Degrees of Difficulty of Water-Consuming Reactions in the Absence of Enzymes

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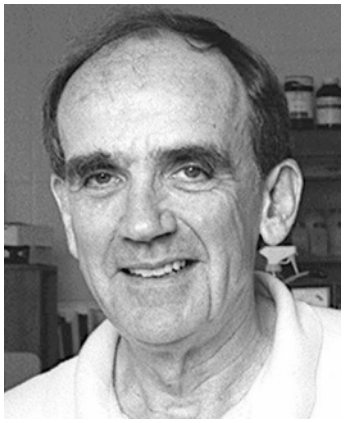
Contents

"A field cannot be well seen from within the field."[1]

1. Introduction

In primitive organisms, enzymes presumably evolved to meet the demand for a molecule with enough catalytic power to turn a substrate over at a rate that conferred some benefit on the host organism under the conditions that prevailed at the time. A second requirement was that this must be accomplished at the very limited concentrations of a complex catalytic molecule that are possible without encroaching on the cell's typical water content of ∼70%. For example, if a 0.1% share of the dry weight of the cell is assigned to a particular enzyme with a molecular weight of 32,000 per active subunit, then the concentration of those subunits is 10^{-5} M. If one supposes that 10,000 molecules of substrate must be turned over by each those subunits within 20 min—the approximate generation time of *E. coli* growing in rich medium—then each subunit must turn over 10 molecules of substrate per second. That value, not greatly exceeded by the actual turnover numbers (k_{cat}) of most modern enzymes that act along the central pathways of metabolism, represents a remarkable achievement. Most enzymes exhibit k_{cat} values between 50 and 5000 s^{-1}, with a typical value in the neighborhood of 500 s^{-1}.



Richard Wolfenden is Alumni Distinguished Professor of Biochemistry, Biophysics and Chemistry at the University of North Carolina at Chapel Hill. He received his Ph.D. from the Rockefeller Institute in 1964 after working with F. A. Lipmann and W. P. Jencks. He has long been fascinated by Michael Polanyi's early realization that catalysis is related to a catalyst's special affinity for the altered substrate in the transition state. Trying to work out some experimental implications of that relationship, he has investigated the design of transition state analogue inhibitors, the structural basis of eyzyme–ligand binding affinities, the physical limits of enzyme–ligand binding discrimination in water, and the actual magnitudes of the factors by which enzymes enhance reaction rates. Although his manual dexterity leaves something to be desired, he continues to enjoy experimental work.

But to appreciate what was accomplished during the evolution of modern enzymes, it is helpful to consider not only the turnover number but also the inherent difficulty of the reaction that each enzyme catalyzes. Until recently, the *increase* in rate that a typical enzyme produces had received relatively little attention from experimentalists. Rate enhancements (k_{cat}/k_{non}) had been estimated as 2×10^6-fold for chorismate mutase,[2] 8×10^6-fold for carbonic anhydrase,[3] and 1×10^9-fold for triosephosphate isomerase.[4] Those values, based on uncatalyzed reaction rates that could be measured over periods of a few seconds to a few days at ambient temperatures, suggested that enzymes might produce a typical rate enhancement in the neighborhood of 10^8-fold.

These experiments involved reactions proceeding at rates that are convenient to follow at room temperature. As these

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apparent constraints of experimental convenience have been abandoned in recent work, it has become evident that the rate enhancements produced by some enzymes are vastly greater than those early experiments seemed to imply.[5] Based on earlier reports, Jencks used a back-of-the-envelope calculation to estimate that urease might produce a rate enhancement as large as 10^{12}-fold.[6] Because there is a difference in mechanism between the catalyzed and uncatalyzed reactions, even Jencks' estimate—which seemed startling at the time—is now known to be exceeded by the actual increase in the rate of urea hydrolysis that urease produces.[7]

Why would one wish to know the rate of a biological reaction in the absence of an enzyme? That information would allow biologists to appreciate what natural selection has accomplished in the evolution of enzymes as proficient catalysts, and it would enable chemists to compare enzymes with artificial catalysts produced in the laboratory. A renewal of interest in enzyme rate enhancements has arisen from efforts to produce synthetic catalysts with enzyme-like properties and to understand and quantify some of the factors that are involved in an enzyme's ability to stabilize the transition state in substrate transformation. A simple algebraic relationship shows that an enzyme's affinity for the altered substrate in the transition state (symbolized here as $S^{\ddagger}$) matches or exceeds the value of k_{cat}/K_m divided by the rate constant for the uncatalyzed reaction in water (Figure 1).[8]

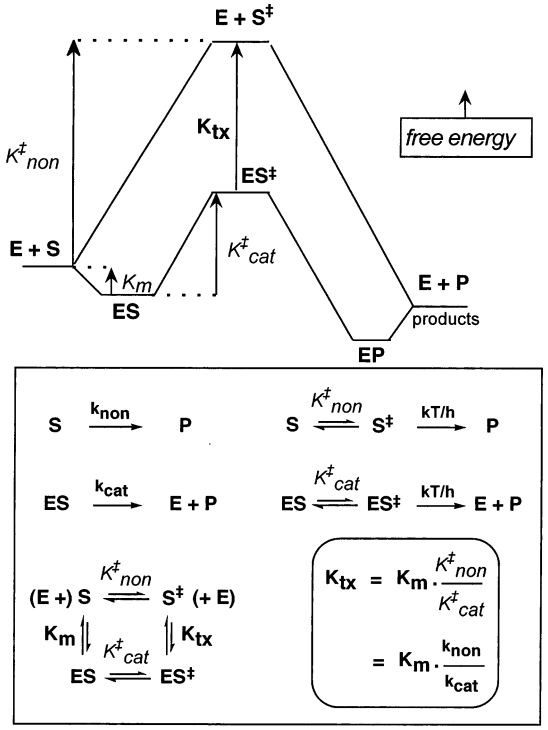

Figure 1. Algebraic relationship describing an enzyme's affinity for the altered substrate in the transition state.

The validity of that relationship does not depend on the detailed mechanism by which any particular enzyme may act. Potential contributions to catalysis by substrate desolvation, by H-bonding and ionic forces of attraction, and by the juxtaposition of several substrates in a configuration appropriate for reaction can all be understood in terms of their contributions to an enzyme's special affinity for the altered substrate in the transition state. Nor is the validity of that relationship affected by whether changes in enzyme conformation occur along the paths to and from the transition state. Indeed, it would be astonishing if progress along the reaction coordinate were not accompanied by changes in enzyme conformation, because of the startling magnitudes of new forces of attraction in the transition state, that were not present in the enzyme−substrate complex in the ground state.[9] Transient changes in the structure of an enzyme's active site, induced by the changing structure of the substrate during its chemical transformation, are probably just as real as the changes in the structure of the substrate that are induced by the enzyme.[10] Moreover, changes in enzyme structure may extend to points that are distant from the active site, if they are connected to the active site by structural elements that are fairly rigid.

In addition to their bearing on mechanistic questions, experiments to establish the rate of benchmark reactions, for comparison with enzyme kinetic constants, are expected to yield practical benefits. First, the greater the rate enhancement that an enzyme produces, the greater is its expected sensitivity to inhibition by a stable molecule that resembles the transition state in structure. That principle (Figure 1) has furnished a basis for the design of transition state analogues, stable molecules exploiting that special affinity. Examples have now been discovered for enzymes of every class, including agents that are currently used to control hypertension, the spread of HIV, the maturation of insects, and the growth of weeds.[11] By allowing "snapshots" of enzymes in action, transition state analogues have also provided valuable tools for investigating enzyme structures and mechanisms, most recently that of the peptide bond-forming center of the ribosome. Those enzymes that produce the largest rate enhancements and transition state affinities should offer the most sensitive targets for inhibitor design. In cases where the rate enhancement is especially large, even a molecule that captures only a small part of that special affinity is expected to be an extremely effective, compared with a conventional substrate analogue. For that reason, the identification of especially proficient enzymes (i.e. enzymes for which $(k_{cat}/K_m)/(k_{non})$ is unusually large) is expected to be valuable in guiding investigators to particularly sensitive targets for inhibitor or drug design. Because they present such a challenge to a catalyst, these very slow reactions also tend to be of greatest mechanistic interest.

Second, in the design of powerful reversible inhibitors, the altered substrate in the transition state represents an ideal ligand for which the enzyme has presumably been optimized by natural selection, but whose binding properties will never be matched exactly by the binding properties of any molecule with a stable geometry. Accordingly, it is not surprising that even the "best" transition state analogues tend to be bound with an affinity (as indicated by their K_i values as competitive inhibitors) that is considerably less than the ideal affinity indicated by comparing the second-order rate constant for the enzyme reaction (k_{cat}/K_m) with the first-order rate constant (k_{non}) for the uncatalyzed reaction in water in the absence of a catalyst. But a few inhibitors do capture much of the negative free energy of binding that would be expected of an ideal transition state analogue. Particularly striking examples are immucillin, an inhibitor of purine nucleoside phosphorylase,[12] and 3,4-dihydrouridine, an inhibitor of cytidine deaminase.[13] In both cases, the inhibitor was designed to resemble an intermediate that approaches the transition state in structure and is bound by the enzyme 10^7−10^8-fold more tightly than the corresponding substrate. The remaining shortcomings of these inhibitors seem likely to

reward closer scrutiny. Concrete information about enzyme rate enhancements makes it possible to estimate the affinity that would be expected of an ideal transition state analogue inhibitor of any particular enzyme reaction, and also furnishes a basis for detailed thermodynamic analysis of the shortcomings of existing inhibitors. When the structural sources of those limitations have been identified, it may become possible to design inhibitors that bear a closer resemblance to the elusive ideal of the transition state itself.[14]

The magnitudes of the rate enhancements that enzymes produce are the subject of this review. Nearly all of the work mentioned here concerns hydrolytic or hydration reactions. Most multisubstrate reactions remain to be explored, and those are expected to differ from single-substrate reactions in the demands that they place on a proficient catalyst. Some of those differences are already apparent in one example that has been described recently: peptidyl transfer from peptidyl-tRNA to the amino group of aminoacyl-tRNA in the ribosome.[15]

Before considering the rates of uncatalyzed reactions, it may be helpful to summarize the state of current information about the rates of enzyme reactions.

2. Rates of Enzyme Reactions

The decomposition of the enzyme−substrate complex can be described by a first-order rate constant (k_{cat}), termed the turnover number:

$$\textit{turnover number}$$
$$k_{cat}\ (s^{-1})$$
$$ES \rightarrow E + P$$
$$d(P)/dt = (k_{cat})(ES)$$

Under physiological conditions, the concentration of the substrate is usually subsaturating, allowing the rate of the enzyme reaction to respond to changing substrate concentrations. The rate of product formation is then described by the second-order rate constant k_{cat}/K_m, usually termed the catalytic efficiency or specificity constant.

$$\textit{catalytic efficiency}$$
$$k_{cat}/K_m\ (s^{-1}\ mol^{-1})$$
$$E + S \rightarrow E + P$$
$$d(P)/dt = (k_{cat}/K_m)(E)(S)$$

The second-order rate constant for enzyme−substrate encounter is probably in the neighborhood of $10^9\ s^{-1}\ M^{-1}$, and that rate constant sets an upper limit on the second-order rate constant (k_{cat}/K_m) for the overall conversion of enzyme and substrate to the enzyme and product.[16]

Many enzymes are highly efficient by that criterion, a property that would be hard to understand if successful encounter required a form of the substrate or the enzyme that was not reasonably populous. When that argument was first advanced,[9] only five values were available for (k_{cat}/K_m), and not all of those had been firmly established. Table 1 presents some of the values of (k_{cat}/K_m) that have been reported during the intervening years, along with information about the physical significance of (k_{cat}/K_m), mainly inferred from the observed effects of viscosogenic agents on (k_{cat}/K_m).[17] Catalytic efficiencies are seen to cluster in the range between 10^5 and $10^9\ M^{-1}\ s^{-1}$, with a mean value in the neighborhood of $10^7\ M^{-1}\ s^{-1}$. The lower values tend to refer to reactions in which k_{cat}/K_m is not diffusion-limited. But most of the higher values apply to reactions for which (k_{cat}/K_m) has been shown to be diffusion-limited, leading one to infer that the chemical transformation of the substrate takes place more rapidly than is indicated by the observed value of (k_{cat}/K_m).

These second-order rate constants, which allow reactions to proceed at useful rates at the limited concentrations at which enzymes are present within the cell, are remarkable in view of the slow rates at which many of the same reactions take place spontaneously in water in the absence of an enzyme.

3. Measuring the Rates of Uncatalyzed Reactions in Water

The characterization of very slow reactions in water presents unusual demands. It is necessary at the outset to choose a model reaction that is uncomplicated by competing side reactions: glycoside cleavage, for example, in a nucleotide whose rate of decarboxylation one wishes to measure.

If a reaction follows simple first-order kinetics, its rate constant can sometimes be obtained from observations at room temperature, using a sensitive method for detecting reaction products in very small amounts.[18−20] Another approach, more general in its application, is to measure reaction rates at a series of elevated temperatures. The resulting Arrhenius plot, if it is linear, can then be extrapolated to 25 °C. How slow a reaction can be measured by this method? According to a "rule of thumb", traceable to some early experiments by Harcourt,[21] reaction rates tend to double with a 10 °C rise of temperature so that "$Q_{10} = 2$". If it were correct, that generalization would lead one to expect a 65-fold increase in rate if the temperature rose from 25 to 100 °C. But in fact, reactions can be conducted at higher temperatures in sealed tubes, and very slow reactions tend to have much larger heats of activation. For example, the rate of a reaction with an E_{act} of 24 kcal/mol ($Q_{10} = 4$)—a value typical of the hydrolysis of C−N bonds in water—increases 3×10^6-fold as the temperature is raised from 25 °C to 200 °C, allowing one month of observation to be compressed into a single second. Moreover, much higher values of E_{act} are often observed in very slow reactions. A practical consequence of that behavior is that even very slow reactions are within reach of experiment somewhere in the range between 0 and 374 °C, over which water exists in liquid form.

