References and Notes

(1) A preliminary communication on this subject has been published: *Nature (London)*, **270**, 223 (1977).
(2) F. Rottman, A. J. Shatkin, and R. P. Perry, *Cell*, **3**, 197 (1974).
(3) Y. Furuichi and K.-I. Miura, *Nature (London)*, **253**, 374 (1975).
(4) Y. Furuichi, M. Morgan, S. Muthukrishnan, and A. J. Shatkin, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 372 (1975).
(5) Y. Furuichi, S. Muthukrishnan, and A. J. Shatkin, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 742 (1975).
(6) T. Urushibara, Y. Furuichi, C. Nishmura, and K.-I. Miura, *FEBS Lett.*, **49**, 385 (1975).
(7) C. M. Wei and B. Moss, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 318 (1975).
(8) G. Abraham, D. P. Rhodes, and A. K. Banerjee, *Nature (London)*, **255**, 37 (1975).
(9) S. Muthukrishnan, G. W. Both, Y. Furuichi, and A. J. Shatkin, *Nature (London)*, **255**, 33 (1975).
(10) Y. Furuichi, M. Morgan, A. J. Shatkin, W. Jenlinek, M. Dalditt-Georgiev, and J. E. Darnell, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 1904 (1975).
(11) J. Adams and S. Cory, *Nature (London)*, **255**, 28 (1975).
(12) S. Muthukrishnan, M. Morgan, A. K. Banerjee, and A. J. Shatkin, *Biochemistry*, **15**, 5761 (1976).
(13) G. W. Both, A. K. Banerjee, and A. J. Shatkin, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 1189 (1975).
(14) Because the 5' terminus of mRNA combines the structures of nucleotide coenzymes and nucleic acids the original investigators[2-13] have employed clever nomenclature that does not conform to IUPAC–IUB recommendations. Thus: m^7G = 7-methylguanosine; $m^7G^{5'}p$ = 7-methylguanosine 5'-monophosphate; $m^7G^{5'}pp$ = 7-methylguanosine 5'-diphosphate; $m^7G^{5'}ppp$ = 7-methylguanosine -triphosphate; $m^7G^{5'}ppp^{5'}AmpA$ = structure in Figure 1; $m^6Am = N^6$-monomethyl-2'-*O*-methyladenosine; N^6'p or p$^{5'}$N, etc., stand for 5'-NMP, etc., where N is A, G, U, or C. Nm is 2'-*O*-methylated nucleoside.
(15) D. Shafritz, J. A. Weinstein, B. Safer, W. Merrick, L. A. Weber, E. D. Hickey, and C. Baglioni, *Nature (London)*, **261**, 291 (1976).
(16) E. D. Hickey, L. A. Weber, and C. Baglioni, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 19 (1976).
(17) E. D. Hickey, L. A. Weber, C. Baglioni, C. H. Kim, and R. H. Sarma, *J. Mol. Biol.*, **109**, 173 (1977).
(18) H. Singh, M. H. Herbut, C. H. Lee, and R. H. Sarma, *Biopolymers*, **15**, 2167 (1976).
(19) D. M. Cheng and R. H. Sarma, *Biopolymers*, **16**, 1687 (1977).
(20) C. H. Lee, F. S. Ezra, N. S. Kondo, R. H. Sarma, and S. S. Danyluk, *Biochemistry*, **15**, 3627 (1976).

