect to reactants, making an exact kinetic analysis difficult. Fortunately convenient model substrates are available, namely dinucleoside phosphates, pyrimidine 2′,3′-cyclic phosphates, and pyrimidine 3′-monophosphates. RNase decomposes the dinucleoside phosphate into the cyclic phosphate and a nucleoside and subsequently hydrolyzes the cyclic phosphate into a 3′-phosphate. This latter reaction is essentially irreversible,[23] so that when equilibrium is reached only the 3′-phosphate is present. Therefore, the interaction of pyrimidine 3′-monophosphates with ribonuclease can be studied with the equilibrium temperature-jump method without interference from the other substrates. Since both the dinucleoside phosphates and pyrimidine cyclic phosphates are stable in the absence of enzyme, the interaction of each of these substrates with RNase can be studied independently with the stopped-flow–temperature jump method. Thus far the breakdown of cytidylyl-3′,5′-cytidine to cytidine 2′,3′-cyclic phosphate and cytidine and the

(10) J. E. Erman and G. G. Hammes, *Rev. Sci. Instr.*, **37**, 746 (1966).
(11) M. Eigen and G. G. Hammes, *Advan. Enzymol.*, **25**, 1 (1963).
(12) G. G. Hammes, *Advan. Protein Chem.*, **23**, 1 (1968).
(13) R. Cathou and G. G. Hammes, *J. Am. Chem. Soc.*, **86**, 3240 (1964).
(14) T. C. French and G. G. Hammes, *ibid.*, **87**, 4669 (1965).
(15) R. Cathou and G. G. Hammes, *ibid.*, **87**, 4674 (1965).
(16) J. E. Erman and G. G. Hammes, *ibid.*, **88**, 5607 (1966).
(17) J. E. Erman and G. G. Hammes, *ibid.*, **88**, 5614 (1966).
(18) C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **235**, 633 (1960).
(19) J. J. Potts, A. Berger, J. Cooke, and C. B. Anfinson, *ibid.*, **237**, 1851 (1962).
(20) D. G. Smyth, W. H. Stein, and S. Moore, *ibid.*, **238**, 227 (1963).
(21) G. Kartha, J. Bello, and D. Harker, *Nature*, **213**, 862 (1967).
(22) H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.*, **242**, 3984 (1967).
(23) J. T. Bahr, R. E. Cathou, and G. G. Hammes, *ibid.*, **240**, 3372 (1965).

hydrolysis of cytidine 2′,3′-cyclic phosphate to cytidine 3′-monophosphate have been investigated with relaxation methods.

Before discussion of the results, a brief introduction to the interpretation of relaxation experiments is given. In relaxation experiments the deviations of concentrations from their equilibrium values are always small. In the neighborhood of equilibrium all rate processes can be described by linear first-order differential equations, the number of independent differential equations being equal to the number of independent concentration variables. Thus for a single chemical reaction, the deviation of the concentration from equilibrium after a stepwise perturbation is $\Delta C_0 e^{-t/\tau}$, where ΔC_0 is the concentration deviation at time $t = 0$ and τ is the relaxation time which is a known function of the rate constants and equilibrium concentrations. If a complex mechanism occurs which involves several coupled reactions, the deviation from equilibrium of any concentration, ΔC_j, after a stepwise perturbation is

$$\sum_{i=1}^{n} A_{ij} e^{-t/\tau_i}$$

where n is the number of independent concentration variables and the A_{ij} are constants which are functions of the starting conditions. Each of the n relaxation times is in general a function of all of the rate constants and equilibrium concentrations associated with the mechanism, rather than being associated with a single step in the mechanism. These "normal-mode" relaxation times are analogous to the normal-mode frequencies of vibrational spectroscopy. The number of relaxation processes observed, that is, the number of relaxation times needed to represent the data, fixes the minimal number of independent concentration variables in a mechanism; for example, in a sequential mechanism the minimal number of reaction intermediates is fixed by the number of observed relaxation times.

The concentration dependence of the relaxation times permits postulation of a reaction mechanism just as the rate law does in ordinary kinetics, since each mechanism will have its own unique set of relaxation times. When a mechanism consistent with the concentration dependence of the relaxation times is found, the rate constants associated with the individual steps usually can be calculated. Finally the dependence of the rate constants on pH, temperature, etc., can be determined to give further information about the mechanism. The two most important advantages of relaxation methods are that all mechanisms can be described by a spectrum of relaxation times (or reciprocal first-order rate constants) and that very fast reactions can be studied.

The relaxation spectra of RNase itself and RNase interacting with its substrates are summarized in Table I. The enzyme undergoes an isomerization with a characteristic relaxation time of about 10^{-3} to 10^{-4} sec.[14] This isomerization apparently involves the catalytic site of the enzyme since the isomerization is not observed when cytidine 3′- and cytidine 2′-monophosphates are bound to the enzyme. However, apparently only a portion of the active site of RNase is involved since sulfate ion has no appreciable effect on the isomerization even though it is known to bind to the catalytic site. A detailed analysis of the pH dependence of the relaxation times permits calculation of the rate constants involved in the isomerization and suggests the involvement of an ionizable group on the enzyme with a pK_A value of about 6.