Reactions in water at elevated temperatures must be conducted in sealed vessels. In our initial experiments on peptide hydrolysis, for example, 0.5 mL of a solution of peptide (0.01 M) in buffer (0.1 M potassium phosphate or potassium acetate, adjusted to ionic strength 2.0 with KCl) was sealed under vacuum in a quartz tube (4 mm internal diameter, 1 mm wall thickness, obtained from G. M. Associates, Inc., Oakland, CA). The sealed tube was incubated in a small oven with an ASTM thermometer for a measured time interval and then cooled on ice and opened. An aliquot (0.2 mL) was evaporated to dryness, D_2O (2 mL) was added, and the sample was evaporated to dryness; this procedure was repeated twice to replace exchangeable protons. The sample was then dissolved in D_2O (0.5 mL), and pyrazine (0.01 M in D_2O, 0.5 mL) was added as an

Table 1. Catalytic Efficiencies (k_{cat}/K_m) at 25 °C Reported for Enzymes of Various Types[a]

enzyme	substrate	k_{cat}/K_m, M^{-1} s^{-1}	rate-det step
superoxide dismutase[b]	superoxide	7×10^9	diffusion
fumarase[c]	fumarate	1×10^9	diffusion
triosephosphate isomerase[d]	glyceraldehyde 3-phosphate	4×10^8	diffusion
β-lactamase[e]	penicillin	1×10^8	partly diff
chymotrypsin[f]	MocTrp *o*-nitrophenyl ester	9×10^7	partly diff
OMP decarboxylase[g,h]	orotidine 5′-phosphate	6×10^7	not diff
cytochrome *c* peroxidase[i]	hydrogen peroxide	5×10^7	not diff
phosphotriesterase[j]	*p*-nitrophenyl phosphate	5×10^7	diffusion
catalase[k]	hydrogen peroxide	4×10^7	partly diff
alkaline phosphatase[l]	4-nitrophenyl phosphate	3×10^7	diffusion
lipoxygenase-1[m]	linoleic acid	3×10^7	partly diff
HIV protease[m]	peptide	2×10^7	not diff
fructose 1,6-bisphosphatase[n]	fructose 1,6-bisphosphate	1.5×10^7	unknown
4-oxalocrotonate tautomerase[o]	4-oxalocrotonate	2×10^7	unknown
carbonic anhydrase[p,q]	carbon dioxide	7×10^6	partly diff
carboxypeptidase a[r]	furylacryloyl-Phe-Phe	7×10^6	diffusion
adenosine deaminase[s]	adenosine	1×10^7	partly diff
staphylococcal nuclease[t]	DNA, pH 9.5	1×10^7	diffusion
acetylcholinesterase[u]	acetyl thiocholine	1×10^7	diffusion
cytidine deaminase[v]	cytidine	3×10^6	not diff
ribonuclease T2[w]	GpC	2×10^6	diffusion
chorismate mutase[x,y]	chorismate	2×10^6	diffusion
mandelate racemase[z]	mandelate	1×10^6	partly diff
ACP synthase[aa]	*S*-adenosylmethionine	1×10^6	diffusion
histamine N-Me transferase[bb]	histamine	7×10^5	unknown
3-chloroacrylate dehalogenase[cc]	3-chloroacrylate	1.2×10^{-5}	not diff
aspartate aminotransferase[dd]	L-aspartate	1×10^5	not diff
threonine dehydrogenase[ee]	L-threonine	9×10^4	unknown
alcohol dehydrogenase[ff]	ethanol	8×10^4	unknown

[a] "Diffusion" indicates those enzymes for which the second-order rate constant (k_{cat}/K_m) appears to be governed by enzyme−substrate complex formation, and "not diff" indicates reactions for which k_{cat}/K_m appears to be limited by a later step rather than by substrate binding. [b] Fielden, E. M.; Roberts, P. B.; Bray, R. C.; Lowe, D. J.; Mautner, G. N.; Rotilio, G.; Calabreses, L. *Biochem. J.* **1974**, *139*, 49. [c] Sweet, W. L.; Blanchard, J. S. *Arch. Biochem. Biophys.* **1990**, *277*, 196. [d] Blacklow, S. C.; Raines, R. T.; Lim, W. A.; Zamore, P. D.; Knowles, J. R. *Biochemistry* **1988**, *27*, 1158. [e] Hardy, L. W.; Kirsch, J. F. *Biochemistry* **1984**, *23*, 1275. [f] Brouwer, A. C.; Kirsch, J. F. *Biochemistry* **1982**, *21*, 1302. [g] Miller, B. G.; Snider, M. J.; Short, S. A.; Wolfenden, R. *Biochemistry* **2000**, *39*, 8113. [h] Porter, D. J. T.; Short, S. A. *Biochemistry* **2000**, *39*, 11788. [i] Loo, S.; Erman, J. E. *Biochem. Biophys. Acta* **1977**, *481*, 279. [j] Caldwell, S. R.; Newcomb, J. R.; Schlecht, K. A.; Raushel, F. M. *Biochemistry* **1991**, *30*, 7438. [k] Ogura, Y. *Arch. Biochem. Biophys.* **1955**, *57*, 288. [l] Simopoulos, T. T.; Jencks, W. P. *Biochemistry* **1994**, *33*, 10375. [m] Szeltner, Z.; Polgar, L. *J. Biol. Chem.* **1996**, *271*, 32180. [n] Kelley, N.; Giroux, E. L. Lu, G.; Kantrowitz, E. R. *Biochem. Biophys. Res. Commun. 219*, 848. [o] Harris, T. K.; Czerwinski, R. M.; Johnson, W. H., Jr.; Legler, P. M.; Abaygudnawardana, C.; Massiah, M. A.; Stivers, J. T.; Whitman, C. P.; Mildvan, A. S. *Biochemistry* **1999**, *38*, 12343. [p] Pocker, Y.; Janjic, N. *Biochemistry* **1988**, *27*, 4114. [q] Pocker, Y.; Janjic, N. *J. Am. Chem. Soc.* **1989**, *111*, 731. [r] Khoshtariya, D. E.; Hammerstad-Perersen, J. M.; Ulstrup, J. *Biochim. Biophys. Acta* **1991**, *1076*, 359. [s] Kurz, L. C.; Weitkamp, E.; Frieden, C. *Biochemistry* **1987**, *26*, 3027. [t] Hale, S. P.; Poole, L. B.; Gerlt, J. A. *Biochemistry* **1993**, *32*, 7479. [u] Bazelyanski, M.; Robey, E.; Kirsch, J. F. *Biochemistry* **1986**, *25*, 125. [v] Snider, M. J.; Gaunitz, S.; Ridgway, C.; Short, S. A.; Wolfenden, R. *Biochemistry* **2000**, *39*, 9746. [w] Steyaert, J.; Wyns, L.; Stanssens, P. *Biochemistry* **1991**, *30*, 8661. [x] Mattie, P.; Kast, P.; Hilvert, D. *Eur. J. Biochem.* **1999**, *261*, 25. [y] Gustin, D. J.; Mattei, P.; Kast, P.; Wiest, O.; Lee, L.; Cleland, W. W.; Hilvert, D. *J. Am. Chem. Soc.* **1999**, *121*, 1756. [z] Kallarakal, A. T.; Mitra, B.; Kozarich, J. W.; Gerlt, G.; Clifton, J.; Petsko, G. A.; Kenyon, G. L. *Biochemistry* **1995**, *34*, 2788. [aa] Li, Y.; Feng, L.; Kirsch, J. F. *Biochemistry* **1997**, *36*, 15477. [bb] Callahan, B. P.; Wolfenden, R. *J. Am. Chem. Soc.* **2003**, *125*, 310. [cc] Horvat, C. M.; Wolfenden, R. *J. Am. Chem. Soc.* **2005**, *45*, 16199. [dd] Goldberg, J. M.; Kirsch, J. F. *Biochemistry* **1996**, *35*, 5280. [ee] Boylan, S. A.; Dekker, E. E. *J. Biol. Chem.* **1981**, *256*, 1809. [ff] Weinhold, E. G.; Benner, S. A. *Protein Eng.* **1995**, *8*, 457.

internal standard for measuring the integrated intensities of nonexchangeable protons by proton NMR. That procedure, dissolving the sample in D_2O only after cooling, was adopted to avoid solvent deuterium effects on reaction rates and to avoid exchange of carbon-bound hydrogen atoms. Experience has shown that the evaporation step is unnecessary for most samples if water peak suppression is used, provided that the resonances being examined are well-separated from the water peak. In most cases, the same goals can be achieved by simply diluting the sample 20-fold with D_2O containing an integration standard. By observing the integrated intensities of signals from carbon-bound protons of peptides and the products of their decomposition, it proved possible to follow the course of each of these reactions by proton NMR, accounting for each of the species arising during the course of peptide hydrolysis. First-order rate constants were obtained by dividing $\log(A_o/A)$ by the time elapsed, where A_o = the starting concentration of peptide and A = (A_o minus the concentration of product formed).

An advantage of using small quartz tubes as reaction vessels is that their contents arrive at thermal equilibrium in the oven within 5 min. A disadvantage is that quartz tubes containing water tend to explode at temperatures above 260 °C. That problem can be circumvented by enclosing each tube in a stainless steel pipe bomb, along with a few milliliters of water. In that way, the vapor pressure is roughly equalized across the walls of the tube, and reaction mixtures can be heated to temperatures up to and exceeding the critical point of water, 374 °C.

But alkaline reaction mixtures dissolve silicic acid, so that these reactions cannot be examined at high temperatures in quartz. At high temperatures, it becomes necessary to conduct alkaline reactions in vessels of PTFE (Teflon) enclosed in a stainless steel bomb (Figure 2). A disadvantage of this approach is that the sample's arrival at thermal equilibrium in the oven is sufficiently gradual, because of the thermal inertia of the steel bomb and Teflon liner, that long reaction times are required. A second limitation is that reactions

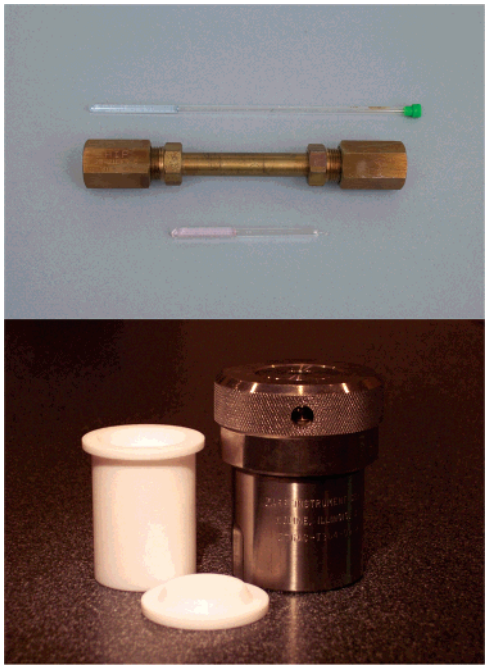

Figure 2. (top) Sealed quartz tube and stainless steel pipe bomb (High Pressure Equipment Co.); (bottom) Teflon-lined stainless steel bomb, 23 mL capacity (Parr Instrument Co. #276AC).

cannot be conducted above 260 °C, because Teflon begins to flow at higher temperatures. In some recent experiments of that kind, reaction mixtures were introduced into Teflon-lined stainless steel bombs (Parr Instrument Co. #276AC) that were placed in convection ovens (Barnstead/Thermolyne Corp. #47900) and maintained at constant temperatures ranging from 150 to 260 °C (temperature variation ±1.5 °C as determined with an ASTM thermometer) for varying periods of time. Approximately 120 min elapsed before the contents of the PTFE-lined reaction vessels arrived within 2 °C of the target temperature, as determined using a bomb and liner that had been drilled to accommodate an ASTM thermometer inserted through the top of the oven. To minimize errors arising from that time lag, all reactions were conducted for a period of at least 16 h, some much longer, and 2 h was subtracted from the total elapsed time in calculating rate constants.

In summary, quartz tubes are useful at all temperatures, but only for reactions conducted at pH values below 9. Teflon reaction vessels are useful at all pH values but become deformed at temperatures above 260 °C. Aqueous reactions above that temperature, at high pH, are not yet accessible to experiment.

To establish the rate of an uncatalyzed reaction in water in the absence of acids, bases, or other catalysts, it is necessary to find conditions under which the rate of reaction does not change with changing pH (Figure 3). Using that precaution, complications are avoided that might have arisen from differing heats of ionization of substrates or buffers. In practice, most simple reactions yield linear Arrhenius plots over a sufficient range to allow the rate constant to be estimated at ambient temperature with reasonable accuracy. Figure 4 compares the results obtained from an Arrhenius plot (upper left corner) with those obtained by direct measurement (lower right corner) for the hydrolysis of a C-terminal peptide bond at room temperature, using a very sensitive fluorimetric method for detecting product formation. Considering the length of the extrapolation and the fact that

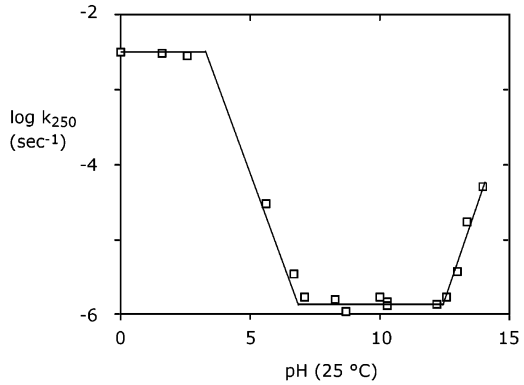

Figure 3. Influence of pH on the hydrolysis of dineopentyl phosphate at 250 °C.

