amide. Thus nearly all of the rate enhancement arises from a bimolecular aminolysis reaction, and only about 6% from the above-mentioned mechanisms for enhanced hydrolysis.

The absence of groups which might stabilize the transition state of the reaction between the long chain species by electronic effects, the likely similarity between ethylamine and decylamine in terms of intrinsic nucleophilicity, and the fact that the reactant concentrations (ester, 4.53 $\mu M$; total amine, 40.8 $\mu M$) are well below the expected critical micelle concentrations (for decylamine, this is 0.04 $M$[25]) limit the possible explanations of this rate enhancement. The factors which affect true rate constants are electronic, steric, and medium effects. Electronic and medium changes will be minimal in going from a reaction involving ethylamine to one involving decylamine, and the effect observed is in the opposite direction from any prediction of steric phenomena. The rise in the observed rate constant is probably best explained by the association of the reactants in solution prior to reaction, so that the system is more appropriately described in Michaelis–Menten terms.

At concentrations of material well below the critical micelle concentration, it is known that molecules containing long alkyl chains associate in aqueous solution. Thus the observed pH of solutions of sodium salts of long chain fatty acids at concentrations as low as $10^{-6}$ $M$ is best accounted for on the basis of the formation of 1:1 complexes between the carboxylic acid and its anion.[26] In the present system, we suggest that such complexes form, and that we are observing the kinetic consequences of this approximation. From our results it is not possible to say whether favorably aligned 1:1 complexes dominate the distribution of complex species at the concentrations used, but we can say that the complexes contain only one kinetically important amine molecule, since the reaction between long chain ester and long chain amine is cleanly first order in amine. The fact that kinetic saturation could not be observed at higher amine concentrations is not surprising (the apparent dissociation constant could easily be as high as $10^{-2}$ or $10^{-2}$ $M$), but this failure precludes any measure of the association constant between reactants, and effectively rules out a direct test of the above description by the determination of the true rate constant for the breakdown of the proposed complex.

When the reactions are carried out in 50% (v/v) aqueous dioxane, the rate enhancement, though it can be estimated only very approximately, disappears. It is known that hydrophobic interactions are seriously disrupted in this medium.[27] In accordance with the interpretation offered for the data of Table I, the ratios $k_{\text{decylamine}}/k_{\text{ethylamine}}$ for the reactions in 50% aqueous dioxane have fallen to values near unity for both esters, compared with values of 6.8 and 317 in more completely aqueous solution (1% (v/v) acetone). This dramatic fall in rate ratio supports the proposal that the ratios in water for both esters are larger than one, due to approximation by hydrophobic forces.

**Acknowledgment.** We gratefully acknowledge the financial support of the Science Research Council.

(26) D. Eagland and F. Franks, *Trans. Faraday Soc.*, 61, 2468 (1965).

(27) C. Tanford, *J. Amer. Chem. Soc.*, 84, 4240 (1962).

# The Kinetic Consequences of Intermolecular Attraction. II. The Hydrolysis of a Series of Fatty Acid *p*-Nitrophenyl Esters Catalyzed by a Series of *N*-*n*-Alkylimidazoles. A Very Simple Esterase Model[1a]

**Carol A. Blyth and Jeremy R. Knowles*[1b]**

*Contribution from the Dyson Perrins Laboratory, University of Oxford, Oxford, England.* *Received September 1, 1970*

**Abstract:** Second-order rate constants have been determined for the *N*-*n*-alkylimidazole catalysis of the hydrolysis of straight chain *p*-nitrophenyl carboxylates, for alkyl chain lengths 2, 4, 6, 8, and 10, at 25°. At concentrations of reactants well below the critical micelle concentrations, larger rate constants are seen for the reactions between long chain reactants despite the expected steric hindrance. Allowing for the steric effect, the rate enhancement for the decanoate ester with *N*-decylimidazole as catalyst is some 550-fold, and is explained on the basis of a hydrophobic interaction between the reactants. This is supported by the smaller size of the enhancement in a "denaturing" medium, 4 *M* aqueous urea. Thermodynamic parameters for typical "short" and "long" pairs indicate that the approximated reaction is favored mainly in the entropic contribution to the activation free energy.