(21) F. S. Ezra, C. H. Lee, N. S. Kondo, S. S. Danyluk, and R. H. Sarma, *Biochemistry*, **16**, 1977 (1977).
(22) R. H. Sarma and R. J. Mynott, *J. Am. Chem. Soc.*, **95**, 1641 (1973).
(23) R. H. Sarma and R. J. Mynott, *J. Am. Chem. Soc.*, **95**, 7470 (1973).
(24) M. Sundaralingam, *Jerusalem Symp. Quantum Chem. Biochem.*, **5**, 417 (1973).
(25) M. Sundaralingam, *Struct. Conform. Nucleic Acids Protein–Nucleic Acid Interact. Proc. Annu. Harry Steenbock Symp., 4th, 1974*, 487 (1976).
(26) C. H. Lee and R. H. Sarma, *J. Am. Chem. Soc.*, **98**, 3541 (1976).
(27) R. H. Sarma, C. H. Lee, F. E. Evans, N. Yathindra, and M. Sundaralingam, *J. Am. Chem. Soc.*, **96**, 7337 (1974).
(28) R. H. Sarma and R. J. Mynott, *Org. Magn. Reson.*, **4**, 577 (1972).
(29) C. H. Lee and R. H. Sarma, *Biochemistry*, **15**, 697 (1976).
(30) F. E. Hruska, *Jerusalem Symp. Quantum Chem. Biochem.*, **5**, 345 (1973).
(31) S. H. Kim, H. M. Berman, N. C. Seeman, and M. D. Newton, *Acta Crystallogr., Sect. B*, **29**, 703 (1973).
(32) J. M. Rosenberg, N. C. Seeman, S. H. Kim, F. L. Suddath, H. B. Nicholas, and A. Rich, *Nature (London)*, **243**, 150 (1973).
(33) B. Hingerty, E. Subramanian, S. D. Stellman, S. B. Broyde, T. Sato, and R. Langridge, *Biopolymers*, **14**, 227 (1975).
(34) J. Rubin, T. Brennan, and M. Sundaralingam, *Biochemistry*, **11**, 3112 (1972).
(35) W. K. Olson, *Biopolymers*, **12**, 1787 (1973).
(36) N. Yathindra and M. Sundaralingam, *Biopolymers*, **12**, 2261 (1973).
(37) S. B. Broyde, R. H. Wartell, D. Stellman, B. Hingerty, and R. Langridge, *Biopolymers*, **14**, 1597 (1975).
(38) H. Berthod and B. Pullman, *Biochim. Biophys. Acta*, **232**, 595 (1971).
(39) C. Giessner-Prettre and B. Pullman, *J. Theor. Biol.*, **65**, 189 (1977).
(40) C. Giessner-Prettre and B. Pullman, *J. Theor. Biol.*, **65**, 171 (1977).
(41) O. Kennard, N. W. Isaacs, W. D. S. Motherwell, J. C. Coppola, D. L. Wampler, A. C. Larson, and D. G. Watson, *Proc. R. Soc. London, Ser. A*, **325**, 401 (1971).
(42) F. E. Hruska, A. Mak, N. Singh, and D. Shugar, *Can. J. Chem.*, **51**, 1099 (1973).
(43) F. E. Evans and R. H. Sarma, *Biopolymers*, **13**, 2117 (1974).
(44) C. Giessner-Prettre, B. Pullman, P. N. Borer, L. S. Kan, and P. O. P. Ts'o, *Biopolymers*, **15**, 2277 (1976).
(45) P. O. P. Ts'o, N. S. Kondo, M. P. Schweizer, and D. P. Hollis, *Biochemistry*, **8**, 997 (1969).
(46) F. E. Evans and R. H. Sarma, *Nature (London)*, **263**, 567 (1976).
(47) R. H. Sarma and S. S. Danyluk, *J. Quantum Chem.*, *QBS* 4, 269 (1977).
(48) C. C. Tsai, S. C. Jain, and H. M. Sobell, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 628 (1975).

Proton Inventories of a Serine Protease Charge-Relay Model in an Aprotic Solvent

John L. Hogg,* Ray Morris III, and Noel A. Durrant

Contribution from the Department of Chemistry, Texas A&M University, College Station, Texas 77843. Received August 1, 1977

Abstract: Solvent isotope effects in H_2O–D_2O mixtures have been used to probe the operation of a catalytic system which has been suggested as a model for the charge-relay chain of serine protease enzymes. The absence of any significant solvent isotope effects coupled with the shapes of the proton inventories rules out any significant proton transfer mechanisms for the imidazole promoted or imidazole–benzoate anion promoted reaction of *p*-nitrophenyl acetate in acetonitrile containing 1 M H_2O. In the present study it was shown that the observed reaction is not hydrolysis as previously suggested. Nucleophilic attack by hydrogen-bonded dimers is proposed as the mechanism of catalysis.

Since the discovery of a catalytic triad of amino acids at the active site of the serine protease, α-chymotrypsin,[1] there have been numerous attempts to verify the operation of the so-called charge-relay system.[2] Attempts to model the aspartic acid-histidine-serine charge-relay chain of the enzymes have met with varying degrees of success.[3,4] Even some enzymatic studies themselves have cast doubt on the operation of the charge-relay chain[5,6] although the interpretation of the proton inventory for the deacetylation of acetyl-α-chymotrypsin has been questioned.[7] Bender et al. have recently reported some success in modeling the intramolecular cooperativity of the carboxylate–imidazole–hydroxyl triad.[8]
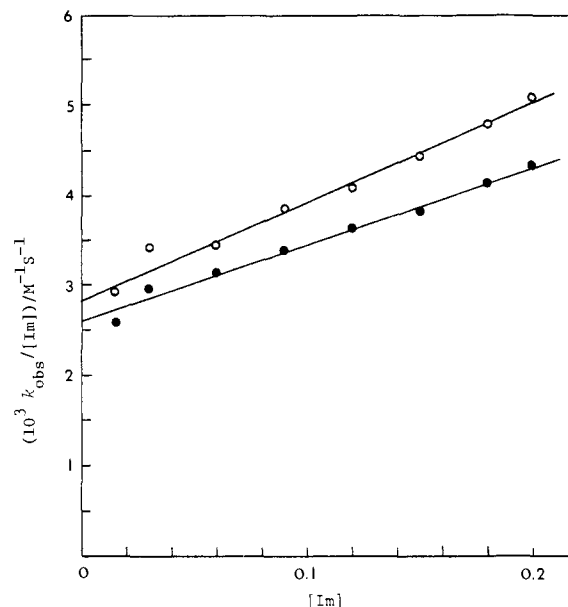
Perhaps the most interesting intermolecular charge-relay model to be studied is that of Haake and coworkers.[9] They investigated the reaction of *p*-nitrophenyl acetate in 1 M water in acetonitrile as a function of imidazole and benzoate ion concentrations. This dipolar, highly aprotic solvent system may mimic the somewhat hydrophobic environment of many enzymes. Their results suggested a high degree of cooperativity between benzoate anion, imidazole, and water in the catalysis.