Table I
Relaxation Spectra of Ribonuclease A

System[a]	No. of relaxation times	Types of reactions
E	1 (τ_1)	Isomerization
E-C3′P	3 (τ_2, τ_3, τ_4)	Bimolecular; two isomerizations
E-C2′3′P	2 (τ_5, τ_6)	Bimolecular; isomerization
E-C3′5′P	2 (τ_7, τ_8)	Bimolecular; isomerization

[a] E = ribonuclease A, C3′P = cytidine 3′-phosphate, C2′3′P = cytidine 2′,3′-cyclic phosphate, C3′5′P = cytidylyl-3′,5′-cytidine.

When cytidine 3′-monophosphate is added to the enzyme a new relaxation process is observed;[15] the associated relaxation time becomes progressively shorter as the substrate concentration is increased, until eventually it is too short to observe with the temperature-jump technique. The concentration dependence of this relaxation time indicates that a bimolecular reaction between enzyme and substrate is occurring and furthermore that one of the isomeric forms of the enzyme binds the cytidine 3′-monophosphate more strongly than the other. When the enzyme is essentially saturated with substrate, two new relaxation processes are observed which are associated with isomerization of the enzyme–cytidine 3′-monophosphate complex. The relaxation times are independent of the concentration of the complex, but are pH dependent.

A similar type of pattern is found for the interaction of RNase with cytidine 2′,3′-cyclic phosphate[16] and cytidylyl-3′,5′-cytidine:[17] one relaxation process in each case can be mainly identified with the bimolecular reaction between enzyme and substrate, while a second relaxation process is associated principally with an isomerization of the enzyme–substrate complex. None of the relaxation processes observed is sufficiently slow to be involved in the rate-determining (probably bond-breaking) steps of the catalysis. The maximal velocities, *i.e.*, the steady-state rates of catalysis when the enzyme is saturated with substrate, are indirect measures of the rate constants involved in the rate-determining steps, and the magnitudes of these constants[24,25] indicate that at least two steps, in addition to those necessary to explain the relaxation spectra, are involved in conversion of the dinucleoside phosphate to the 3′-phosphate.

If a sequential mechanism is assumed, which is the simplest possible assumption, the minimal possible reaction mechanism is shown schematically in Figure

(24) D. G. Herries, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, **85**, 127 (1962).
(25) H. Witzel, *Progr. Nucleic Acid Res.*, **2**, 221 (1963).

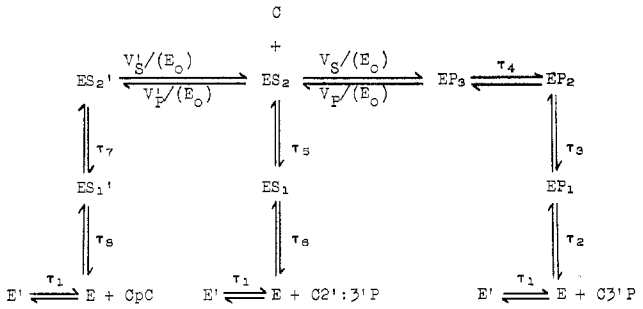


Figure 2. Schematic mechanism for the hydrolysis of cytidylyl-3′,5′-cytidine by ribonuclease. Symbols used: CpC, cytidylyl-3′,5′-cytidine; C2′,3′P, cytidine 2′,3′-cyclic phosphate; C3′P, cytidine 3′-phosphate; C, cytidine; E′ and E, isomers of native ribonuclease; ES_1' and ES_2', ribonuclease–cytidylyl-3′,5′-cytidine 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 can be approximately associated with the individual steps in the mechanism as indicated.

2. The time constants which can be roughly associated with the individual steps are also indicated; this association is not exact because the relaxation times are actually normal-mode parameters, as previously explained, and the steady-state parameters are complex (but known) functions of the rate constants. The large number of reaction intermediates required is only the minimum number since faster processes may occur which could not be observed. At least eight different states of the enzyme exist in the over-all reaction described in Figure 1. This number is fixed only by the *number* of experimentally observed time constants, *i.e.*, relaxation times and steady-state maximal velocities. The initial binding of all of the substrates is followed by an isomerization of the complex with a characteristic time constant of the order of magnitude of 10^3 to 10^4 sec^{-1}.

Information about the ionizable groups on the enzyme involved in the reaction mechanism can be inferred from the pH dependence of the kinetic parameters. The protolytic reactions themselves occur too rapidly for direct kinetic study with the temperature-jump method. A detailed analysis has been carried out for the hydrolysis of cytidine 2′,3′-cyclic phosphate to cytidine 3′-monophosphate.[15] For this reaction, the pH dependence of five relaxation times (excluding the isomerization of the enzyme itself which can be interpreted independently) and four steady-state parameters[16,24] (the maximal velocities and Michaelis constants for the forward and reverse reactions) are known. In addition, the pH dependence of some of the individual rate constants can be determined by consideration of the concentration dependence of the relaxation times. The simplest possible mechanism consistent with all of the data implicates three ionizable groups on the free enzyme with approximate pK_A values of 5, 6, and 6.7 at 25°. Since the kinetic studies could only be carried out in the pH range 5–8 no information about the involvement in the mechanism of groups with pK_A values lower than 5 or greater than 8 could be obtained.