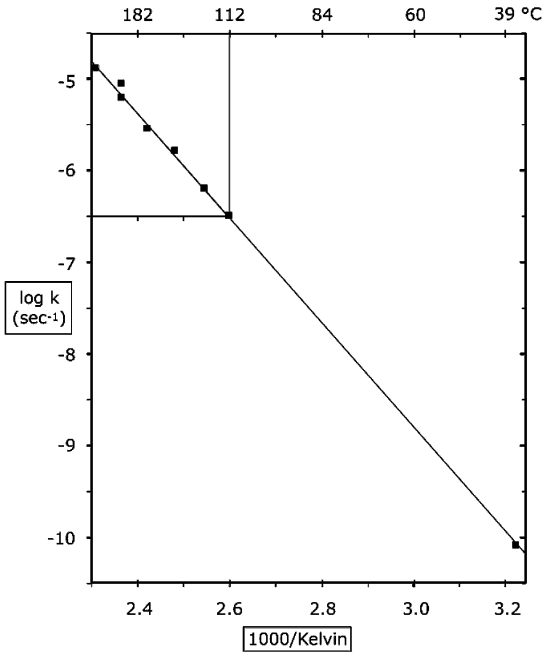

Figure 4. Arrhenius plot of rate constants for peptide hydrolysis observed at elevated temperatures and at ambient temperature.

these measurements were made by different methods in different laboratories, the agreement is satisfactory. The Arrhenius plot of the observed rate constants appears to be linear, within experimental error, over a range of 5 orders of magnitude.

4. Rate Constants for Hydrolysis: A Preview

Before considering the rates of individual biological reactions in neutral aqueous solution in the absence of a catalyst, it may be helpful to place these reactions in context. Figure 5 displays a logarithmic scale of apparent first-order rate constants and half-times for water-consuming reactions proceeding in dilute solution at or near pH 7, at 25 °C. The observed range of values, spanning 14 orders of magnitude, gives some sense of the relative degrees of difficulty of the tasks faced by biological catalysts.

The present review is confined to water-consuming reactions, proceeding at pH 7. Reactions at other pH values are not included in Figure 5, nor are nonhydrolytic reactions that involve a single substrate or multiple substrates other than water. The reaction of water with the dianion of phosphate monoesters, for example, is the reaction catalyzed by many phosphatases. That uncatalyzed reaction is vastly slower than the reaction of the monoanion with water near neutrality and

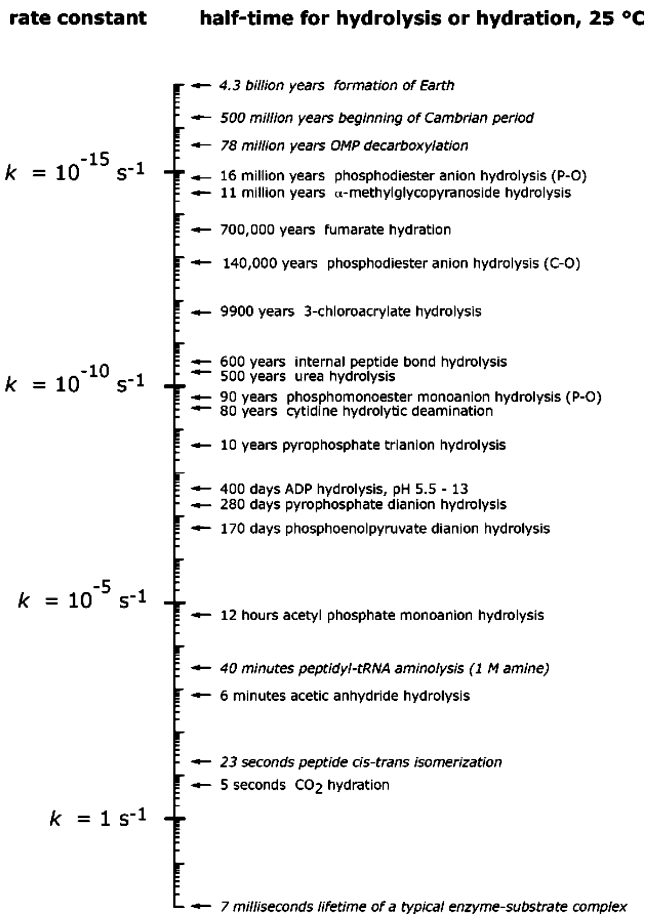

Figure 5. Logarithmic scale of rate constants and half-times for hydrolysis and hydration reactions, extrapolated to 25 °C.

can only be observed at very high pH, where it proceeds with an estimated half-time of roughly 10^{12} years. Most reactions that involve two or more substrates, not including water, remain to characterized in this way.

5. Hydrolysis of Peptides

In biological systems, peptide bonds are cleaved by two types of hydrolases that act by different mechanisms. The first group, typified by chymotrypsin and papain, employs an active site nucleophile (the side chain of serine or cysteine) to displace the amine leaving group from the substrate to form a covalently bound acyl-enzyme intermediate that is subsequently hydrolyzed. Rate enhancement by enzymes that act through a double-displacement mechanism is not simple to analyze in terms of transition state affinity or transition state stabilization,[22] although rates of reaction with model nucleophiles can be used to estimate equilibrium constants for transition state interchange between an enzyme and the model nucleophile.[23] A second group of C−N hydrolases, typified by carboxypeptidases and the angiotensin-converting enzyme, catalyzes direct attack by water on carbon to displace the leaving amine, and therefore lends itself directly to the estimation of transition state affinities.

The susceptibility of peptide hydrolysis to catalysis by acids, bases, and metal complexes is well established: glycylglycine, for example, is hydrolyzed with a half-time of approximately 2 days in 1 M NaOH and 150 days in 1 M HCl, at 25° C,[24] and unactivated peptide bonds are cleaved efficiently in the presence of complexes of cobalt,[25] palladium,[26] and copper.[27] Enzymes catalyzing the hydrolysis of peptide bonds have been characterized extensively with respect to their catalytic efficiencies (k_{cat}/K_m), structures, and sensitivities to reversible and irreversible inhibitors. To appreciate the abilities of peptide hydrolases to enhance the rates of hydrolysis of peptide bonds, it would be desirable to have information concerning the rate constants (k_{non}) of nonenzymatic reactions corresponding to those catalyzed by carboxypeptidases, endopeptidases, aminopeptidases, and dipeptidases, under comparable conditions.

The uncatalyzed hydrolysis of peptide bonds had received relatively little attention until Kahne and Still, using a resin-bound peptide with ^{14}C-labeled glycine at its C-terminus, were able to monitor the release of small quantities of glycine with an extrapolated half-time of several years, throughout the pH range near neutrality.[19]

We examined the cleavage of glycine-containing peptides of various types by analyzing the contents of sealed quartz tubes that had been exposed to elevated temperatures for various intervals.[28] Based on the integrated intensities of carbon-bound proton resonances observed by high-field NMR, it proved possible to account for all species arising during the cleavage of simple peptides and to determine first-order rate constants for peptide cleavage. Although susceptible to decarboxylation at higher temperatures, glycine itself underwent less than 1% decomposition under the most extreme conditions used in these experiments. Rate constants obtained at elevated temperatures were then extrapolated to room temperature. First-order rate constants for hydrolysis of bonds joining two glycine residues were determined for (1) glycylglycine, representing the substrate of a generic dipeptidase, (2) *N*-acetylglycylglycine, representing the substrate of a generic carboxypeptidase, and (3) *N*-acetylglycylglycine *N*′-methylamide, representing an internal peptide bond, and the results were extrapolated to 25 °C. The rate of hydrolysis of the peptide bond in glycylglycine *N*-methylamide, which might have represented the substrate of a generic endopeptidase, could not be measured because of its rapid cyclization to form diketopiperazine.

To monitor the hydrolysis of glycylglycine (GG), correction was made for the rapid equilibration of GG with the cyclic dipeptide diketopiperazine (G=G) at elevated temperatures, to obtain the actual concentration of GG that was present under each set of reaction conditions.[29−31] When that precaution was adopted, hydrolysis of glycylglycine (GG) to glycine (G) followed satisfactory first-order kinetics over the temperature range from 120 to 200 °C, as indicated by linear semilogarithmic plots of A_o/A as a function of the time elapsed. To determine the rate constant for hydrolysis of GG to glycine, allowance was made for the "buffering" effect of the relatively rapid equilibrium between GG and G=G described above, by expressing rates of product formation as a function of the reduced concentrations of GG that were actually present at equilibrium under the reaction conditions. In 0.1 M potassium acetate and phosphate buffers in the range from pH 4.2 to pH 7.8, hydrolysis of GG was found to proceed with a rate constant of 9×10^{-6} s^{-1} at 150 °C. Rates of reaction did not change when buffer concentrations were varied in the range from 0.1 to 0.3 M, nor was the rate of reaction affected by variations of the ionic strength from 0.5 to 2, adjusted by adding KCl to 0.1 M potassium acetate buffer (pH 4.7) or potassium phosphate buffer (pH 6.8). Apparent rate constants in 0.1 M potassium acetate, pH 5.0, plotted as a logarithmic function of $1/T$, yielded $\Delta H^{\ddagger} = +22.4$ kcal/mol, for the conversion of GG to G + G in the

neutral pH range, and $k_{25°C} = 6.3 \times 10^{-11}$ M^{-1} s^{-1}. At pH 7, the rates of acid- and base-catalyzed hydrolysis of GG reported earlier,[24] extrapolated to pH 7, were found to be much too slow to contribute significantly to the rate of hydrolysis of GG observed at pH 7. Accordingly, that value appears to represent the typical rate constant for uncatalyzed hydrolysis of a simple dipeptide.

Acetylglycylglycine (AcGG), with a peptide bond resembling those cleaved by a carboxypeptidase, was found to decompose by hydrolysis of its peptide bond, to yield (1) AcG + G as the major products and (2) acetate + GG as minor products. At 150 °C, for example, AcGG decomposed with a first-order rate constant of 1.1×10^{-5} s^{-1}, while acetylglycine (AcG) accumulated with an apparent first-order rate constant of 5.1×10^{-6} s^{-1}, and GG and G=G accumulated together with a combined apparent rate constant of 3×10^{-6} s^{-1}. Separate experiments showed that the product AcG is hydrolyzed with a rate constant of 2×10^{-6} s^{-1} under these conditions and that a similar product ratio is maintained at lower temperatures. After subtracting the rate of disappearance of the product AcG, the rate constant for cleavage of the peptide bond of AcGG was estimated from the rate of appearance of AcG, as approximately 6×10^{-6} s^{-1} at 150 °C, in the pH range between 4.7 and 7.8. Rates of reaction did not vary when the concentrations of acetate or phosphate buffers varied in the range from 0.1 to 0.3 M, nor was the rate of hydrolysis affected by variations in ionic strength produced by addition of KCl, from 0.3 to 2.0. Rate constants for AcGG hydrolysis, observed over the temperature range between 110 and 170 °C, yielded a linear Arrhenius plot with $\Delta H^{\ddagger} = +24.4$ kcal/mol for the conversion of AcGG to AcG + G in the neutral pH range. The rate constant for hydrolysis of the peptide bond in AcGG, obtained by extrapolation to 25 °C and pH 7, was 2×10^{-11} s^{-1}, considerably lower than the rate constant (3×10^{-9} s^{-1}) reported earlier by Kahne and Still for release of C-terminal glycine from a tetrapeptide (Phe-Phe-Phe-Gly) joined through its N-terminus to a polyacrylamide resin.[18] The nature of the peptide bond undergoing cleavage is similar in these two reactions, and steric hindrance, if any, seems more likely to have been present in the case of the resin-bound peptide. That suggested the possibility that attack by water on the resin-bound peptide might have been assisted by its proximity to the polymer matrix, directly or through some effect on the solvent environment.

Acetylglycylglycine *N*-methylamide (AcGGNHMe), with a peptide bond resembling those that are cleaved by endopeptidases, undergoes nonenzymatic hydrolysis of its peptide bond to yield AcG and GNHMe as the major products (>90%), with a rate constant of 6.0×10^{-6} s^{-1} at 150°, in 0.1 M potassium acetate and imidazole-HCl buffers in the range from pH 4.2 to pH 7.8. When buffer concentrations were varied, no evidence of catalysis by acetate or imidazole buffers was observed in the range from 0.1 to 0.3 M. Unlike hydrolysis of GG or AGG, however, hydrolysis of AGGNHMe was found to be subject to significant catalysis by potassium phosphate buffers ($k_2 = 5.5 \times 10^{-5}$ s^{-1} M^{-1} at pH 6.8, 150 °C). An Arrhenius plot of rate constants observed for the uncatalyzed reaction in potassium acetate buffer, pH 5.0, over a temperature range between 100 and 170 °C, yielded $\Delta H^{\ddagger} = +22.9$ kcal/mol for the conversion of AcGGNHMe to AcG + GNHMe in the neutral pH range.