The observed rate constant, eq 1, contained terms suggesting modes of catalysis such as those represented in eq 2-4. The
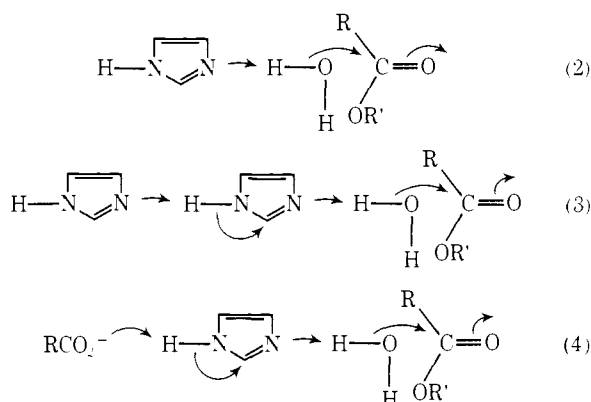
$$k_{obsd} = k_1[\text{Im}] + k_2[\text{Im}]^2 + k_3[\text{Im}][\text{RCO}_2^-] \qquad (1)$$
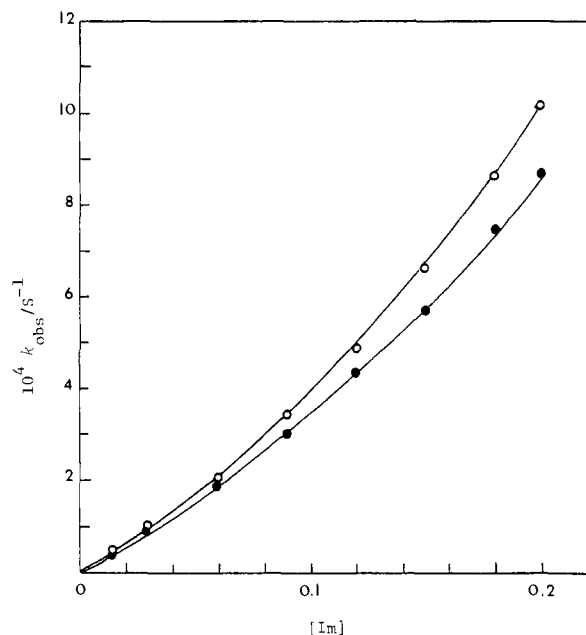
Figure 1. Plot of k_{obsd} vs. the concentration of imidazole (Im) for the reaction of p-nitrophenyl acetate in acetonitrile with either 1 M protium oxide (O) or 1 M deuterium oxide (●) present. The concentration of tetra-n-butylammonium perchlorate was kept at 10^{-2} M. Reactions were followed at 314 nm at 30.1 °C. See text for additional details.

Figure 2. Plot of $k_{obsd}/$[Im] vs. concentration of imidazole (Im). Prepared from the data of Figure 1 and Table I. The open circles (O) represent runs with 1 M protium oxide present while the filled circles (●) represent runs with 1 M deuterium oxide present.

$$H—N\overset{\frown}{\underset{\smile}{N}} \rightarrow H—O \overset{R}{\underset{\overset{|}{H}\,\overset{.}{O}R'}{C}}{=}O \qquad (2)$$

$$H—N\overset{\frown}{\underset{\smile}{N}} \rightarrow H—N\overset{\frown}{\underset{\smile}{N}} \rightarrow H—O \overset{R}{\underset{\overset{|}{H}\,\overset{.}{O}R'}{C}}{=}O \qquad (3)$$

$$RCO_2^- \overset{\frown}{} H—N\overset{\frown}{\underset{\smile}{N}} \rightarrow H—O \overset{R}{\underset{\overset{|}{H}\,\overset{.}{O}R'}{C}}{=}O \qquad (4)$$
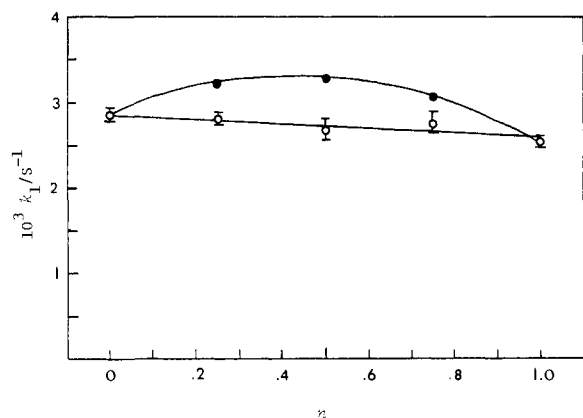
Results