The results of the kinetic studies can be correlated with the molecular structure of the enzyme. Ribonuclease is a compact kidney-shaped molecule with the active site located along a groove.[21,22] Phosphate ion and uridylic 2′(3′)-monophosphate are bound to the enzyme near two histidine residues (no. 12 and 119 of the amino acid sequence). Previous chemical evidence has also suggested the presence of these residues at the catalytic site.[26] The imidazole side chains of these two histidines can be identified with two of the ionizable groups inferred to be present at the active site from kinetic studies. The third ionizable group suggested by the kinetic work could be the imidazole side chain of histidine 48, which although not at the active site is located at the "hinge" of the groove and could be important in maintaining the conformations of the enzyme. In the X-ray structure of native ribonuclease the imidazole of histidine 48 is somewhat buried, whereas in ribonuclease S, which is fully active, but has one peptide linkage cleaved, it is quite exposed; the relaxation process associated with isomerization of the free enzyme is not seen with ribonuclease S, which suggests the imidazole of histidine 48 may be identified with the group of $pK_A = 6$ found to be associated with the isomerization. A number of carboxyl groups and an ϵ-amino group are also located near the active site and may interact with the bound substrate. The ring of the pyrimidine also appears to interact with side chains of the enzyme; some evidence for base specificity has been found from binding studies.[27] Finally, mention should be made of the fact that no tyrosine residues are at the active site, yet evidence exists that the environment of some of the tyrosine residues is changed when cytidine monophosphates bind to the enzyme[28] and iodination of the tyrosines changes the catalytic efficiency of the enzyme.[29] Thus the over-all conformation of the protein is apparently important for the catalytic process as well as the specific groups at the substrate binding site.

The chemical mechanism of action of ribonuclease in terms of specific ionizable groups on the enzymes is still not certain. Several mechanisms have been proposed,[12,25,30] and one which is consistent with all known facts is shown in Figure 3. In this mechanism one of the imidazole groups serves as a positively charged binding locus while another serves first as a base, then as an acid. No attempt has been made to depict all of the isomeric states of the enzyme–substrate complexes inferred from the kinetic data and other protein side-chain groups at the active site. The possible

(26) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 2421 (1963).
(27) D. G. Anderson, G. G. Hammes, and F. G. Walz, Jr., *Biochemistry*, **7**, 1637 (1968).
(28) R. E. Cathou, G. G. Hammes, and P. R. Schimmel, *ibid.*, **4**, 2687 (1965).
(29) G. G. Hammes and F. G. Walz, Jr., in preparation.
(30) D. Findlay, D. G. Herries, A. P. Mathias, B. R. Rabin, and C. A. Ross, *Biochem. J.*, **85**, 152 (1962).

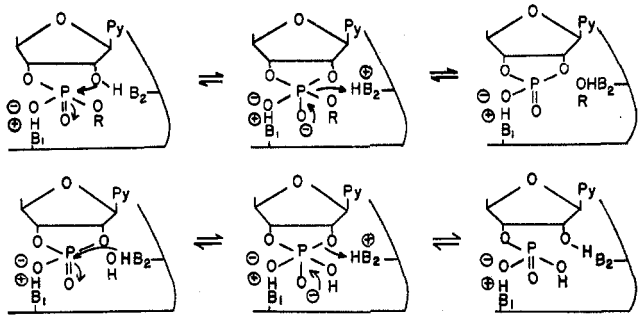


Figure 3. A possible chemical mechanism for the action of ribonuclease. No attempt is made to depict the many conformational states inferred from the kinetics. The bases B_1 and B_2 can be identified with the imidazole side chains of histidine residues 12 and 119. An ϵ-amino group (lysine 41) is probably also near the active site.

role of multiple conformations in the catalytic process is considered in a later section. A recent physical organic study of the general species catalyzed hydrolyses of a model of a dinucleoside phosphate, the phenyl ester of *cis*-tetrahydrofuran-3,4-diol phosphate, is consistent with a mechanism of the type shown in Figure 3.[31] Thus the kinetic and structural investigations of ribonuclease are gradually merging into a molecular mechanism.

The enzyme aspartate aminotransferase which catalyzes the reversible transfer of an amino group between aspartic acid and ketoglutaric acid to give glutamic acid and oxalacetate has also been studied in considerable detail with relaxation methods.[32-34] Although the detailed results will not be discussed here, mention should be made of the fact that a large number of intermediate states (at least 15!) are again inferred to be present in the over-all catalysis.