Hydrolysis of the peptide bond in glycylglycine *N*-methylamide (GGNHMe) might in principle have served as a model for the reaction catalyzed by an aminopeptidase. But that reaction could not be observed because GGNHMe undergoes rapid intramolecular cyclization to form diketopiperazine (G=G), with displacement of methylamine. No trace of any other product was observed. In GGNHMe, diketopiperazine formation proceeded to completion as a simple first-order process, generating linear semilogarithmic plots of A_o/A as a function of time elapsed. At 80 °C, a rate constant of 6.2×10^{-7} s^{-1} was obtained in potassium phosphate and imidazole-HCl buffers at pH 6.8. Rate constants were measured in 0.1 M potassium phosphate buffer, pH 6.8, over the temperature range between 37 and 130 °C, with $\Delta H^{\ddagger} = 16.6$ kcal/mol for cyclization of GGNHMe. At 37 °C, the rate constant for this process was 3.2×10^{-7} s^{-1}, corresponding to a half-time of 25 days. The observed route by which GGNHMe decomposes, via rapid formation of diketopiperazine, agrees with earlier observations of a similar route of degradation of glycylglycine amide at 130 °C[32] and of several peptides that are degraded by elimination of diketopiperazines from the N-terminal position at 100 °C.[33] Unfortunately, for purposes of comparison with the reaction catalyzed by an aminopeptidase, GGNHMe cyclizes too rapidly to permit measurement of the rate of the competing hydrolytic cleavage of the glycyl−glycine bond in GGNHMe, and G=G is the only observable product. With a half-time of approximately 35 days at pH 7 and 37 °C, diketopiperazine formation is rapid enough to pose an apparent threat to the stability of proteins and to suggest a possible evolutionary reason for the posttranslational N-acetylation of proteins that has been observed in most proteins in higher organisms.[34] It seems reasonable to speculate that, in the relatively long-lived cells of eukaryotes, N-acetylation has evolved as a mechanism for protecting proteins against spontaneous degradation. Short generation times may alleviate this problem in prokaryotic organisms, in which mechanisms for N-acetylation appear to be absent.

Almost simultaneously, Bryant and Hansen[20] reported results employing a sensitive fluorimetric method for following peptide hydrolysis, by reacting the liberated amino groups with naphthalenedialdehyde in the presence of cyanide. That method, capable of detecting as little as 2×10^{-13} moles of amine, permitted them to follow the hydrolysis of benzoylglycylphenylalanine at 25 °C over a period of 50 days, at which time the extent of hydrolysis was 0.09%. Using the same method, Smith and Hansen[35] observed rate constants of 9×10^{-11} s^{-1} for the hydrolysis of phenylacetylglycine (PAG) and 8×10^{-11} s^{-1} for glycylvaline (GV) at 37 °C, with estimated errors of ±50%. Figure 3 compares an Arrhenius plot of the rate constants obtained by Radzicka and Wolfenden at elevated temperatures, for AGG, with the rate constants obtained independently by Smith and Hansen at 37 °C, in neutral solution, for PAG and GV. Considering the length of the extrapolation and the fact that these measurements were made by different methods in different laboratories, the agreement is satisfactory and implies that the Arrhenius plot is linear, within experimental error, over a range of 5 orders of magnitude in rate constants.

6. Hydrolysis of Urea, Carboxylic Acid Amides, and Dihydroorotic Acid

Conventional peptide bonds undergo spontaneous hydrolysis in neutral aqueous solution with a half-time of ~500 years, and typical proteases cleave these bonds with half-times in the neighborhood of 10 ms, increasing the rate of water attack on the peptide bond by a factor of roughly 10^{12}. It would be of interest to know how these rate enhancements compare with those produced by other mechanistically related ═C—N— hydrolases. But in the case of urease, well-characterized in many respects, the meaning of that comparison is clouded by a disparity between the mechanisms of the catalyzed and uncatalyzed reactions.

At the active site of urease, urea appears to undergo nucleophilic attack by water.[36,37] But in solution[38] and in the presence of an artificial dinickel catalyst,[39,40] urea decomposes by elimination of ammonia to form cyanate. The half-time for uncatalyzed elimination is 40 years at 25 °C.[16] It is clear that the half-time for spontaneous hydrolysis of urea must be substantially greater than 40 years, but the actual rate of spontaneous hydrolysis was unknown because that reaction has never been detected experimentally under any conditions.

The results of quantum mechanical simulations, undertaken to approach that question, have been interpreted as indicating that urea hydrolysis may be very slow indeed. Thus, at 25 °C, $t_{1/2}$ was predicted to be ~10^{19} years.[41,42] If that inference were correct, then urease would surpass proteases, and indeed all other enzymes including phosphomonesterases, in its power to enhance the rate of reaction. But in view of the similarity between urea[43,44] and conventional amides[45] in their bond lengths and bond angles, and their implied similarity in resonance energies, that conclusion seemed surprising. If urea hydrolysis were extremely difficult, then a fully methylated derivative of urea that could undergo hydrolysis, but from which elimination could not occur, would be expected to be extremely stable to decomposition in water.

Callahan et al. tested that possibility by examining the hydrolysis of 1,1,3,3-tetramethylurea (Me_4U), comparing its rate constant and heat of activation with those of related compounds including formamide and acetamide.[7] Each compound yielded only the products expected as a result of its hydrolysis; and the rate of hydrolysis of Me_4U was invariant between pH 4 and 10, as had been observed earlier for peptide hydrolysis. When the logarithm of the rate of Me_4U hydrolysis was plotted as a function of the reciprocal of the absolute temperature, extrapolation of the results yielded a rate constant of 4.2×10^{-12} s^{-1} at 25 °C and $\Delta H^{\ddagger} = 22.9$ kcal/mol. These findings, and the results of similar experiments on related compounds, show that the rate constants and thermodynamics of activation for hydrolysis of Me_4U are similar to the values for hydrolysis of structurally related compounds but are very different from the values for NH_3 elimination from urea.

Is urea likely to resemble Me_4U in its susceptibility to hydrolysis? N-Methylation and N-dimethylation are known to influence the measured free energies of solvation of acetamides by water,[46] and those differences would be expected to change to some extent in the transition states for hydrolysis. But solvation and electronic effects are unlikely to be very significant, as indicated by the fact that acetamide undergoes spontaneous hydrolysis only a little more rapidly than *N*,*N*-dimethylacetamide at 25 °C. If that factor is applied to the observed rate constant for hydrolysis of Me_4U, then the estimated first-order rate constant for spontaneous hydrolysis of urea would be 1.2×10^{-11} s^{-1} at 25 °C, quite similar to the average of values for related compounds.

These results led to the inference that the rate enhancement produced by urease is not markedly greater than the rate enhancement produced by hydrolases that bring about water attack on similar bonds, typified by carboxypeptidase B and *E. coli* cytidine deaminase. Within this group of enzymes, which differ in their overall folding and active site topology, urease retains the distinction of catalyzing the hydrolysis of so simple a substrate as urea, with protease-like proficiency. That rate enhancement (3×10^{15}-fold) seems especially remarkable in view of the absence from urea of the molecular complexity ordinarily present in the substrates of proteases, which presumably helps proteases to distinguish between the substrate in the ground state and the transition state for hydrolysis. Urease appears to be unique among ═C—N— hydrolases in containing two nickel atoms, which presumably assist the enzyme in grappling effectively with its unusually simple substrate.

Very recently, the hydrolysis of dihydroorotic acid has been examined for comparison with the reaction catalyzed by dihydroorotases from hamster and a thermophilic bacterium.[47] The pseudo-first-order rate constant for spontaneous hydrolysis at pH 4, where the reaction is pH-independent, is 3.2×10^{-11} s^{-1} at 25 °C, and the associated thermodynamics of activation for this reaction are $\Delta H^{\ddagger} = 24.7$ kcal/mol and $T\Delta S^{\ddagger} = -6.9$ kcal/mol. The magnitudes of these values are comparable with those mentioned above for the uncatalyzed hydrolysis of similar bonds that join the elements of formamide, acetamide, *N*-methylacetamide, urea, and simple peptides,[7] and they are also comparable with those for the exocyclic amino group of cytidine, mentioned below.

7. Hydrolytic Deamination of Cytidine

Cytidine undergoes conversion to uridine by a simple first-order process at temperatures between 80 and 157 °C.[48,49] The results yielded a satisfactory Arrhenius plot, indicating that $\Delta H^{\ddagger} = 22.1$ kcal/mol and $T\Delta S^{\ddagger} = 8.3$ kcal/mol at 25 °C. The rate constant of the uncatalyzed reaction, extrapolated to 25 °C, is 3×10^{-10} s^{-1}. Under the conditions of those experiments, glycoside cleavage and opening of the pyrimidine ring occur at slower rates than deamination (G. Schroeder and R. Wolfenden, manuscript in preparation).

8. Hydrolysis of Phosphoric Acid Monoesters

In biological systems, phosphate monoesters are cleaved by two types of monoesterases that act by different mechanisms. (1) The first group of phosphomonoesterases, typified by bacterial alkaline phosphatase[50] and protein tyrosine phosphatases,[51] employs an active site nucleophile to displace the alcohol leaving group from the substrate to form a phosphoryl-enzyme intermediate which is subsequently hydrolyzed. Rate enhancement by enzymes that act through a double-displacement mechanism is not simple to analyze in terms of transition state affinity or transition state stabilization,[52] although rates of reaction with model nucleophiles can be used to estimate equilibrium constants for transition state interchange between an enzyme and the model nucleophile.[53] (2) A second group of phosphatases catalyzes direct attack by water on phosphorus to displace the leaving alcohol and, therefore, lends itself directly to the estimation

of transition state affinities. Enzymes that bring about direct water attack on phosphate monoesters include protein phosphatase-1, of which numerous variants with differing specificities have been found within the human proteome; fructose 1,6-bisphosphatase, which catalyzes the rate-determining step in gluconeogenesis; and inositol 1-phosphatases, which are involved in the transmission of hormonal signals.

The spontaneous hydrolysis of methyl phosphate (MeP) is relatively rapid at low pH values, where it is present mainly as the monoanion, but proceeds more slowly with increasing pH as the abundance of the monoanion (MeP^-) declines.[54] Initial experiments in sealed quartz tubes[55] showed that the rate of hydrolysis of methyl phosphate appeared to reach a constant value above pH 10, corresponding to the reaction of the dianion (MeP^{2-}) with water. Slow as it was, that reaction proceeded considerably more rapidly than had been expected by extrapolation of rate constants for hydrolysis of monoester dianions with much better leaving groups.[56,57]

Reinvestigation of that discrepancy revealed that the spontaneous hydrolysis of MeP^{2-} proceeds more slowly than we had reported, because of undetected leaching of silicic acid from the quartz reaction vessels by the alkaline solutions. To circumvent that difficulty, the hydrolyses of methyl phosphate and 3-(4-carboxy)-2,2-dimethylpropyl phosphate (CDPP) were conducted in PTFE reaction vessels at high pH over the temperature range 160−240 °C.[58] In 1 M KOH, the rate of hydrolysis was much slower than that at pH 10, showing that the earlier data had indeed led to overestimation of the rate of the dianion reaction. From the new Arrhenius plot, the enthalpy of activation is 50 kcal/mol and the entropy of activation is 23 cal mol^{-1} $degree^{-1}$ for the hydrolysis of CDPP. The results obtained for methyl phosphate were similar. Extrapolating those data to 39 °C yielded a rate constant of 2×10^{-17} s^{-1}, somewhat higher than predicted by the linear free energy relationship of Kirby and Varvoglis[57] but no longer in serious disagreement. The rate of reaction varied inversely with the concentration of KOH, indicating that, even in 1 M KOH, the reaction that is observed is specific acid catalyzed and that MeP^- (not the dianion) is the kinetically active species.

Because the earlier linear free energy relationship[57] included only leaving groups with pK_a values of 7 or less, the range of experiments was extended to include the less acidic alcohols that are typically present in biological phosphate esters. The hydrolysis of phenyl phosphate in 0.1, 0.3, and 1.0 M KOH showed no differences in rate within experimental error, indicating that these data represent the pH-independent reaction of the phenyl phosphate dianion. The enthalpy of activation was 38 kcal/mol, and the entropy of activation was small and positive (7 cal mol^{-1} $degree^{-1}$), consistent with unimolecular decomposition via a loose transition state, a mechanism that has been widely accepted for phosphate monoester dianions.[59,60] Extrapolation of these data yielded a value of 1×10^{-12} s^{-1} for phenyl phosphate at 39 °C, in satisfactory agreement with the earlier linear free energy relationship.

Since the results obtained for methyl phosphate and CDPP established only an upper limit for dianion reactivity, it was of interest to inquire how much lower the actual rate constant for MeP^{2-} hydrolysis is likely to be. The free energy relationship led to the prediction that k_{obs} should be 5×10^{-19} s^{-1} at 39 °C for a leaving alcohol with a pK_a value of 15.5. To estimate the rate at 25 °C, the entropy of activation for spontaneous hydrolysis of MeP^{2-} was assumed to be equivalent to the value determined above for the phenyl phosphate dianion. With an estimated enthalpy of activation of 47 kcal mol^{-1}, the rate would be expected to be approximately 30-fold slower at 25 °C than at 39 °C. Thus, the rate constant for dianion hydrolysis at 25 °C was estimated as 2×10^{-20} s^{-1}, a value in reasonable agreement with the upper limit established by the earlier work[57] and with an earlier estimate based on thermodynamic data.[61]

These results imply that simple phosphate monoesters are much less reactive and that phosphatases are correspondingly more proficient catalysts than had been recognized.[55] Comparisons with kinetic constants that have been reported for type 1 protein phosphatase acting on phosphoryl-phosphorylase *a*,[62] fructose-1,6-bisphosphatase acting on fructose-1,6-bisphosphate,[63] and inositol phosphatase acting on myo-inositol 1-phosphate[64] indicate that each of these enzymes, which catalyze direct water attack on the substrate,[65,66] enhances the rate of water attack on the dianionic substrate by a factor of approximately 10^{21} and binds the altered substrate in the transition state with a formal dissociation constant of approximately 10^{-26} M.