Every attempt was made to duplicate the kinetic procedures of Haake and coworkers as exactly as possible.[9] Initial experiments were conducted in the absence of benzoate anion in order to evaluate k_{obsd} and, subsequently, k_1 and k_2 in protium oxide, deuterium oxide, and protium oxide–deuterium oxide mixtures of atom fraction deuterium n in acetonitrile. Figure 1 shows the nonlinear dependence of k_{obsd} upon imidazole concentration in pure protium oxide ($n = 0$) and pure deuterium oxide ($n = 1.0$). Table I lists the values of k_1 and k_2 obtained from these and additional protium oxide–deuterium oxide mixtures. Values of k_1 and k_2 were obtained from plots of $k_{obsd}/$[Im] vs. [Im] as the intercept and slope, respectively. Figure 2 illustrates the linearity of these plots for $n = 0$ and 1.0.

Our values of 2.88×10^{-3} M^{-1} s^{-1} and 1.07×10^{-2} M^{-2} s^{-1} are in reasonable agreement with the values of 3.1×10^{-3} M^{-1} s^{-1} and 1.3×10^{-2} M^{-2} s^{-1} obtained for k_1 and k_2, respectively, by Haake and coworkers in 1 M protium oxide in acetonitrile.[9] The solvent isotope effects on k_1 and k_2 were determined in the present study to be $k_1^{H_2O}/k_1^{D_2O} = 1.11 \pm 0.03$ and $k_2^{H_2O}/k_2^{D_2O} = 1.24 \pm 0.09$, respectively. Plots of k_1 and k_2 vs. the atom fraction of deuterium in the solvent are shown in Figures 2 and 3. The significance of the small isotope effects and shape of the plots will be discussed in the following section.

It was shown that the observed product spectrum (λ_{max} at 314 and 410 nm) could be duplicated by omitting the p-nitrophenyl acetate and substituting equivalent concentrations of p-nitrophenol and 1-acetylimidazole.

Two separate experiments in which the imidazole concentration was held constant and the concentration of benzoate anion was varied were carried out in order to evaluate k_3 of eq 1. Plots of $k_{obsd}/$[Im] vs. [RCO$_2^-$] yield k_3 as the slope. Because of the importance of this experiment it was done twice by two different people using independently prepared solutions. The values of $k_3^{H_2O}$ and $k_3^{D_2O}$ obtained in these two separate experiments by measuring k_{obsd} at eight different benzoate concentrations are shown in Table II. Considering the complexity of the experiment and the data treatment involved the agreement between the two experiments is reasonable. The

dependence of k_{obsd} on the water concentration was not determined. The magnitude of the k_3 term led to the suggestion that this term represented a charge-relay mode of catalysis as represented by eq 4 although the role of the water molecule was not investigated by Haake and coworkers.[9] We wish to report here our investigations into the role of proton transfers in this reaction and on the influence of the water concentration on k_{obsd}. We have shown that the observed reaction is not hydrolysis but a nucleophilic reaction to give 1-acetylimidazole as the sole product.

Table I. Values of k_1 and k_2 for the Imidazole-Promoted Reaction of p-Nitrophenyl Acetate in Acetonitrile Containing 1 M Water of Atom Fraction Deuterium n^a

n	$10^3 k_1/$M^{-1} s^{-1}	$10^3 k_2/$M^{-2} s^{-1}	
0.00^b	2.88 ± 0.07	10.72 ± 0.57	$k_1^{H_2O}/k_1^{D_2O} = 1.11 \pm 0.03$
0.25	2.84 ± 0.05	9.81 ± 0.38	
0.50	2.72 ± 0.13	10.36 ± 1.12	
0.75	2.81 ± 0.11	8.11 ± 0.76	
1.00^c	2.60 ± 0.05	8.68 ± 0.38	$k_2^{H_2O}/k_2^{D_2O} = 1.24 \pm 0.09$

^{a} Reactions were followed at 30.1 °C at 314 nm. See text for a discussion of how the k_1 and k_2 values were obtained. ^{b} 1 M H$_2$O in acetonitrile. ^{c} 1 M D$_2$O in acetonitrile.

Figure 3. Plot of k_1 vs. the atom fraction of deuterium, n, in the solvent (open circles). The values of k_1 were obtained from the intercepts of plots like those shown in Figure 2. The theoretical line drawn through the filled circles was calculated from eq 5 in the text and represents the expected inventory for opposing isotope effect contributions.

Figure 4. Plot of k_2 vs. the atom fraction of deuterium, n, in the solvent (open circles). The values of k_2 were obtained from the slopes of plots like those shown in Figure 2. The theoretical line drawn through the filled circles was calculated from eq 6 in the text and represents the expected inventory for opposing isotope effect contributions.