Elementary Steps in Enzyme Mechanisms

Certain types of reactions are an inevitable part of any enzyme mechanism: an enzyme–substrate complex is first formed, the complex then isomerizes, and finally the product is released. Some types of elementary steps important in enzyme catalysis are now considered, namely formation of enzyme–substrate complexes, isomerizations involving noncovalent interactions, and proton transfers. Some aspects of these elementary steps were discussed in an earlier review.[11]

The formation of enzyme–substrate complexes has been studied in several cases with relaxation methods. In many cases, enzyme–substrate complex formation is associated with two relaxation times and can be depicted as a two-step process such as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2$$

where E designates enzyme, S substrate, and X_1 and X_2 are two different complexes. A summary of results from relaxation studies is given in Table II, where k_1 and the order of magnitude of $k_2 + k_{-2}$ are given. In two cases no relaxation process which can be identified with isomerization of the enzyme–substrate complex is observed; however the associated relaxation time may be too short to measure. For physiological substrates of the enzyme, the value of k_1 approaches, but is usually somewhat less than, the maximum possible value of about $10^9\ M^{-1}\ \text{sec}^{-1}$ expected for a diffusion-controlled reaction.[35] Steric requirements are apparently severe since, for example, α-methylaspartate reacts with aspartate aminotransferase with a characteristic rate constant about 10^{-4} smaller than that for the analogous reaction with aspartate. Nevertheless, formation of the initial enzyme–substrate complex is generally very rapid for physiological substrates—at concentrations of about $10^{-4}\ M$ enzyme and substrate, the reaction half-times are typically in the range of 10–100 μsec.

The isomerization following formation of the initial complex generally appears to have a time constant of about $10^4\ \text{sec}^{-1}$. Thus formation of the enzyme–substrate complex is not likely to be rate limiting at usual substrate concentrations. The functional role of a two-step mechanism (*if* this mechanism is catalytically important) is not clear; one possibility is that the first step is concerned mainly with structural specificity in combining the enzyme with substrate, while the second step is more specifically concerned with the catalytic process. Structural changes of the macromolecule may accompany substrate binding. For example, optical rotatory dispersion studies of ribonuclease suggest that this is the case when the enzyme combines with cytidine monophosphates.[28]

Isomerizations of enzyme–substrate complexes must involve structural change, although they may be very subtle, and thus may be termed conformational changes. Both covalent and noncovalent changes may be involved. However, the covalent changes are specific for each reaction, whereas the noncovalent interactions involving the protein backbone and side chains and water are probably similar in nature in all cases. These include charge–charge interactions, hydrogen bonding, protein–water interactions, and hydrophobic interactions.

The kinetics of formation of several hydrogen-bond-stabilized complexes in nonaqueous solvents have been studied, mainly with ultrasonic techniques. A summary of the measured rate constants is given in Table III. In all cases studied thus far, independent of the solvent, the magnitude of the equilibrium constant, or the type of hydrogen bonds formed, the characteristic rate constant for formation of the complex, k_f, is about $10^9\ M^{-1}\ \text{sec}^{-1}$, which is the value expected if reaction occurs at every collision. Therefore the dissociation rate constant, k_r, is a direct measure of the stability of the hydrogen bonds. The fact that association is diffusion controlled means that the rate constant for

(31) D. G. Oakenfull, D. I. Richardson, Jr., and D. A. Usher, *J. Am. Chem. Soc.*, **89**, 5491 (1967).
(32) P. Fasella and G. G. Hammes, *Biochemistry*, **6**, 1798 (1967).
(33) G. G. Hammes and J. L. Haslam, *ibid.*, **7**, 1519 (1968).
(34) G. G. Hammes and J. L. Haslam, in preparation.
(35) R. A. Alberty and G. G. Hammes, *J. Phys. Chem.*, **62**, 154 (1958).

Table II
Enzyme–Substrate Complex Formation

Enzyme	Substrate	$10^{-9}k_1$, M^{-1} sec^{-1}	$\sim(k_2 + k_{-2})$, sec^{-1}
Ribonuclease (pH 6.0)	Cytidylyl-3′,5′-cytidine[17]	0.014	10^4
	Cytidylyl 2′,3′-cyclic phosphate[16]	0.02–0.05	10^4
	Cytidine 3′-phosphate[15]	0.06	10^3–10^4
	Cytidine 2′-phosphate[a]	0.01	10^3
Aspartate aminotransferase	Aspartate, glutamate ketoglutarate, oxalacetate[32]	>0.1	*g*
	α-Methylaspartate[33]	1.2×10^{-5}	10^2
	β-*erythro*-Hydroxyaspartate[34]	3.1×10^{-3}	10^3
Lactate dehydrogenase	Reduced nicotinamide–adenine dinucleotide[b]	~1	10^3
Chymotrypsin	Proflavin[c]	0.1	10^4
	Furylacryloyl-L-tryptophanamide[d]	0.04	10^4
Creatine kinase[e]	Adenosine triphosphate	>0.01	10^4
	Adenosine diphosphate	0.022	10^4
Glyceraldehyde phosphate dehydrogenase[f]	Nicotinamide–adenine dinucleotide	0.019, 0.0014	*g*

[a] G. G. Hammes and C. D. Hubbard, unpublished results. [b] G. Czerlinski and G. Schreck, *J. Biol. Chem.*, **239**, 913 (1964). [c] B. H. Havsteen, *ibid.*, **242**, 769 (1967). [d] G. P. Hess, private communication. [e] G. G. Hammes and J. Hurst, in preparation. [f] K. Kirschner, M. Eigen, R. Bittman, and B. Voigt, *Proc. Natl. Acad. Sci. U. S.*, **56**, 1661 (1966). [g] Not observed.