As noted above, transition state affinity cannot be evaluated directly for those phosphatases that react through a covalent phosphoryl-enzyme intermediate. But for that group of phosphatases, a recent investigation of *E. coli* alkaline phosphatase, acting on methyl phosphate under conditions where product inhibition was carefully avoided,[67] has shown that k_{cat}/K_m is similar in magnitude to those of PP1, FBPase, and IMPase. Thus, APase matches the enzymes mentioned above in its formal proficiency ($[k_{cat}/K_m]/k_{non}$) as a catalyst. Among enzymes of this latter type, a high value of k_{cat} (1.5×10^4 s^{-1}) has been recorded for a human protein tyrosine phosphatase, acting on a model peptide through a phosphocysteine intermediate.[68] Comparison with the present rate constant for spontaneous hydrolysis of the phenyl phosphate dianion indicates that PTP enhances the rate of hydrolysis of phosphorylated tyrosine residues by roughly 17 orders of magnitude.

9. Hydrolysis of Phosphodiesters

Phosphoric acid diesters are, in general, exceedingly unreactive in water,[69−71] so that the phosphodiester linkages that join the nucleotides of DNA are highly resistant to spontaneous hydrolysis. By extrapolation of earlier model experiments at elevated temperatures, the uncatalyzed hydrolysis of dimethyl phosphate in neutral solution was found to proceed with an estimated rate constant of $\sim 2 \times 10^{-13}$ s^{-1} at 25 °C, corresponding to a half-time of 100,000 years. But that reaction was found to proceed at least 99% by C−O cleavage, suggesting an upper limit of $\sim 1 \times 10^{-15}$ s^{-1} at 25 °C on the rate constant for spontaneous P−O cleavage of a phosphodiester anion, the reaction that is catalyzed by many phosphodiesterases.[55]

To resolve that discrepancy and to evaluate the approximate rate enhancement produced by phosphodiesterases such as staphylococcal nuclease,[72] we recently examined the rate of spontaneous hydrolysis of dineopentyl phosphate (Np_2P), in which P−O cleavage occurs but C−O cleavage is sterically hindered, and the reactivity of methyl triester analogues.[73]

Rate constants were obtained for Np_2P (0.01 M) hydrolysis at 250 °C in 0.1 M buffers, in anion-forming buffers (potassium formate, acetate, phosphate, borate, and carbon-

ate) whose pH had been determined at 25 °C, and also in solutions containing HCl (0.1−1.0 M) and KOH (0.1−1.0 M). For dineopentyl phosphate (Np_2P), C−O cleavage is sterically precluded and only P−O cleavage occurs, as was demonstrated by mass spectrometric analysis of the products of hydrolysis in $H_2{}^{18}O$. Very similar rate constants were obtained for hydrolysis over the range from pH 6.5 to 13. Extrapolation of the Arrhenius plot indicated that, at 25 °C, the apparent first-order rate constant is 7×10^{-16} s^{-1} for hydrolysis of the Np_2P anion and that $\Delta H^{\ddagger} = 29.5$ kcal/mol for this reaction and $T\Delta S^{\ddagger} = -8.5$ kcal/mol.

In strongly alkaline solution, hydroxide ion catalysis became apparent at KOH concentrations higher than 0.1 M. The results of an Arrhenius plot of rate constants obtained in 1 M KOH were extrapolated to give $k_{25} = 7 \times 10^{-16}$ s^{-1} for this reaction, with $\Delta H^{\ddagger} = 29.5 \pm 0.7$ kcal/mol and $T\Delta S^{\ddagger} = -8.5 \pm 1.0$ kcal/mol. That rate is closely comparable with the rate of hydrolysis in 1 M KOH of $Np^*{}_2P$, in which the leaving alcohol similarly prevents C−O cleavage but P−O cleavage is expected to be unimpeded.[74] The rate of Np_2P hydrolysis also increased at pH values below 6, consistent with water attack on uncharged Np_2P.

The similarity between the extrapolated value of $k_{25} = 7 \times 10^{-16}$ s^{-1} for Np_2P and the approximate *upper* limit on the rate constant for P−O cleavage of dimethyl phosphate ($\sim 1 \times 10^{-15}$ s^{-1} at 25 °C), that was indicated by earlier experiments on dimethyl phosphate (1), suggests that P−O cleavage is not sterically impeded in dineopentyl phosphate. It seems reasonable to infer that the extrapolated rate constant of $k_{25} = 7 \times 10^{-16}$ s^{-1}, equivalent to a half-time of 31,000,000 years at 25 °C, can be considered typical of apparent water attack on the phosphorus atom of simple dialkyl phosphate anions through the range between pH 6.5 and 13.

As to the actual mechanism by which Np_2P is hydrolyzed, it is of interest that the rate constant for Np2P hydrolysis at 100 °C, extrapolated from the present results, is larger by 3 orders of magnitude than would be expected by extrapolation to pK_a 15.5 of a Brønsted plot based on the rates of hydrolysis of anions of diaryl phosphate esters of alcohols with pK_a values ranging from 4 to 8.5.[71] Possibly the dialkyl diester is not hydrolyzed through water attack on the monoanion but through the kinetically equivalent mechanism of hydroxide attack on the neutral diester. One can estimate the rate of the latter reaction by assuming that the methyl triester of $Np^*{}_2P$ is a reasonable model for the neutral diester; at 25 °C, the rate of reaction of $Np^*{}_2P$ with the hydroxide ion is 1×10^{-6} M^{-1} s^{-1}. (The displacement of ethanol from the related triester methyl diethyl phosphate is only 8-fold faster, showing that there is not a significant difference in the combined electronic and steric differences between ethanol and neopentanol as a leaving group.) Taking into account the unfavorable equilibrium ($K \sim 10^{-13}$) for transfer of a proton from water to the diester anion, the predicted rate for the reaction through this mechanism is about 10^{-19} s^{-1}. These comparisons and the associated errors do not allow a clear distinction to be made between the two mechanisms.

It is of interest to consider the bearing of these observations on the lifetime of the backbone of DNA. Because C−O cleavage competes effectively with P−O cleavage, it seems reasonable to suppose that the predominant mode of phosphodiester hydrolysis of DNA might involve C−O cleavage by water (or hydroxide) attack at the relatively unhindered 5′-carbon atom of the nucleoside, to which the phosphoryl group is attached. The possibility of testing that conjecture by experiment is clouded, however, by the likelihood that other modes of decomposition transpire far more rapidly than ester hydrolysis, as discussed below.

A relatively rapid rate of internucleotide hydrolysis ($k = 6 \times 10^{-7}$ s^{-1}) has recently been reported for thymidylyl-3′-5′-thymidine at 80 °C in 1 M.[75] In 1 M KOH at 80 °C, TpT was reported to decompose approximately 5 orders of magnitude more rapidly than would be expected from the present findings for Np_2P. To investigate the source of that discrepancy, we examined the reaction of TpT in 1 M KOH at 80 °C and also in potassium phosphate buffer (0.1 M, pH 6.8). We were able to duplicate the findings of Takeda et al., observing decomposition of TpT at 80 °C in 1 M KOH. We also observed decomposition, proceeding at a similar rate, in potassium phosphate buffer (0.1 M, pH 6.8) at 80 °C. We found, however, that, under both sets of conditions, thymidine itself is unstable and that both thymidine and TpT decompose at similar rates, in such a way as to obscure the rate of cleavage (if any) of the phosphodiester bond of TpT. Moreover, in 1 M KOH, both thymidine and TpT decompose at 80 °C, with virtually identical pseudo-first-order rate constants and activation parameters, to roughly the same set of products, as indicated by proton NMR analysis. UV analysis indicated that opening of the pyrimidine ring proceeds with the same rate constant. Because both thymidine and TpT decompose at similar rates in 1 M KOH at 80 °C, whereas dineopentyl phosphate is hydrolyzed 5 orders of magnitude more slowly under the same conditions, it seems reasonable to infer that ring-opening in base may open elimination pathways for further decomposition (e. g. by aldol cleavage) that were not available before ring-opening and are not available to a simple phosphodiester.

If the rate constant for the uncatalyzed hydrolysis of Np_2P^- anion, extrapolated to 25 °C (7×10^{-16} s^{-1}), is compared with k_{cat} for staphylococcal nuclease at 25 °C (95 s^{-1}),[1] the rate enhancement produced by staphylococcal nuclease is 1.4×10^{17}-fold, corresponding to a 23.2 kcal/mol reduction in $\Delta G^{\ddagger}$. That value is comparable with the rate enhancements produced by OMP decarboxylase (1.4×10^{17}-fold)[5] and β-amylase (7×10^{17}-fold)[76] and is at present exceeded only by the values that have been recorded for fructose 1,6-bisphosphatase (1.1×10^{21}-fold)[58] and arginine decarboxylase (7×10^{19}-fold).[77]

Comparison of $\Delta H^{\ddagger} = 27.8$ kcal/mol for water attack on the Np_2P anion with $\Delta H^{\ddagger} = 10.8$ kcal/mol for reaction of the enzyme−substrate complex of staphylococcal nuclease indicates that most of the 23.2 kcal/mol reduction in $\Delta G^{\ddagger}$ by staphylococcal nuclease is accounted for by a reduction in the heat of activation for phosphodiester hydrolysis. That tendency, also observed in the other slow reactions mentioned in the previous paragraph, seems understandable in view of the fact that most of the very high activation barrier to product formation is enthalpic rather than entropic in these reactions.[78] Moreover, the entropic benefits to be gained by desolvation or juxtaposition of the substrate, as for example in the catalysis of peptidyl transfer by the ribosome,[15] appear to be very limited, especially in one-substrate or hydrolytic reactions in which the second substrate, water, is present in abundance. But a major reduction in enthalpy of activation might be expected for a reaction in which polar forces of attraction, especially ionic H-bonds,[79,80] are at work between an enzyme and the altered substrate in stabilizing the transition state. That is presumably the case in most reactions

that involve general acid or general base catalysis. In the particular case of staphylococcal nuclease,[72] ionized functional groups have been shown to line the active site cavity.[81]

10. Hydrolysis of RNA

RNA phosphodiester hydrolysis, the reaction catalyzed by pancreatic ribonuclease and most ribozymes, does not proceed by direct water attack under any conditions that are presently known (for a recent review, see ref 82). Instead, intramolecular attack by the neighboring 2′-OH group, to form a 2′,3′-cyclic phosphate that undergoes subsequent hydrolysis, appears to be nearly universal, illustrating the advantages of proximity in promoting alkoxide attack. Reported rate constants are 1.3×10^{-8} s^{-1} for the cleavage of 3′,5′-UpU at 80 °C and pH 7[83] and 5×10^{-9} s^{-1} for the cleavage of 3′,5′-UpA at 25 °C and pH 6.[84] A thorough study of 3′,5′-linked ribonucleotides, embedded in the interior of a sequence of deoxynucleotides, indicates a rate constant of 2.2×10^{-11} s^{-1} for the cleavage of 3′,5′-ApG at 23 °C and pH 6.[85] Effects of base composition on the rate of the cyclization reaction appear to be minor (4-fold at most).[85] Even at pH 6, the hydrolysis observed appeared to be base-catalyzed, involving attack by the 2′-alkoxide group.

Once formed, the 2′,3′ cyclic phosphate intermediate undergoes hydrolysis at a rate that reaches a minimum, at which it is apparently free of acid or base catalysis, near pH 6. At that pH, the rate constant is roughly 4×10^{-8} s^{-1} at 50 °C. If we assume that the heat of activation is ∼20 kcal/mol, as in other hydrolytic reactions that proceed at similar rates, that would be equivalent to a rate constant of 4×10^{-8} s^{-1} or a half-time of ∼8 years at 25 °C. Because its hydrolysis is somewhat faster than the rate of its formation, the cyclic intermediate does not accumulate in significant quantities.[86] This special route of decomposition, which is more efficient for ribonucleoside diesters than for compounds that are closely related in structure,[87] resembles the mechanism that is believed to be involved in the action of pancreatic ribonuclease, which should make it possible in principle to compare the thermodynamics of activation for the catalyzed and uncatalyzed reactions.[88]

11. Hydrolysis of Carboxylic and Phosphoric Acid Anhydrides

Carboxylic acid anhydrides are notoriously unstable. Thus, the half-time for hydrolysis of acetic anhydride is 6 min.[89] The reactivity of a biologically important anhydride, acetyl phosphate, was first established by Koshland in 1952.[90] He showed that the acetyl phosphate monoanion, with a pK_a value of 4.5, is hydrolyzed about half as rapidly as the dianion, exhibiting a half-time of 3 h at 39 °C. Both reactions of acetyl phosphate resulted in cleavage of the P−O bond, with acid- and base-catalyzed C−O bond cleavages intervening only in strong acid or base.

Inorganic pyrophosphate is much more stable, especially in forms that carry 2 or more negative charges (pK_a values = 1.5, 2.4, 6.6, 9.25). In a careful early study, Campbell and Kilpatrick established that at 70 °C the dianion is hydrolyzed with a rate constant of 6×10^{-4} s^{-1}, and the trianion with one of 7×10^{-5} s^{-1}.[91] These findings have been confirmed in the author's laboratory (Fergus, M.; Wolfenden, R. Unpublished), along with activation energies that indicate half-times of 9 months for the dianion and 10 years for hydrolysis of the trianion, at 25 °C. The tetraanion is at least 5 orders of magnitude less reactive than the trianion. Thus, the trianion accounts for all the hydrolysis that is observed, even in 1 M KOH.