Table II. Values of k_3 for the Imidazole–Benzoate Promoted Reaction of p-Nitrophenyl Acetate in Acetonitrile Containing 1 M Protium Oxide or Deuterium Oxide[a]

	$k_3{}^b$/$M^{-2}\ s^{-1}$	$k_3{}^c$/$M^{-2}\ s^{-1}$
1 M H_2O	0.678 ± 0.041	0.627 ± 0.035
1 M D_2O	0.465 ± 0.054	0.481 ± 0.022
$k_3{}^{H_2O}/k_3{}^{D_2O}$	1.46 ± 0.19	1.30 ± 0.09

[a] Reactions were followed at 30.1 °C at 314 nm. See text for a discussion of how k_3 was determined. [b] Experiment number 1 (see text). [c] Experiment number 2 (see text).

values of $k_3{}^{H_2O}$ of 0.627 and 0.678 $M^{-2}\ s^{-1}$ are somewhat lower than the literature value of 1.0 $M^{-2}\ s^{-1}$ but we are confident of our values.[9] These values set limits of 1.30 ± 0.09 to 1.46 ± 0.19 for the value of $k_3{}^{H_2O}/k_3{}^{D_2O}$. Because of the complexity of the experiment and the relatively large errors involved no attempt was made to evaluate k_3 in mixtures of protium oxide and deuterium oxide.

Figure 4 shows the dependence of k_{obsd} upon the concentration of water at constant imidazole concentration in the absence of benzoate anion. The significance of this result in view of the proposed mechanism will be discussed.

Discussion

The utility of solvent isotope effects in helping to delineate the role of proton transfers is well documented.[10] Recent interest in this area has helped to spur the development of the proton inventory technique which involves the measurement of reaction rates in mixtures of protium oxide and deuterium oxide.[11] This technique allows one to specify with some certainty the number of protons making contributions to the observed solvent isotope effect as well as the magnitude of the individual contributions. Several reviews and papers have recently appeared which outline the theory of the proton inventory technique.[10-12] For this reason the theory will not be discussed here. In greatly simplified terms, curvature in plots of rate constants vs. the atom fraction of deuterium in the solvent, n, results from multiple contributions to the observed solvent isotope effect. Linear plots will result when only a single proton contributes the entire isotope effect except under highly fortuitous circumstances.[7,10a]

It is generally accepted that solvent isotope effects in the range of 2–3 are consistent with hydrolysis mechanisms involving general base catalyzed attack by water as represented by eq 2.[11] Recently, the water-promoted hydrolyses of acetylimidazolium ion and a related compound have been shown
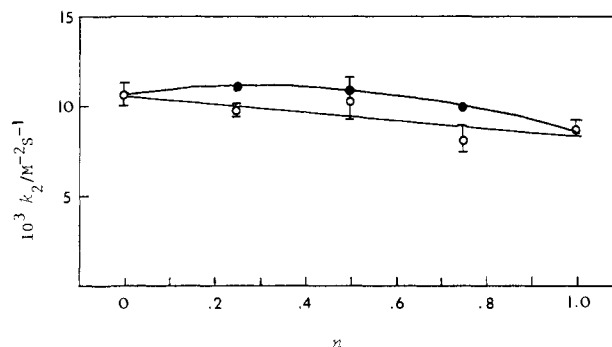
to be subject to a similar type of catalysis in which one molecule of water serves as a general base to catalyze attack by another water molecule.[12]

For catalysis by any of the mechanisms shown in eq 2–4 one would expect significant solvent isotope effects to be incurred if either a water proton or imidazole proton or both are transferred. Furthermore, mechanisms 3 and 4 should result in nonlinear proton inventories since multiple proton transfers are involved. Mechanism 4, if truly operative, would be expected to give an isotope effect similar to the enzymatic systems. The deacetylation of acetyl-α-chymotrypsin gives a solvent isotope effect of $k^{H_2O}/k^{D_2O} = 2.4$.[6]

The magnitude of the solvent isotope effect on k_1 and k_2 is clearly too low to suggest any significant proton transfer unless some inverse isotope effect of unknown origin is also present to diminish the observed effect. Although it is difficult to imagine how such an inverse effect might arise in this system it is useful to consider the shape of the proton inventories to be expected for k_1 and k_2 if such a mechanism is operating.

For the k_1 term it is likely that a mechanism operating as shown in eq 2 would give a normal solvent isotope effect of about 2.5 for the transferring proton. Such a proton would exhibit an isotopic fractionation factor of 0.4. If an inverse solvent isotope effect of unknown origin and having a fractionation factor of 2.25 is incurred then this two-proton transition-state model would still give the observed solvent isotope effect. However, the proton inventory for such a model, represented by eq 5, would exhibit an upward bulge as shown in

$$k_n = k_0(1 - n + 0.4n)(1 - n + 2.25n) \qquad (5)$$

Figure 3. The absence of any such curvature in the observed inventory confirms that no unusual effects, however unexpected, are occurring for this system. Such an analysis further illustrates the additional information that can be gained by doing a complete proton inventory as opposed to measuring a simple solvent isotope effect. In other words, the absence of a significant solvent isotope effect does not necessarily rule out proton transfers in the transition state of a reaction.