Table III
Rate Constants for Hydrogen Bond Dimerization

Reactants	Solvent	$10^{-9}k_f$, M^{-1} sec^{-1}	$10^{-7}k_\tau$, sec^{-1}	Temp, °C
Benzoic acid[a,b]	CCl_4	5	0.073	25
	Toluene	1.6	0.37	25
	Hexane	8.1	0.022	20
	C_6H_5Cl	5.1	0.21	20
	$CHCl_3$	4.7	0.75	20
ε-Caprolactam[c]	CCl_4	5.5	4.6	22
	Benzene	6.5	26	22
2-Pyridone[d]	Dioxane	2.1	13	25
1-Cyclohexyluracil[36]	$CHCl_3$	1.5	25	25
1-Cyclohexyluracil-9-ethyladenine[36]	$CHCl_3$	4.0	3.2	20

[a] W. Maier, *Z. Elektrochem.*, **64**, 132 (1960). [b] L. Borucki, *Ber. Bunsenges. Physik. Chem.*, **71**, 504 (1967). [c] K. Bergmann, M. Eigen, and L. de Maeyer, *ibid.*, **67**, 8119 (1963). [d] G. G. Hammes and H. O. Spivey, *J. Am. Chem. Soc.*, **88**, 1621 (1966).

Table IV
Water–Solute Interactions

Molecular species	$1/\tau$[d] or k_D,[e] sec^{-1}
$NH_3 \cdot H_2O$[37]	2.2×10^{11} [e]
$(C_6H_5CH_2)_2NCH_3 \cdot H_2O$[37]	2.7×10^9 [e]
Dioxane$(H_2O)_2$[a]	2.8×10^8 [e]
(Dioxane)$_2(H_2O)_2$[a]	1.0×10^8 [e]
Diglycine$(H_2O)_n$[b]	3.8×10^8 [d]
(Polyethylene glycol)$(H_2O)_n$[c]	1.6×10^8 [d]
Polyglutamate$(H_2O)_n$[43]	$\sim 10^8$ [d]

[a] G. G. Hammes and W. Knoche, *J. Chem. Phys.*, **45**, 4041 (1966). [b] G. G. Hammes and N. C. Pace, *J. Phys. Chem.*, **72**, 2227 (1968). [c] G. G. Hammes and T. B. Lewis, *ibid.*, **70**, 1610 (1966). [d] $1/\tau$. [e] k_D.

formation of the first hydrogen bond is greater than the rate constant for diffusion apart of the reactants, that is, $>10^{10}$ sec^{-1}, while the reverse rate constant is essentially determined by the equilibrium partitioning between formation of the intermolecular hydrogen bond and solvent–hydrogen-bond-site interactions.[36] This mechanism is quite consistent with the available data, even in such polar solvents as water–dioxane mixtures. Unfortunately, study of the formation of hydrogen-bond-stabilized complexes in water has not been possible; it is possible that the rate of formation of dimers is no longer diffusion controlled in aqueous solutions.

The interaction of water with solutes containing hydrogen-bonding sites has also been studied; nuclear magnetic resonance and ultrasonic techniques have been used. Some of the results obtained are presented in Table IV. Either the dissociation rate constant characteristic of water leaving the aggregate or the reciprocal relaxation time, which is the sum of the association and dissociation rate constants, is determined. For the ammonia–water aggregate, the rate of dissociation is essentially controlled by how fast the two molecules can diffuse apart. However, when bulky nonpolar groups are substituted for the hydrogens in ammonia, the dissociation rate constant drops by as much as two orders of magnitude. An explanation is that water molecules tend to form "icebergs" around nonpolar substances,[37] that is, the water dipoles line up around the nonpolar group to form a sheath. This sheath may include one or more layers of water molecules. The water which dissociates is now part of this sheath, whose structure is considerably tighter than that of bulk water, and therefore the rate constant is decreased relative to that in bulk water. (An explanation of this phenomenon in terms of dispersion forces has also been proposed.[38]) The relative slowness of the dissociation of water from dioxane–water aggregates can be explained on a similar basis; as might be predicted the rate constant is smaller when two

(36) G. G. Hammes and A. C. Park, *J. Am. Chem. Soc.*, **90**, 4151 (1968).

(37) H. S. Frank and W. Y. Wen, *Discussions Faraday Soc.*, **24**, 133 (1957).

(38) E. Grunwald and E. K. Ralph, III, *J. Am. Chem. Soc.*, **89**, 4405 (1967).

dioxane molecules, rather than one, are part of the aggregate. Although an interaction between water and diglycine has been observed, the stoichiometry could not be defined, and only the relaxation time could be measured rather than the individual rate constants. This interaction probably involves the charged groups as well as "iceberg" formation. The interactions with polyethylene glycol and polyglutamate are more complex, both structurally and kinetically.

If several noncovalent interactions are made or broken virtually simultaneously, the over-all process is termed "cooperative." More precisely, a cooperative change implies that the making or breaking of the first linkage occurs considerably less readily than the making or breaking of the second linkage, and so on, so that a cascade effect occurs. Good examples of cooperative processes are the transitions from helix to random coil in polypeptides.[39] These transitions occur over narrow ranges of pH, temperature, and solvent composition and involve the breaking of a large number of hydrogen bonds. A statistical mechanical treatment of the kinetics indicates that the "average" reciprocal relaxation time when the polymer is half helix and half coil is equal to the rate constant for formation of an intramolecular hydrogen bond times a factor which is a measure of the relative difficulty of breaking the first hydrogen bond.[40] The rate constant is known to be about 10^{11} sec^{-1} from the arguments given above, and the other factor is known to be about 10^{-4} from equilibrium measurements. Thus the expected characteristic time constant is about 10^{7} sec^{-1}, which is considerably less than the rate constants associated with simple hydrogen bonding.