The time required for spontaneous hydrolysis of ATP and ADP in neutral solution is intermediate between those for di- and trianions of pyrophosphate. That reaction, investigated systematically by Tetas and Lowenstein,[92] was found to be quite insensitive to changing pH in the neutral range. Somewhat surprisingly, the influence of magnesium concentration on the nonenzymatic hydrolysis of ADP and ATP was also found to be almost negligible, even though magnesium exhibits a substantial affinity for ATP in water and is almost invariably involved in the enzyme-catalyzed reactions of ATP. Cleland has shown that Mg-ATP is the true substrate of yeast hexokinase.[93] The absence of any substantial effect of magnesium on the uncatalyzed hydrolysis of ATP would seem to imply that magnesium, although not a catalyst by itself, serves as a link between the enzyme and ATP that becomes more effective in the transition state than in the ground state. Admiraal and Herschlag[94] used Brønsted analysis to show that the bond between phosphorus and the leaving group is largely broken, but the bond to the attacking nucleophile is only just beginning to be formed, in the transition state for phosphoryl transfer.

12. Glycoside Hydrolysis

Polysaccharides account for most of the carbon in the biosphere. The stability of these polymers in water renders them suitable for the storage of metabolic energy in amylose and glycogen and for the maintenance of physical structures supported by cellulose and chitin. The O-glycosidic bonds joining these polymers are readily hydrolyzed in the presence of acids or glycosidases,[95] but the rate of the uncatalyzed hydrolysis of a simple, unactivated, glycoside was not examined until recently.[96,97] When methyl glucopyranosides and methyl ribofuranosides (0.05−0.1 M) were dissolved in potassium acetate, phosphate, or carbonate buffers (0.05−0.2 M), β-methyl-D-glucopyranoside was found to undergo hydrolysis at 220 °C with rate constants approaching a value that remains constant at pH values above 7, as expected for an uncatalyzed reaction with water, and that rate did not vary significantly with changing buffer concentration. When hydrolysis was conducted at pH 10 in the presence of $H_2{}^{18}O$ (70 atom % excess), GC-MS analysis of the methanol produced showed that this reaction occurs >99% by cleavage between the glycosidic oxygen atom and C-1 of glucose (exchange of ^{18}O from water to methanol does not occur to a detectable extent under these conditions). Arrhenius plots of rate constants obtained from 180 to 260 °C (Figure 2), in potassium carbonate buffer (0.1 M, pH 10.6 measured at room temperature), indicate that the enthalpy of activation for spontaneous hydrolysis of β-methyl glucopyranoside is 29.7 kcal/mol. Unlike the acid-catalyzed hydrolysis of β-methyl glucopyranoside (with a positive entropy of activation, as expected for unimolecular decomposition of its conjugate acid[98]), the uncatalyzed hydrolysis of β-methyl glucopyranoside proceeds with a negative entropy of activation (−24 cal degree^{-1} mol^{-1}), consistent with bimolecular attack by water.

In both the ribofuranosides and glucopyranosides, the β-anomer was found to be roughly twice as reactive as the

α-anomer, and removal of the 2′-hydroxyl group enhances the rate of spontaneous hydrolysis by a factor of ~10^3. In those respects, the uncatalyzed hydrolysis of glycosides resembles the acid-catalyzed reactions,[99] despite the difference in mechanism noted above. Methyl ribofuranosides are somewhat more reactive than glucopyranosides, a difference similar to that observed earlier for phenoxide departure from tetrahydrofuran[100] and tetrahydropyran.[101]

When these rate constants for spontaneous cleavage of glycosides were compared with those of other covalent bonds in biological materials, it became evident that the uncatalyzed hydrolysis of O-glucopyranosides proceeds far more slowly ($t_{1/2} \sim (5-8) \times 10^6$ years) than hydrolysis of bonds that join other biological polymers including DNA ($t_{1/2} \sim 10^5$ years), proteins ($t_{1/2} \sim 400-500$ years), or RNA ($t_{1/2} \sim 4$ years). Extrapolation of the observed rate constants to room temperature yielded a first-order rate constant of 1.9×10^{-15} s^{-1} for uncatalyzed water attack on α-methyl glucopyranoside at 25 °C. Sweet potato β-amylase (1,4-α-D-glucan maltohydrolase; EC 3.2.1.2) generates maltose from starch[102] by an "inverting" mechanism involving direct water attack,[103] with $k_{cat} = 1360$ s^{-1} and $K_m = 7 \times 10^{-5}$ M. Comparison of these rate constants indicates that this enzyme enhances the rate of water attack on α-methyl glycoside by a factor of more than 10^{17} and that it places an upper limit of 10^{-22} M on the formal dissociation constant of this enzyme's complex with the altered substrate in the transition state. Evidently, glycosidases, like phosphatases, phosphodiesterases, and orotidine 5′-phosphate decarboxylase, are unusually proficient catalysts and sensitive targets for inhibitor design.

These findings have an unexpected bearing on efforts to preserve paper books and other documents against deterioration with time. The alum-rosin sizing process, that has been in use for more than a century, yields book papers with average pH values in the neighborhood of 4.8, in contrast to the higher pH values of ancient book papers that are better preserved. Because of the known susceptibility of polysaccharides to acid-catalyzed hydrolysis,[99] modern conservation methods have been designed to produce "mass deacidification" by treatment of documents with diethyl zinc vapor, magnesium methyl carbonate, basic metal oxide particles, or ammonia vapor.[104] It has not been established, however, that acidity is the actual cause of paper deterioration. Extrapolated to room temperature, the present experiments indicate a half-time of ~5000 years at pH 4.8 and 25 °C for hydrolysis of the β-glycosidic linkages of cellulose: somewhat longer than the time that has elapsed since the invention of paper in China. Thus, it seems reasonable to infer that factors other than simple acidity may be responsible for the instability of modern documents.

The rate of spontaneous hydrolysis of cellulose, as Baty has noted,[104] may be underestimated by comparison with β-methylglucopyranoside, since the latter molecule is less strained thermodynamically by roughly 0.7 kcal/mol, corresponding to a factor of 3. Moreover, the 4-O^- group of the terminal glucose residue of cellulose, whose conjugate acid has an approximate pK_a value of 14, is expected to be a roughly 30-fold better leaving group than methoxide, whose conjugate acid has an approximate pK_a value of 15.5. To investigate the properties of a compound that might serve as a model for the spontaneous breakdown of cellulose in the kraft pulping process, he examined the behavior of 1,5-anhydrocellobiitol, a dimer of β-linked glucosepyranoside residues in which the 1-hydroxyl group at the reducing end has been replaced by a hydrogen atom. The observed rate of hydrolysis was somewhat faster than that of 1-methyl-β-glucopyranoside, and this is consistent with the view that hydrolysis might be the rate-limiting step in cellulose digestion in the kraft pulping process (170−180 °C for 90 min).

Of special interest from the standpoint of paper preservation is the finding (for both β-methylglucopyranoside and 1,5-anhydrocellobiitol) that glycoside hydrolysis exhibits a larger enthalpy of activation (~30 kcal/mol) than has been observed for other biological polymers. At 30 °C, a 10° reduction in temperature reduces the rate of hydrolysis by a factor of 6. Thus, refrigeration may furnish a particularly effective means of preserving valuable paper documents against the ravages of time.

13. Hydration Reactions of Fumarate and 3-Chloroacrylate

Fumarase catalyzes the reversible hydration of fumarate to (*S*)-malate with high efficiency under physiological conditions, acting on its substrate with an apparent second-order rate constant that approaches the rate of encounter between enzyme and substrate in solution.[105] To determine the rate of fumarate hydration in the absence of enzyme, Bearne and Wolfenden measured the nonenzymatic rate of fumarate hydration in neutral solution in sealed quartz tubes at elevated temperatures, obtaining by extrapolation a rate constant of 2.5×10^{-13} s^{-1} for the nonenzymatic reaction at pH 6.8 and 37 °C.[106] Comparison with the turnover number ($k_{cat} = 4.5 \times 10^3$ s^{-1}) reported for fumarase[107] indicates a rate enhancement of 1.8×10^{16}-fold at pH 6.8 and 37 °C. Interestingly, the formal equilibrium of transition state binding by fumarase appears to be accompanied by a gain in entropy, which may seem surprising in view of the loss of translational and rotational freedom that is expected when two molecules combine. But in aqueous solution, compensating entropic effects may arise from changing solvation. Electrostatic interactions, like hydrophobic interactions, probably involve a release of ordered water molecules from the region between the interacting groups, resulting in a positive contribution to the observed ΔS; complete protonation of the dianion of fumarate, for example, is accompanied by a gain of entropy of 37 cal mol^{-1} K^{-1}.[108] In the ground state, the binding of fumarate by fumarase has been shown to be accompanied by a small increase in entropy, and electrostatic stabilization of the greater negative charge, developed by fumarate in a carbanionic intermediate that approaches the transition state, could explain the much greater gain in entropy that is observed for binding the altered substrate in the transition state relative to the ground state.

A somewhat similar set of observations has been made on the reaction catalyzed by 3-chloroacrylate dehalogenase, a hydrolytic enzyme whose action almost certainly involves addition of water to 3-chloroacrylic acid followed by elimination of HCl.[109] The soil of potato fields in the Netherlands harbors bacteria with this enzyme, which confers on these bacteria the ability to metabolize 3-chloroacrylic acid, generated by the degradation of a pesticide (1,3-dichloropropene) that entered the environment in 1946. Examination of rate constants at elevated temperatures led to the inference that the half-time at 25 °C for *spontaneous* hydrolytic dechlorination of *trans*-3-chloroacrylic acid is 10,000 years, several orders of magnitude longer than the

Table 2. Apparent First Order Rate Constants and Thermodynamics of Activation for Water-Consuming Reactions at 25 °C, with the Activity of Pure Water Taken as Unity[a]

substrate (electrostatic charge)	k_{25}, s^{-1}	$\Delta G^{\ddagger}_{25}$, kcal/mol	$\Delta H^{\ddagger}$, kcal/mol	$T\Delta S^{\ddagger}_{25}$, kcal/mol
methyl phosphate dianion (−2)	2.0×10^{-20}	44.3	47.0	+2.7
dineopentyl phosphate anion (−1)	7.0×10^{-16}	38.1	29.5	−8.6
1,5-anhydrocellobiitol	1.2×10^{-15}	37.7	35.2	−2.5
α-1-methylglucopyranoside	1.9×10^{-15}	37.4	30.3	−7.1
β-1-methylglucopyranoside	4.7×10^{-15}	36.8	29.7	−7.1
α-1-methylribofuranoside	1.9×10^{-14}	36.1	31.3	−4.8
fumarate, pH 6.8 (−2)	3.5×10^{-14}	35.7	28.9	−6.8
β-1-methylribofuranoside	3.7×10^{-14}	35.7	30.9	−4.8
dimethyl phosphate anion (−1)	1.6×10^{-13}	34.9	25.9	−9.0
3-chloroacrylate (−1)	2.2×10^{-12}	33.3	26.7	−6.5
tetramethylurea	4.2×10^{-12}	32.9	22.3	−10.6
N,*N*-dimethylacetamide	1.8×10^{-11}	32.0	22.5	−9.5
acetylglycyl-glycine (−1)	4.4×10^{-11}	31.5	24.4	−7.1
acetylglycyl-glycine *N*-methylamide	3.6×10^{-11}	31.5	22.9	−8.6
acetamide	5.1×10^{-11}	31.4	23.8	−7.6
α,*β*-1-methyldeoxyribofuranoside	3.7×10^{-11}	31.4	27.4	−4.2
5,6-dihydroorotate (−1)	3.2×10^{-11}	30.7	24.7	−6.9
urea	1.2×10^{-11}	31.1	22.9	−8.2
formamide	1.1×10^{-10}	30.9	21.9	−9.0
methyl phosphate monoanion (−1)	2.4×10^{-10}	30.6	30.0	−0.6
cytidine	2.7×10^{-10}	30.4	22.1	−8.3
N-methylacetamide	4.6×10^{-10}	30.1	22.5	−7.6
pyrophosphate trianion (−3)	2.2×10^{-10}	29.2	28.7	−0.4
trimethyl phosphate	2.0×10^{-8}	28.1	22.6	−5.5
acetyl phosphate dianion (−2)	2.7×10^{-8}	27.7	26.6	−1.1
pyrophosphate dianion (−2)	2.9×10^{-8}	27.7	26.0	−1.7
ADP (or ATP) (−2 or −3)	4.0×10^{-8}	27.5	22.9	−4.5
phosphoenolpyruvate (−2)	1.2×10^{-7}	26.8	25.7	−1.1
acetyl phosphate monoanion (−1)	1.9×10^{-3}	21.1	22.1	+1.0

[a] For references, see text. Because the activity coefficient of water is temperature-dependent, it is worth noting that the effect of the customary convention, of taking water activity as unity, is itself temperature-dependent. That convention is expected to affect comparisons of the thermodynamics of activation for reactions whose rates vary with the activity of water (most of the reactions in this list) with those of other reactions whose rates do not vary with the activity of water. Those latter include reactions in which water does not participate as a reactant and reactions in which water does participate but in such as way that the rate does not depend on water concentration (e.g. methyl phosphate monoanion hydrolysis).

half-times for spontaneous decomposition of other environmental pollutants, such as 1,2-dichloroethane (72 years), paraoxon (13 months), atrazine (5 months), or aziridine (52 h). With thermodynamic parameters for activation similar to those for the spontaneous hydration of fumarate at pH 6.8, this slow reaction proceeds at a constant rate through the pH range between 2 and 12. But at the active site of the enzyme 3-chloroacrylate dehalogenase (CaaD), isolated from a pseudomonad growing in these soils, hydrolytic dechlorination proceeds with a half-time of 0.18 s. Neither k_{cat} nor k_{cat}/K_m is reduced by increasing solvent viscosity with trehalose, implying that the rate of enzymatic dechlorination is controlled by chemical events in catalysis rather than by diffusion-limited substrate binding or product release. CaaD achieves a $\sim 10^{12}$-fold rate enhancement, matching or surpassing the rate enhancements produced by many enzymes that act on more conventional biological substrates.