A similar analysis for the k_2 term is now considered. The mechanism of eq 3 would involve two "in-flight" protons each of which might be expected to make an equal contribution to an observed solvent isotope effect of about 2.5. Each would exhibit an isotopic fractionation factor of $(2.5)^{-0.5}$ or 0.63. Assuming again an offsetting inverse effect of unknown origin we arrive at eq 6. Such a model generates the proton inventory

$$k_n = k_0(1 - n + 0.63n)^2(1 - n + 2.04n) \qquad (6)$$

with the upward bulge in Figure 4. Again the observed inventory rules out any such unusual transition state.

The proton inventories of Figures 3 and 4 can be generated equally well by the models represented by eq 7 and 8. Equation

$$k_n = k_0(1 - n + n\phi_i{}^*) \tag{7}$$

$$k_n = k_0(k_{1.0}/k_0)^n \tag{8}$$

7 is a simple linear model in which the entire effect comes from a single transition-state proton having the fractionation factor required to generate the observed solvent isotope effect for the k_1 and k_2 terms. The fractionation factors would be 0.903 and 0.81 for k_1 and k_2, respectively. It has been shown that for a transition state in which a very large number of protons each contribute a very small normal isotope effect eq 8 predicts the shape of the proton inventory.[10e] The product of the ratio of the rate constant in D_2O to the rate constant in H_2O raised to the n power, where n is the atom fraction of deuterium in the solvent, times the rate constant in H_2O represents a "medium" effect. Such an effect represents a net loosening of the binding for a very large number of bulk solvent protons. Either of the models represented by eq 7 or 8 is consistent with the small isotope effects and the observed proton inventories for the k_1 and k_2 terms. In both cases the calculated inventories (not shown) fall within the error bars for the experimental points.

The above considerations clearly rule out the operation of mechanisms 2 and 3 as written. The isotope effect on k_3 is somewhat larger but it is still well below the range to be expected for a multiple proton transfer mechanism as shown in eq 4. Although no proton inventory was done for this term we feel the arguments presented above are equally applicable here. Taken alone these results suggest that either the water is not involved or that it is involved as a simple nucleophilic catalyst. Evidence for the former possibility is presented below.

Thus, mechanisms involving no significant proton transfers but still accounting for all the terms of the kinetic rate law must be formulated. D'Andrea and Tonellato have recently postulated carboxylate ion–imidazole dimers as the effective catalytic species in a similar hydrolysis in benzene.[13] We would like to propose a similar mechanism for the present system. We have shown that the product of this reaction is acetylimidazole which undergoes no further reaction. This was proven to be the case since the observed product spectrum in the imidazole-promoted reactions could be duplicated by adding, instead of p-nitrophenyl acetate, equivalent concentrations of p-nitrophenol and acetylimidazole. No detectable change in this spectrum was detected as a function of time. It was also shown that imidazole did not catalyze the hydrolysis of initially added 1-acetylimidazole under the kinetic conditions. Likewise, initially added 1-acetylimidazole would not catalyze any reaction of p-nitrophenyl acetate in the absence of imidazole. Thus, it was shown that 1-acetylimidazole did not undergo solvolysis to generate any imidazole which would then be expected to catalyze the p-nitrophenyl acetate reaction. It was necessary to rigorously purify the 1-acetylimidazole used in these experiments since trace imidazole impurities initially gave confusing results. Thus, convincing evidence against the involvement of water directly in the reaction and evidence in favor of a nucleophilic role for imidazole or some imidazole–benzoate dimers has been obtained.

It is not surprising that the rate constant for the term involving catalysis by benzoate and imidazole is ~100× more effective than that for catalysis by two molecules of imidazole in view of the work done on acid–base relationships and solvation in acetonitrile.[14] Acetonitrile solvates anions very poorly and, thus, benzoate anion would be expected to be more likely to form hydrogen-bonded dimers than would imidazole molecules. There is a considerable amount of evidence in the literature which supports the existence of the hydrogen-bonded dimers postulated to occur in this system.[14,15]
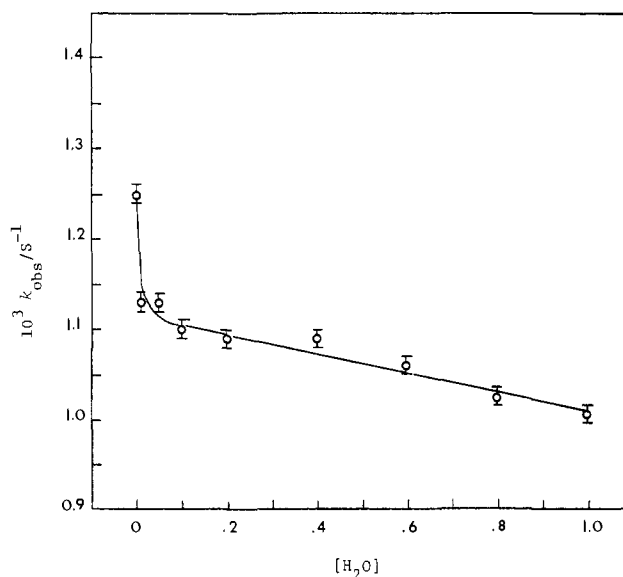
Figure 5. Plot of k_{obsd} vs. the concentration of water for the reaction of p-nitrophenyl acetate with 0.2 M imidazole and 10^{-2} M tetra-n-butylammonium perchlorate in acetonitrile at 30.1 °C.