The "average" reciprocal relaxation time at the midpoint of the transition for poly-L-ornithine in water–methanol has recently been measured and is 5.9×10^{7} sec^{-1}.[41] The analogous time constant for poly(L-glutamic acid) in aqueous solution has been ascertained to be in the range 10^{5}–(2×10^{7}) sec^{-1},[42,43] in good agreement with theory. Sharp changes in the relaxation time suggestive of cooperative transitions are also observed when urea or guanidine hydrochloride is added to solutions of poly(ethylene glycol), providing the polymer has a molecular weight greater than $\backsim$ 3000.[44–46] Thus in this case a minimum polymer size and associated water of solvation are required before a cooperative process can occur. This size requirement for cooperativity has obvious implications for proteins.

Although conformational changes of proteins can in principle occur within the period of a few molecular vibrations, the studies on model compounds cited above suggest that, if rearrangement of water and polymer structure occur, the time constants should be less than $\sim 10^{8}$ sec^{-1}, which is considerably greater than typical substrate turnover numbers of enzymes. If cooperative processes are involved, considerably smaller time constants are expected. The relative smallness of the time constants for isomerizations given in Table II suggests that the isomerizations are highly cooperative processes.

The fact that the rates of most enzymatic reactions are dramatically dependent on pH suggests that acid–base catalysis is an important part of enzymatic mechanisms. Proton-transfer reactions have been extensively studied, and implications for enzymatic systems of the results obtained are discussed elsewhere.[11] Only a brief resumé is presented here. Since an enzyme must begin and end in the same ionization state for every catalytic cycle, both protonation and deprotonation of any group involved in acid–base catalysis must occur. Since the reactions of protons and hydroxyl ions with normal acids and bases are diffusion controlled, the characteristic rate constant being about 10^{10} M^{-1} sec^{-1}, the rate of deprotonation of an acid by dissociation is approximately $10^{10}K_A$ and the rate of protonation of a basic site by water is approximately $10^{10}K_W/K_A$, where K_A is the ionization constant of the conjugate acid of the basic site and K_W is the self-ionization constant of water. At 25° these two rates are optimized at about 10^{3} sec^{-1} when the pK_A is about 7. This is the pK_A value of imidazole, and this group has been implicated in many enzyme mechanisms. For larger pK_A values the protonation rate constant increases, but the deprotonation rate constant decreases, while the reverse situation prevails for smaller pK_A values.

Enzyme configurational changes could alter the pK_A values of a particular group so as to enhance the rate constant for protonation or deprotonation. Interactions with neighboring groups, for example, metal ions and charge clusters, can also cause shifts in the acid dissociation constants. If changes in pK_A's are attributed to substrate binding, the maximum changes possible are limited by the free energy of binding and would not be expected to exceed more than several units. Moreover, because an enzyme reaction is a cyclic process, the principle of detailed balance places considerable restriction on how much the over-all rate is enhanced. For example, a shift in pK_A favoring proton transfer generally implies an unfavorable shift of other equilibria. However, by such considerations over-all rate constants for protonation–deprotonation reactions of the order of 10^{4}–10^{5} sec^{-1} might be anticipated.

In principle intramolecular proton transfers not involving solvent molecules could occur very rapidly. The rate constant for intramolecular proton transfer in a hydrogen-bonded network when the proton acceptor is a weaker acid than the donor is about 10^{13}

(39) G. D. Fasman in "Poly-α-Amino Acids," G. D. Fasman, Ed., Marcel Dekker, Inc., New York, N. Y., 1967.
(40) G. Schwarz, *J. Mol. Biol.*, **11**, 64 (1965).
(41) G. G. Hammes and P. D. Roberts, in preparation.
(42) R. Lumry, R. Legare, and W. G. Miller, *Biopolymers*, **2**, 489 (1964).
(43) J. J. Burke, G. G. Hammes, and T. B. Lewis, *J. Chem. Phys.*, **42**, 3520 (1965).
(44) G. G. Hammes and P. R. Schimmel, *J. Am. Chem. Soc.*, **89**, 442 (1967).
(45) G. G. Hammes and J. C. Swann, *Biochemistry*, **6**, 1591 (1967).
(46) G. G. Hammes and P. B. Roberts, *J. Am. Chem. Soc.*, in press.

sec^{-1}.[9] Therefore, the rate constant for the reverse intramolecular proton transfer is approximately 10^{13} K_D/K_A sec^{-1}, where K_D is the acid dissociation constant of the proton donor, K_A is the acid dissociation constant of the proton acceptor, and $K_D < K_A$. For example, the intramolecular transfer of a water proton to a base of $pK_A = 7$ would have a rate constant of about 2×10^4 sec^{-1} at 25° (assuming the acid dissociation constants measured in aqueous solution are still appropriate).