One of those enzymes is 4-oxalocrotonate tautomerase (4-OT), with which CaaD appears to share a common evolutionary origin. CaaD produces a rate enhancement (2×10^{12}-fold) that much exceeds the rate enhancement produced by 4-OT (2×10^{7}-fold). Those are not the properties expected of a newly evolved enzyme. Because the structure of the active center of CaaD lacks any apparent leaving group site for chloride, and for other reasons, it has been suggested that the apparently novel catalytic activity of CaaD may be a fortuitous side reaction of an enzyme that evolved earlier to act on a natural substrate that remains to be identified.[110] That possibility would be strengthened if the gene for CaaD were found in pseudomonads from fields never treated with pesticide.

14. Rate Enhancements Produced by Hydrolytic and Hydrating Enzymes

Table 2 shows that the spontaneous rate constants (k_{non}) of the biological hydrolysis and hydration reactions examined above span a range of 14 orders of magnitude, with half-times ranging from 5 s for the hydration of CO_2[111] to 30 million years for the cleavage of phosphodiesters.[73] Table 3 compares these values with kinetic constants that have been observed for hydrolytic and hydrating enzymes that catalyze the same reactions. This table is confined to enzymes that do not appear to proceed by double displacement mechanisms,[103] because comparisons of enzymatic and nonenzymatic reaction rates do not permit straightforward estimation of transition state affinity for reactions of that type.[22,23] The enzymes in Table 3 can be seen to vary over a range of 10^{14}-fold in the rate enhancements that they produce.

As a measure of catalytic proficiency[5] and a benchmark against which to test the merits of potential transition state analogues as enzyme inhibitors, the value of K_{tx} is of greater interest. The value of K_{tx} sets an upper limit on the dissociation constant of the enzyme's complex with the altered substrate in the transition state.[8] That value is obtained by dividing the rate constant of the uncatalyzed reaction (k_{non}) by k_{cat}/K_m, as shown in Figure 1. The findings reviewed here are summarized in Table 3 and Figure 7, in which the length

Table 3. Turnover Numbers (k_{cat}), Catalytic Efficiencies (k_{cat}/K_m), Rate Enhancements (k_{cat}/k_{non}), and Values of K_{tx} ($k_{non}/(k_{cat}/K_m)$) for Water-Consuming Enzyme Reactions at 25 °C, Obtained by Comparison of Enzyme Reaction Rate Constants with Rate Constants for the Corresponding Uncatalyzed Reactions (k_{non})

enzyme	k_{non},[a] s^{-1}	k_{cat}, s^{-1}	k_{cat}/K_m, s^{-1} M^{-1}	k_{cat}/k_{non}	K_{tx}, M
fructose-1,6-bisphosphatase[b]	2.0×10^{-20}	21	1.5×10^{7}	1.05×10^{21}	7×10^{-26}
staphylococcal nuclease[c]	7.0×10^{-16}	95	1.0×10^{7}	1.4×10^{17}	7×10^{-23}
β-amylase[d]	1.9×10^{-15}	1.4×10^{3}	1.9×10^{7}	7.2×10^{17}	1.0×10^{-22}
fumarase (37 °C)[e]	3.5×10^{-14}	880	2.4×10^{8}	3.5×10^{15}	1.0×10^{-21}
jack bean urease[f]	1.2×10^{-11}	3.6×10^{4}	9×10^{6}	3×10^{15}	1.25×10^{-18}
chloroacrylate dehalogenase[g]	2.2×10^{-12}	3.8	1.2×10^{5}	1.8×10^{12}	1.8×10^{-17}
carboxypeptidase b[h]	4.4×10^{-11}	240	6×10^{6}	1.3×10^{13}	3.3×10^{-17}
E. coli cytidine deaminase[i]	2.7×10^{-10}	300	2.7×10^{6}	1.1×10^{12}	1.0×10^{-16}
phosphotriesterase[j]	2.0×10^{-8}	2.1×10^{3}	4.0×10^{7}	1.8×10^{11}	1.9×10^{-16}
hamster dihydroorotase[k]	3.2×10^{-11}	1.2	1.1×10^{5}	3.7×10^{10}	2.9×10^{-16}
carbonic anhydrase[l]	0.13[m]	1.0×10^{6}	1.2×10^{6}	7.7×10^{6}	1.1×10^{-9}

[a] Except for carbonic anhydrase, k_{non} values are from Table 2. [b] Kelley, N.; Giroux, E. L.; Lu, G.; Kantrowitz, E. R. *Biochem. Biophys. Res. Commun.* **1996**, *219*, 848. [c] Serpersu, E.; Shortle, D.; Mildvan, A. S. *Biochemistry* **1987**, *26*, 1289. [d] Balls, A. K.; Walden, M. K.; Tompson, R. R. *J. Biol. Chem.* **1948**, *173*, 9. [e] Brant, D. A.; Barnett, L. B.; Alberty, R. A. *J. Am. Chem. Soc.* **1963**, *85*, 2204. [f] Laidler, K. J.; Hoare, J. P. *J. Am. Chem. Soc.* **1950**, *72*, 2489. [g] Horvat, C. M.; Wolfenden, R. *Proc. Natl. Acad. Sci., U.S.A.* **2005**, *102*, 16199. [h] Radzicka, A.; Wolfenden, R. *J. Am. Chem. Soc.* **1996**, *118*, 6105. [i] Snider, M. J.; Gaunitz, S.; Ridgway, C.; Short, S. A.; Wolfenden, R. *Biochemistry* **2000**, *39*, 9746. [j] Dumas, D. P.; Caldwell, S. R.; Wild, J. R.; Raushel, F. M. *J. Biol. Chem.* **1989**, *264*, 19659. [k] Huang, T.; Kaplan, J.; Menz, R. I.; Katis, V. L.; Wake, R. G.; Wolfenden, R.; Christopherson, R. I. *Biochemistry*, published online June 15, 2006, http://dx.doi.org/10.1021/bi060595w. [l] Steiner, H.; Jonsson, B.-H.; Lindskog, S. *Eur. J. Biochem.* **1975**, *59*, 253. [m] Roughton, J. F. W. *J. Am. Chem. Soc.* **1941**, *63*, 2930.

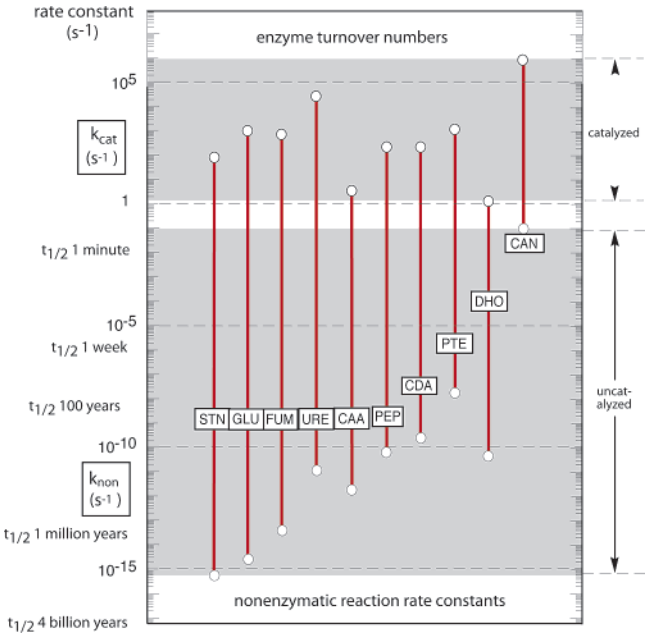

Figure 6. Logarithmic scale of k_{cat} and k_{non} values for hydrolysis and hydration reactions at 25 °C (Table 3). Abbreviations: STN = staphylococcal nuclease; GLU = β-amylase; FUM = fumarase; URE = urease; CAA = 3-chloroacrylate dehalogenase; CDA = cytidine deaminase; PTE = phosphotriesterase; DHO = hamster dihydroorotase; CAN = carbonic anhydrase.

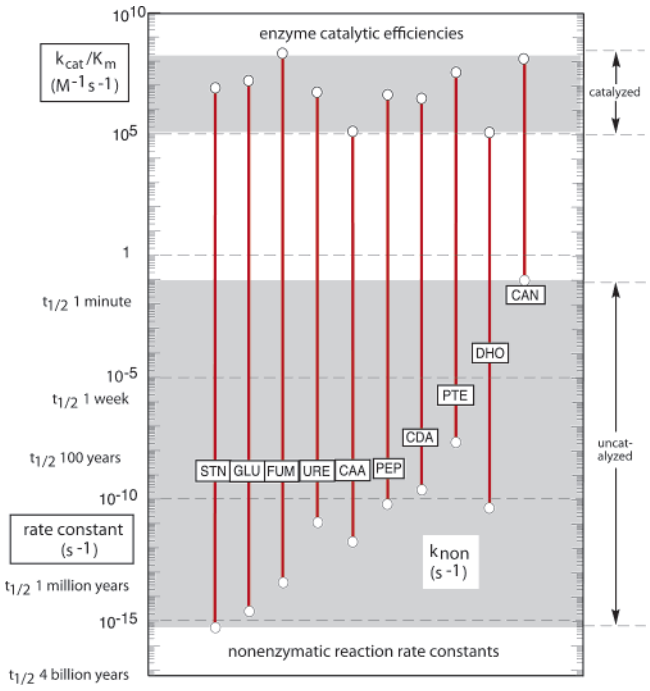

Figure 7. Logarithmic scale of k_{cat}/K_m and k_{non} values for hydrolysis and hydration reactions at 25 °C (Table 3). For abbreviations, see Figure 6.

of each vertical bar represents the value of K_{tx}, on a logarithmic scale. For hydrolases and hydratases, that value spans a range of more than 17 orders of magnitude.

15. Thermodynamic Origins of Catalysis by Hydrolytic and Hydrating Enzymes

The power of an enzyme as a catalyst depends on its ability to discriminate between the substrate in the ground state and in the altered form that is present in the transition state, binding only the latter species very tightly. In seeking to understand the origins of these remarkable powers of attraction and binding discrimination, it seems reasonable to inquire whether the rate enhancement produced by an enzyme, and the corresponding increase in affinity, tends to be mainly enthalpic or entropic in origin. Earlier, an apparent answer to that question seemed to be suggested by two familiar generalizations: (1) The rates of chemical reactions increase with increasing temperature, tending to double in rate ("$Q_{10} = 2$") as the temperature is adjusted from 20 to 30 °C.[112] (2) Many enzyme−substrate complexes form products at rates that roughly double as the temperature is increased by 10 °C.[113] If both these generalizations were true, then heats of activation would be similar for enzymatic and nonenzymatic reactions, indicating that the catalytic effect of an enzyme usually arises from its ability to increase a reaction's entropy of activation.

But the findings reviewed here furnish a broadly distributed set of benchmarks against which that hypothesis can be tested, and it is clear that uncatalyzed reactions tend to

Table 4. Comparison of First Order Rate Constants and Thermodynamics of Activation for Uncatalyzed Water-Consuming Reactions at 25 °C (Table 2), with k_{cat} Values and Thermodynamics of Reactions Catalyzed by the Correspond Enzymes (Shown in Italics)

substrate (electrostatic charge)	k_{25}, s^{-1}	$\Delta G^{\ddagger}_{25}$,kcal/mol	$\Delta H^{\ddagger}$, kcal/mol	$T\Delta S^{\ddagger}_{25}$, kcal/mol
dineopentyl phospate anion (−1)	7.0×10^{-16}	38.1	29.5	−8.6
staphylococcal nuclease[a]	*95*	*14.7*	*10.8*[b]	*−3.9*
urea	1.2×10^{-11}	31.1	22.9	−8.2
jack bean urease[c]	*3.6×10^{4}*	*22.1*	*9.9*[b]	*−12.2*
3-chloroacrylate (−1)	2.2×10^{-12}	33.3	26.7	−6.5
chloroacrylate dehalogenase[d]	*3.8*	*16.6*	*9.4*	*−7.2*
cytidine	2.7×10^{-10}	30.4	22.1	−8.3
E. coli cytidine deaminase[e]	*3×10^{2}*	*14.0*	*14.9*	*+0.9*
5,6-dihydroorotate (−1)	3.2×10^{-11}	30.7	24.7	−6.9
hamster dihydroorotase[f]	*1.2*	*17.5*	*12.3*	*−5.2*

[a] Serpersu, E.; Shortle, D.; Mildvan, A. S. *Biochemistry* **1987**, *26*, 1289. [b] Wolfenden, R.; Snider, M.; Ridgway, C.; Miller, B. *J. Am. Chem. Soc.* **1999**, *121*, 7419 [c] Laidler, K. J.; Hoare, J. P. *J. Am. Chem. Soc.* **1950**, *72*, 2489. [d] Horvat, C. M.; Wolfenden, R. *Proc. Natl. Acad. Sci., U.S.A.* **2005**, *102*, 16199. [e] Snider, M. J.; Gaunitz, S.; Ridgway, C.; Short, S. A.; Wolfenden, R. *Biochemistry* **2000**, *39*, 9746. [f] Huang, T.; Kaplan, J.; Menz, R. I.; Katis, V. L.; Wake, R. G.; Wolfenden, R.; Christopherson, R. I. *Biochemistry* **2006**, *45*, accpeted.