Such dimers will be influenced by the water present in this system to an unknown extent. The influence of the water concentration on k_{obsd} for a constant imidazole concentration is shown in Figure 5. A dramatic decrease in rate is observed upon the initial addition of water followed by a more-or-less gradual leveling of the water effect. It is suggested that this rate decrease probably reflects a decrease in k_2 with increasing water concentration since water should compete with imidazole for hydrogen bonding sites. This effect should be even more dramatic for the k_3 term since water should effectively solvate the benzoate anion and reduce its ability to form the reactive imidazole–benzoate dimers.

An attempt to measure the effectiveness of N-methylimidazole as a catalyst was carried out at 50 °C. Even at this elevated temperature the observed rate constant at 2×10^{-1} M N-methylimidazole was approximately 100× lower than for an equivalent imidazole concentration at 30.1 °C. This is the expected result since N-methylimidazole is incapable of forming hydrogen-bonded dimers and the only possible kinetic role would be a nucleophilic mode of catalysis by a single molecule.

Conclusion

Although an initially attractive proposal it appears that the system under study does not operate by a charge-relay mechanism. Efficient nucleophilic catalysis by imidazole–imidazole and benzoate–imidazole dimers involving no significant proton transfer appears to account for the observed kinetics and isotope effects. Additional proton inventories on charge-relay models such as those recently proposed by Bender and co-workers[8] should yield valuable information on the feasibility of such mechanisms in enzymic systems.

Experimental Section

Materials. p-Nitrophenyl acetate was prepared from acetyl chloride and p-nitrophenol and had mp 76.5–77.5 °C (lit.[16] 77.5–78.0 °C). Imidazole (Eastman) was 3× recrystallized from benzene and had mp 89.5–90.5 °C (lit.[17] 90–91 °C). Benzoic acid (Mallinckrodt) was twice recrystallized from water and had mp 121–122 °C (lit.[17] 122.4 °C). Tetra-n-butylammonium perchlorate (Eastman) was recrystallized from absolute ethanol and dried in vacuo before use. Acetonitrile was purified as previously reported.[12] Tap-distilled water was redistilled from an all-glass apparatus before use. Deuterium oxide (Aldrich; 99.8 atom % deuterium) was purified via distillation. 1-Acetylimidazole (Aldrich) was recrystallized from isopropenyl acetate

and dried in vacuo over phosphorus pentoxide and had mp 101.5–102.0 °C (lit.[18] 101.5–102.5 °C with bath preheated to 95 °C). *N*-Methylimidazole (Sigma) was purified by distillation before use.

N^1-Imidazole-*d* was prepared according to the procedure of Garfinkel and Edsall.[19] Estimation of the deuterium content by Mr. Josef Nemeth[20] indicated that deuteration had occurred at more than one site during the exchange process. It had been spectroscopically verified that the N_1H undergoes essentially quantitative exchange under the conditions of the preparation with some C_2H exchange occurring.[19] Based on these observations we estimated the N_1H site to be at least 95% deuterated.

Benzoic-acid-*d* was prepared according to the procedure of More O'Ferrall et al.[21] Mr. Josef Nemeth[20] determined the acid to be 95.1% deuterated.

Tetramethylammonium benzoate was prepared according to the procedure of Kolthoff and Chantooni for a similar compound.[22] These hygroscopic crystals were stored over magnesium perchlorate in vacuo.

Kinetics. Every attempt was made, where applicable, to duplicate the conditions specified by Haake and coworkers in their study of this system.[9] Stock solutions of the various reactants in acetonitrile were prepared and appropriate volumes were pipetted into a 1-cm quartz UV cell to give a total volume of 3.00 mL. The total salt concentration was kept at 10^{-2} M using tetra-*n*-butylammonium perchlorate and tetramethylammonium benzoate. In the imidazole experiments the only salt present was tetra-*n*-butylammonium perchlorate. The concentration of water was kept at 1.0 M. The concentration of *p*-nitrophenyl acetate was about 6.5×10^{-5} M and the reactions were followed at 30.1 °C.

The imidazole concentration was varied from 1.5×10^{-2} to 2×10^{-1} M in those experiments done to outline its role in the catalysis. Benzoate catalysis was studied with 2×10^{-1} M imidazole present. The concentration of benzoate anion was varied from 10^{-3} to 9×10^{-3} M. An equivalent amount of benzoic acid was added in the benzoate studies.

Reactions were monitored by following the increase in absorbance at 314 nm using a Cary 118C spectrophotometer. All reactions were followed for at least 3 half-lives with infinity values being taken at 10 half-lives. The system followed good pseudo-first-order kinetics for at least 3 half-lives as evidenced by plots of ln $(A_\infty - A_t)$ vs. time. The reported rate constants were calculated using a nonlinear least-squares computer program. Equally good kinetics were obtained at 410 nm (absorbance increase) or 270 nm (absorbance decrease).