Proton transfers to and from the substrate are the slowest to be expected since most substrates are very weak acids and bases, although the enzyme might enhance their acidity and basicity. If the ratio K_D/K_A is small, this also implies that the equilibrium concentration of the reactive species is small. For example, if K_D/K_A is $\sim 10^{-4}$, the rate constant would be $\sim 10^9$ sec^{-1}, but the equilibrium concentration of the reactive species would at best be 10^{-4} of the total enzyme concentration. In view of the normal acidity and basicity of most substrates, K_D/K_A would have to be unusually large to exceed the previously derived limits of 10^4–10^5 sec^{-1} for the maximum over-all rate of protonation–deprotonation. The apparent first-order rate constant for conversion of substrate to product when the enzyme is saturated is about 10^3 sec^{-1} for most enzymes and very rarely exceeds 10^5 sec^{-1}.[11] Thus the maximal over-all rate constants for protonation–deprotonation are similar in magnitude to those associated with the over-all rate constants (turnover numbers) of enzyme reactions.

Considerations such as the above can also be used to infer information about rates of other individual steps in enzymatic mechanisms. For example, consider the proposed mechanism for ribonuclease action given in Figure 3. If association and dissociation of the substrates and products are neglected, then the mechanism for transesterification can be envisaged as transfer of the 2′-hydroxyl hydrogen to B_2, formation and breakdown of the pentacoordinated phosphorus intermediate, and transfer of the proton from B_2 to RO^-. If the ratio of the acid dissociation constant of the 2′-hydroxyl and ROH to that of B_2 is estimated as $\sim 10^{-7}$, the initial proton transfer has a rate constant of $\sim 10^6$ sec^{-1} and the species formed is 10^{-7} of the total enzyme species. Inasmuch as the over-all first-order rate constant for transesterification of dinucleosides is as large as 10^3–10^4 sec^{-1},[25] the formation and breakdown of the pentacoordinate intermediate must have rate constants greater than 10^{10}–10^{11} sec^{-1}. (The final proton transfer would have a rate constant of 10^{13} sec^{-1}.) Similarly if the hydrolysis of the cyclic phosphate is assumed to be initiated by intramolecular hydrolysis of water by B_2 with a rate constant of 2×10^4 sec^{-1}, as estimated above, the concentration of the reactive species would only be 2×10^{-9} of the total enzyme species. The over-all rate constant for this process is about 10^2 sec^{-1};[24] this implies that formation and breakdown of the pentacoordinate intermediate is associated with rate constants greater than 5×10^{10} sec^{-1}, which is similar to the value found for the transesterification step.

The above calculations are naive and are based on a very simple model. The role of the enzyme in altering pK_A values and assisting in concerted processes and the formation and breakdown of enzyme–substrate complexes are entirely ignored, as is the explanation for the formation and breakdown of the pentacoordinate intermediate occurring as rapidly as 10^2–10^3 molecular vibrations. Nevertheless, such calculations serve to illustrate the possibilities. Similar considerations of intramolecular proton transfer for enzymic mechansims, particularly for chymotrypsin, have been recently presented.[47]

Mechanism of Enzyme Action

Consideration of the elementary steps in enzyme reactions provides some insight into the types and rates of reactions which are of importance, but the problem of putting these reactions together to obtain the tremendous catalytic efficiency of enzymes remains. Some possibilities are now considered, but this discussion must be regarded as quite speculative. No attempt is made to give an encyclopedic coverage of the many speculations which appear in the literature. The experimental work discussed earlier suggests that three important features of enzymic catalysis are: (1) the catalytic reaction is divided into a large number of discrete steps, (2) cooperative conformational changes of the enzyme occur, and (3) acid–base catalysis is important in most enzyme reactions. A fourth point is that a macromolecule is necessary to achieve high catalytic rates: attempts to hydrolyze off large portions of enzymes lead to inactivation and no small molecules synthesized thus far approach the catalytic efficiency of enzymes.

An important feature of enzyme catalysis is almost certainly precise orientation of the substrate at the active site. Precise orientation of the substrate with respect to acid–base groups of the enzyme can appreciably aid proton transfer. In addition, substrate interactions with charged groups on the enzyme and other noncovalent interactions (*e.g.*,hydrogen bonding) can cause bond polarization in the substrate which could appreciably aid bond breaking and could alter the acidity or basicity of the substrate, thus facilitating proton transfer (*cf.* the preceding section). Noncovalent interactions, *e.g.*, charge–dipole, dipole–dipole, are extremely orientation dependent. The very fact that a stable enzyme–substrate complex is formed can be thought of as raising the effective concentration of the catalyst and as orienting the reactants so as to restrict the large number of possible conformations and positions available in solution. If the enzyme may act as a polyfunctional catalyst, that is, if several catalytic groups interact simultaneously with the substrate, catalysis relative to simple molecules may be facilitated. The last three effects are primarily

(47) J. H. Wang and L. Parker, *Proc. Natl. Acad. Sci. U. S.*, **58**, 2451 (1967).

entropic in nature and have been discussed by several authors;[48] however they do not appear sufficient to account for the high rates associated with enzyme catalysis.