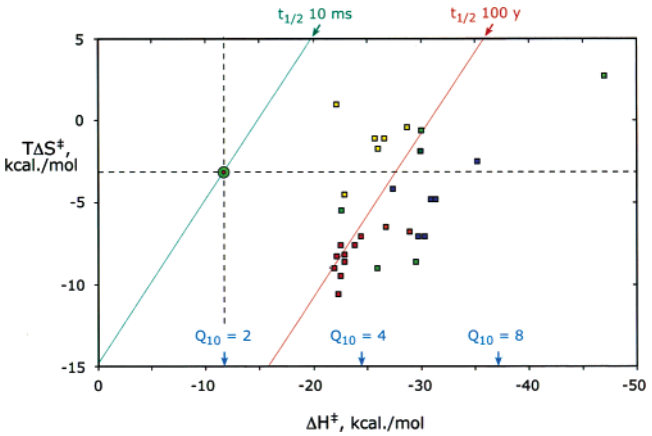

Figure 8. Heats and entropies of activation of hydrolysis and hydration reactions, compared with k_{cat} for a typical hydrolase (see Table 2 and text). Color code: red = urea or peptide cleavage; blue = glycoside cleavage; green = phosphoric acid ester cleavage; yellow = phosphoric anhydride cleavage. The green circle represents a hydrolase with an average of the properties of the enzymes in Table 4.

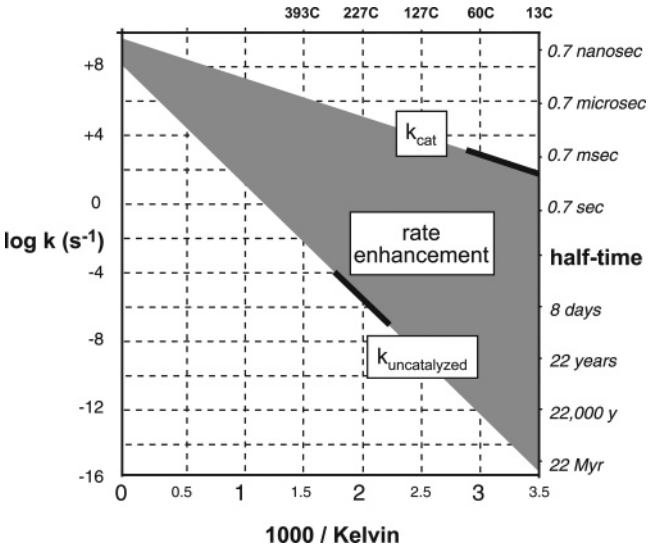

Figure 9. Increase in rate enhancement with decreasing temperature, by a $\Delta H^{\ddagger}$-reducing enzyme, as illustrated by the rates of hydrolysis of glycosides in the presence and absence of bacterial α-glucosidase. Dark lines show the actual ranges of temperature over which enzymatic and uncatalyzed reaction rate constants were observed.[114] Reprinted with permission from ref 114. Copyright 1999 American Chemical Society.

be very much more sensitive to temperature than the first of these generalizations would imply.[114] Each of the reactions described in this review followed simple first-order kinetics to completion, and each reaction yielded a linear Arrhenius plot. The energy of activation (E_{act}) was obtained by plotting the logarithm of observed rate constants as a function of the reciprocal of absolute temperature, and $T\Delta S^{\ddagger}_{25}$ was obtained by subtracting $\Delta G^{\ddagger}_{25}$ from $\Delta H^{\ddagger}$ (equivalent to $E_{act} - RT$).

Not one of the k_{non} values for the reactions in Table 2 exhibits a $\Delta H^{\ddagger}$ value as low as 12 kcal/mol, corresponding to a Q_{10} value of 2. Instead, the values of $\Delta H^{\ddagger}$ range from 22 to 47 kcal/mol, corresponding to Q_{10} values ranging from 4 to 16. For the corresponding enzyme reactions, in contrast, $\Delta H^{\ddagger}$ values are relatively tightly clustered (Table 4), with an average value of 11.5 kcal/mol, in accord with the second generalization above.

That behavior is of interest in considering the forces of attraction that are chiefly responsible for catalysis, and the thermodynamic properties expected of transition state analogue inhibitors.

Figure 8 shows the values of $T\Delta S^{\ddagger}$ (vertical axis) and $\Delta H^{\ddagger}$ (lower horizontal axis) for k_{non} of the uncatalyzed reactions in Table 1. These values may be compared with the values for the turnover number of a hypothetical ES complex (circled) with the average properties of the hydrolases in Table 4 (mean $\Delta H^{\ddagger} = 11.5$ kcal/mol, mean $T\Delta S^{\ddagger} = -5.5$ kcal/mol at 25 °C). Table 4 also allows the thermodynamics of activation for several enzymatic and nonenzymatic reactions to be compared individually.

In Figure 8, all values for uncatalyzed reactions fall to the right of the "Harcourt line",[112] representing $Q_{10} = 2$ ($\Delta H^{\ddagger} = 12$ kcal/mol). Variations along the horizontal axis of Figure 8 show that these hydrolytic enzymes invariably lower the reaction's enthalpy of activation substantially, by an amount that largely determines the effectiveness of the enzyme as a catalyst. Variations along the vertical axis show that the entropy of activation is sometimes raised and sometimes lowered, usually by an amount that is less than that of the change in $\Delta H^{\ddagger}$. As a result of this tendency, the corresponding rate enhancements (k_{cat}/k_{non}) increase acutely as the temperature is lowered, as illustrated in Figure 9 by the example of α-glucosidase.

As a result of this general tendency, enzyme affinities for transition state analogue inhibitors are also expected to increase sharply with decreasing temperature, relative to their affinities for conventional substrates or substrate analogues. That behavior is exemplified by the much more negative enthalpy of binding of 1,6-dihydroinosine (−18 kcal/mol)

by adenosine deaminase, than that for binding of either the substrate adenosine (−7.7 kcal/mol) or 1,6-dihydronebularine, a substrate analogue (−8.3 kcal/mol). Correspondingly, $\Delta H^{\ddagger}$ is roughly 8 kcal/mol more favorable for the enzyme-catalyzed reaction than for the uncatalyzed reaction.[115] Similarly, the binding of a transition state analogue of cytidine deaminase, like that of the transition state itself, is much more sensitive to temperature than that of the substrate or product (see below).

This sharp temperature dependence of K_i furnishes a new criterion for testing potential transition state analogue inhibitors, and it may have a significant bearing on the practical uses of transition state analogues as enzyme antagonists in medicine and agriculture.

What is the source of the misleading generalization that rates of spontaneous chemical reactions in water tend to double with a 10 °C rise of temperature? Reaction rates are relatively easy to measure if half-times fall in the range between 1 min and 1 day, with corresponding $\Delta G^{\ddagger}_{25}$ values that range between 20 and 24 kcal/mol at 25 °C. If $T\Delta S^{\ddagger}$ falls in the range between 0 and −10 kcal/mol, as is the case for most spontaneous reactions in water (Figure 8), then $\Delta H^{\ddagger}$ must fall in the range between 10 and 24 kcal, corresponding to Q_{10} values of 2−4, in the low range observed by Harcourt[112] and later investigators. When these unrecognized constraints of experimental convenience are abandoned, it becomes evident that values of $\Delta H^{\ddagger}$ for spontaneous reactions seldom or never fall near the "Harcourt line" ($Q_{10} = 2$) but are distributed over a much wider range. Thus, enthalpies of activation, not entropies of activation, tend to govern the variation in the rates of spontaneous reactions in water.

These findings suggest that the rate enhancement, and hence the corresponding increase in affinity as the ES complex proceeds from the ground state to the transition state, is largely enthalpic in origin. Thus, the increase in substrate affinity tends to be accompanied by a substantial release of enthalpy. That would be consistent with the formation of new electrostatic and hydrogen bonds that can act synergistically,[115] for whose existence much evidence exists in the structures of enzymes crystallized with transition state analogue inhibitors.[116] Figure 2 suggests a possible evolutionary advantage of that release of enthalpy. At the elevated temperatures at which the earliest organisms may have arisen, even a weak catalyst would have allowed the reaction to occur at a substantial rate. Such a catalyst, if it chanced to share the $\Delta H^{\ddagger}$-reducing character of modern enzymes, might have conferred an ever-increasing advantage on the host organism as the surroundings cooled, by enhancing the rate of the desired reaction relative to the rates of other reactions.[78] Among those undesirable side reactions would be reactions leading to destruction of the catalyst itself. For example, the hydrolysis of proteins[28] and nucleic acids[76] is known to be extremely temperature-dependent.

16. Thermodynamic Analysis of Transition State Binding by Cytidine Deaminase: The Apparent Contribution of a Single Substituent

In addition to enhancing our appreciation of the extent to which enzymes enhance reaction rates, comparison of the rates of spontaneous and enzyme-catalyzed reactions can be used to analyze the thermodynamic basis of transition state affinity and the shortcomings of existing transition state analogue inhibitors. That approach can be illustrated by reference to cytidine deaminase from *E. coli*, which has been analyzed in some detail.

Substrate binding by cytidine deaminase occurs with a favorable change in enthalpy ($1/K_s$, $\Delta H = -13$ kcal/mol, $T\Delta S = -8$ kcal/mol), and comparison of the temperature dependence of the enzymatic and nonenzymatic reactions (both pH-independent in the neutral range) suggests that additional heat is released upon binding of the altered substrate in the transition state ($1/K_{tx}$, $\Delta H = -20$ kcal/mol, $T\Delta S = +2$ kcal/mol). Perhaps, in the ground state ES complex (but not in the transition state complex), one excess water molecule is present at the attacking group site. Thus, the relatively favorable entropy of transition state binding might be attributed, at least in part, to the formal release of bound water from the active site into bulk solvent.[117]

The binding of 3,4-dihydrouridine (DHU, or zebularine 3,4-hydrate), a potential transition state analogue, is accompanied by a favorable change of enthalpy ($1/K_s$, $\Delta H = -21$ kcal/mol), almost equivalent to that of the altered substrate in the transition state ($1/K_{tx}$, $\Delta H = -20$ kcal/mol). But the binding of THU is entropically unfavorable ($T\Delta S = -5$ kcal/mol), unlike the binding of the transition state itself ($T\Delta S = +2$ kcal/mol). X-ray analysis suggests that bonding is similar in the two complexes, except that a water molecule may be trapped in the complex of the slightly smaller transition state analogue, with a corresponding reduction in entropy. Mass spectrometric experiments, using FT-ICR, show that cytidine deaminase, nebulized from the mildly acidic solution in which it is active, remains dimeric in the vapor phase. The exact mass of the presumably native dimer has been used to infer the presence at each active site of a single water molecule, trapped in place by the transition state analogue.[118]

If the 4-OH group is removed from the transition state analogue DHU, the heat released upon binding is much reduced ($\Delta H = -10$ kcal/mol) compared with the heat released upon binding DHU itself ($\Delta H = -21$ kcal/mol). But the entropy of binding is hardly affected by removal of the 4-OH group ($T\Delta S = -3$ kcal/mol, versus −5 kcal/mol for DHU), consistent with the continued presence of a single water molecule (now split between the zinc atom and a catalytic glutamate residue) at the active site. The shortcomings of DHU, in capturing all the free energy expected of an ideal transition state analogue, may arise from its truncated structure and consequent inability to displace water from the leaving group site. A more perfect inhibitor might fill that space.

17. Summary

Many enzyme reactions proceed with k_{cat}/K_m values in the neighborhood of 10^7 M^{-1} s^{-1}, and these reactions appear to be similarly efficient by that criterion. But to assess the proficiency of an enzyme as a catalyst, and its corresponding affinity for the altered substrate in the transition state, it is useful to compare k_{cat}/K_m with the rate constant of the corresponding reaction under the same conditions in the absence of a catalyst. In contrast to the relatively narrow range of values of k_{cat}/K_m observed for enzyme-catalyzed reaction rates, the rates of these uncatalyzed reactions span a range of at least 19 orders of magnitude. Thus, differences in enzyme proficiency tend to reflect differences between the rates of the uncatalyzed reactions rather than differences between the rates of the enzyme-catalyzed reactions. As a

result, enzymes differ greatly from one another in their prowess as catalysts. Rate enhancements, by those enzymes that catalyze hydrolytic and hydration reactions, are largely achieved by lowering the enthalpy of activation. The effects of these enzymes on the entropy of activation tend to be relatively small and variable.

Experiments that establish reaction rate constants in water in the absence of a catalyst, as benchmarks for comparison with the rate constants of enzyme reactions, are expected to yield two practical benefits. First, the greater the rate enhancement an enzyme produces, the greater is its expected sensitivity to inhibition by an ideal transition state analogue. Since enzyme reaction rates tend to be relatively invariant, information about the rate of the uncatalyzed reaction can be used to identify especially sensitive enzyme targets for inhibitor and drug design. Second, concrete information about the rate enhancement that an enzyme produces makes it possible to estimate the affinity that is expected of an ideal transition state analogue inhibitor and to perform a detailed thermodynamic analysis of the shortcomings of existing inhibitors. If the structural sources of those limitations can be identified, it may become possible to design inhibitors that bear a closer resemblance to the elusive ideal of the transition state itself.

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