For runs with deuterium oxide and/or protium oxide–deuterium oxide mixtures separate stock solutions of the isotopically labeled reagents were prepared in acetonitrile. Appropriate volumes of these "isotopically pure" stock solutions were added to the UV cell for a given run. Thus, the isotopic composition of exchangeable protons which could contribute to any isotope effects in each run was maintained exactly at the desired value.

When it was necessary to characterize reactions as a function of starting material spectrum, product spectrum, etc., an automatic repetitive scan accessory was used.

Product Characterization. A kinetic solution containing no initially added *p*-nitrophenyl acetate but containing 6.5×10^{-5} M *p*-nitrophenol and 6.5×10^{-5} M 1-acetylimidazole was found to duplicate the observed product spectrum for the imidazole-promoted runs. There was no observable change in this spectrum as a function of time.

Under the reaction conditions, *p*-nitrophenyl acetate did not undergo any observable reaction if the imidazole was omitted and replaced by an equivalent amount of 1-acetylimidazole.

Reference and Notes

(1) D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature (London)*, **221**, 337 (1969).
(2) M. L. Bender, "Mechanisms of Homogeneous Catalysis from Protons to Proteins", Wiley-Interscience, New York, N.Y., 1971, Chapter 16 and references therein; D. M. Blow, *Acc. Chem. Res.*, **9**, 145 (1976).
(3) T. H. Fife, *Adv. Phys. Org. Chem.*, **11**, 29–63 (1975).
(4) G. A. Rogers and T. C. Bruice, *J. Am. Chem. Soc.*, **96**, 2473 (1974); T. C. Bruice, *Annu. Rev. Biochem.*, **45**, 331 (1976).
(5) H. T. Wright, *J. Mol. Biol.*, **79**, 1, 13 (1973).
(6) E. Pollock, J. L. Hogg, and R. L. Schowen, *J. Am. Chem. Soc.*, **95**, 968 (1973).
(7) A. J. Kresge, *J. Am. Chem. Soc.*, **95**, 3065 (1973).
(8) M. Komiyama, E. J. Breaux, and M. L. Bender, *Bioorg. Chem.*, in press; M. Komiyama and M. L. Bender, *ibid.*, **6**, 13 (1977).
(9) G. Wallerberg, J. Boger, and P. Haake, *J. Am. Chem. Soc.*, **93**, 4938 (1971).
(10) (a) R. L. Schowen in "Isotope Effects on Enzyme-Catalyzed Reactions", W. W. Cleland, M. H. O'Leary, and D. B. Northrop, Ed., University Park Press, Baltimore, Md., 1977, and references therein; (b) V. Gold, *Adv. Phys. Org. Chem.*, **7**, 259 (1969); (c) A. J. Kresge, *Pure Appl. Chem.*, **8**, 243 (1964); R. L. Schowen, *Prog. Phys. Org. Chem.*, **9**, 275 (1972); (d) W. J. Albery in "Proton-Transfer Reactions", E. Caldin and V. Gold, Ed., Chapman and Hall, London, 1975; (e) W. J. Albery and M. H. Davies, *Trans. Faraday Soc.*, **65**, 1059 (1969).
(11) S. S. Minor and R. L. Schowen, *J. Am. Chem. Soc.*, **95**, 2279 (1973).
(12) (a) J. L. Hogg, M. K. Phillips, and D. E. Jergens, *J. Org. Chem.*, **42**, 2459 (1977); (b) J. L. Hogg and M. K. Phillips, *Tetrahedron Lett.*, 3011 (1977).
(13) F. D'Andrea and U. Tonellato, *J. Chem. Soc., Chem. Commun.*, 659 (1975).
(14) J. F. Coetzee, *Prog. Phys. Org. Chem.*, **4**, 45 (1967).
(15) M. R. Grimmett, *Adv. Heterocycl. Chem.*, **12**, 104 (1970), and references therein; C. M. W. Anderson, J. L. Duncan, and F. J. C. Rossotti, *J. Chem. Soc.*, 140, 2165, 4201 (1961); S. N. Vinogradov and R. H. Linnell in "Hydrogen Bonding", Van Nostrand-Reinhold, New York, N.Y., 1971; B. H. Robinson in "Proton-Transfer Reactions", E. Caldin and V. Gold, Ed., Chapman and Hall, London, 1975.
(16) M. L. Bender and K. Nakamura, *J. Am. Chem. Soc.*, **84**, 2577 (1962).
(17) Merck Index, Eighth Edition.
(18) J. H. Boyer, *J. Am. Chem. Soc.*, **74**, 6274 (1952).
(19) D. Garfinkel and J. T. Edsall, *J. Am. Chem. Soc.*, **80**, 3807 (1958).
(20) 303 W. Washington, Urbana, Ill. 61801.
(21) R. A. More O'Ferrall, W. K. Kwok, and S. I. Miller, *J. Am. Chem. Soc.*, **86**, 5553 (1964).
(22) I. M. Kolthoff and M. K. Chantooni, *J. Phys. Chem.*, **70**, 856 (1966).