A possible explanation for the macromolecular nature and unusual catalytic efficiency of enzymes is based on the *conformational adaptability* of enzymes. An enzyme can divide the catalytic process into a number of discrete steps, and the structure can be optimized for each step by small alterations in the macromolecular structure. The possibility that conformational changes increase proton-transfer rates has been mentioned earlier. The large number of intermediate states observed with relaxation methods is consistent with this sort of mechanism. Conformational changes may play a more dynamic role in catalysis by lowering the free energy of activation *via* a "compensation effect."[49] For example, the activation energy for decomposition of H_2 by an inert gas is greater than 100 kcal/mol, whereas that for the reaction $H_2 + I_2 \rightleftharpoons 2HI$ is only about 40 kcal/mol. In both reactions an H_2 bond is broken, but the activation energy is lower in the second case because two H–I bonds are also formed. In an enzyme reaction the formation of protein side-chain bonds (*e.g.*, hydrogen bonding and hydrophobic interactions) might lower the free energy of activation for the making and breaking of substrate bonds. This type of compensation could alter both the enthalpy and the entropy of activation.[50]

Although the *conformational adaptability* of the protein appears to be a reasonable explanation of the catalytic efficiency of enzymes, direct verification by experiments is currently not possible. However, the combination of detailed kinetic studies of enzymes with fast reaction techniques and structural information about enzymes obtained by X-ray techniques promises to yield an increasingly detailed picture of the mechanism of enzyme catalysis.

I am indebted to my many coworkers who carried out the research discussed here and to the National Institutes of Health for their financial support.

(48) D. E. Koshland, Jr., *J. Theoret. Biol.*, **2**, 75 (1962); T. C. Bruice and V. K. Pandit, *J. Am. Chem. Soc.*, **82**, 5858 (1960); T. C. Bruice and S. L. Benkovic, *ibid.*, **86**, 418 (1964); F. Westheimer, *Advan. Enzymol.*, **24**, 441 (1962); M. L. Bender, F. J. Kézdy, and C. R. Gunter, *J. Am. Chem. Soc.*, **86**, 3714 (1964).

(49) G. G. Hammes, *Nature*, **204**, 342 (1964).

(50) For some other proposed models of enzyme action see: D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U. S.*, **44**, 98 (1958); R. Lumry and H. Eyring, *J. Phys. Chem.*, **58**, 110 (1954); W. P. Jencks in "Current Aspects of Biochemical Energetics," N. V. Kaplan and E. P. Kennedy, Ed., Academic Press, New York, N. Y., 1966, p 273

Electronic Spectroscopy of Arylmethylenes

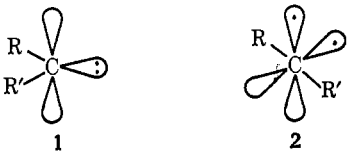
ANTHONY M. TROZZOLO

Bell Telephone Laboratories, Inc., Murray Hill, New Jersey

Received April 15, 1968

The tetravalency of carbon lies at the very foundation of the structural theory of organic chemistry. However, the most important reaction intermediates in organic chemistry are those in which at least one carbon atom is in a valence state other than the normal tetravalent one. Three types of such intermediates are *trivalent* carbon derivatives, namely, carbonium ions, carbanions, and free radicals.

The study of *divalent* carbon species, RĊR′, has received considerable attention in recent years.[1] Of particular interest is the assignment of the spin multiplicity of these intermediates since, unlike carbonium ions, carbanions, or free radicals, where the spin state is usually obvious, there are two possible electronic configurations which can occur readily in divalent carbon species: (a) a singlet configuration (**1**) in which the unshared electrons are paired in an sp^2 orbital, leaving an empty p orbital, or (b) a triplet configuration (**2**) in which the two unshared electrons are unpaired, each in a p orbital, and in which the RĊR′ angle is



1 **2**

more nearly linear than when the molecule is in configuration **1**.

A number of chemical criteria have been developed to assist in the assignment of the spin state.[2] The application of these criteria is logically an inductive process in which the spin state is inferred from chemical evidence. For example, Skell, *et al.*,[3] studied the photolysis of diphenyldiazomethane (**3**) in *cis*- and *trans*-2-butene. From the fact that the addition was not completely stereospecific, they concluded that diphenyl-

(1) (a) J. Hine, "Divalent Carbon," Ronald Press, Inc., New York, N. Y., 1964; (b) W. Kirmse, "Carbene Chemistry," Academic Press Inc., New York, N. Y., 1964; (c) G. G. Rozantsev, A. A. Fainzil'berg, and S. S. Novikov, *Usp. Khim.*, **34**, 177 (1965); *Russ. Chem. Rev.*, 69 (1965).

(2) (a) P. S. Skell and R. C. Woodworth, *J. Amer. Chem. Soc.*, **78**, 4496 (1956); (b) R. C. Woodworth and P. S. Skell, *ibid.*, **81**, 3383 (1959); W. von E. Doering and P. LaFlamme, *ibid.*, **78**, 5447 (1956). For a review, see P. P. Gaspar and G. S. Hammond, Chapter 12 of ref 1b.

(3) R. M. Etter, H. S. Skovronek, and P. S. Skell, *J. Amer. Chem. Soc.*, **81**, 1009 (1959).