The catalytic importance of enzyme–substrate binding has long been realized,[2–5] but assessment of the contribution of such binding to the reaction rate is bedevilled by lack of unambiguous model systems. In

(1) (a) For a preliminary report of some of this work, see J. R. Knowles and C. A. Parsons, *Nature (London)*, **221**, 53 (1969). (b) Address correspondence to this author.

(2) D. E. Koshland, *J. Theoret. Biol.*, 2, 75 (1962).
(3) F. H. Westheimer, *Advan. Enzymol.*, **24**, 441 (1962).
(4) W. P. Jencks, *Annu. Rev. Biochem.*, 32, 639 (1963).
(5) T. C. Bruice and S. Benkovic, "Bioorganic Mechanisms," Vol. 1, W. A. Benjamin, New York, N. Y., 1966.

a simple bimolecular reaction, part of the overall free energy of activation comes from the loss of independent translational modes when two molecules come together to form a complex. Bringing the two reactants into close proximity (at a surface or by direct prereaction binding) should therefore lower the overall free energy of activation Systems containing functional neighboring groups (in which the approximating force is a covalent bond) have been studied,[5,6] and while these are excellent models for reactions occurring within covalent intermediates in enzyme-catalyzed reactions, they are not appropriate for the formation and initial reaction of the Michaelis complex. Various weak interactions have been employed to simulate this situation, but generally in systems which are multifunctional,[7] or which are complicated by other effects (*e.g.*, micellar,[8] polymer–monomer,[9] and polymer–polymer[10] interactions, $\pi$ complexes[11]).

The high dielectric constant of water reduces the catalytic utility of monomeric electrostatic attractions and intersolute hydrogen bonds, both of which may be more important on protein surfaces than in free aqueous solution. By contrast, a hydrophobic interaction as a means of conferring catalytic specificity is amenable to investigation in model systems. That such interactions do occur is shown by the formation of small oligomers of carboxylic acids and their anions, the existence of which has been used to explain anomalies in the electrical properties of very dilute soap solutions.[12] We have previously reported the kinetic effect of extending the chain lengths of *n*-alkylamines and *p*-nitrophenyl carboxylates from two to ten carbon atoms.[13] However, this reaction was not an entirely suitable model for the importance of prereaction binding in a *catalyzed* process, and the high amine p$K_a$'s necessitate work at alkaline pH where hydrolysis rates are high.

We now report the extension of this principle to a system showing turnover, involving the catalysis of the hydrolysis of *p*-nitrophenyl esters by *N*-alkylimidazoles. The relatively low p$K_a$ values of *N*-alkylimidazoles (7.1 ± 0.2 at 25°) make these materials particularly useful nucleophiles or general bases at physiological pH values, and in the present system facilitate kinetic studies in the virtual absence of the conjugate acids, in a pH range where uncatalyzed hydrolysis rates are low.

(6) B. Capon, *Quart. Rev., Chem. Soc.*, **18**, 45 (1964).
(7) T. E. Wagner, C-J. Hsu, and C. S. Pratt, *J. Amer. Chem. Soc.*, **89**, 6366 (1967); R. G. Shorenstein, C. S. Pratt, C-J. Hsu, and T. E. Wagner, *ibid.*, **90**, 6199 (1968); C. Aso, T. Kunitake, and S. Shinkai, *Chem. Commun.*, 1483 (1968).
(8) See, *e.g.*, T. C. Bruice, J. Katzhendler, and L. R. Fedor, *J. Amer. Chem. Soc.*, **90**, 1333 (1968); R. B. Dunlap and E. H. Cordes, *ibid.*, **90**, 4395 (1968); C. A. Bunton, E. J. Fendler, L. Sepulveda, and K-U. Yang, *ibid.*, **90**, 5512 (1968); C. Gitler and A. Ochoa-Solano, *ibid.*, **90**, 5004 (1968).
(9) R. L. Letsinger and I. S. Klaus, *ibid.*, **87**, 3380 (1965); G. P. Royer and I. M. Klotz, *ibid.*, **91**, 5885 (1969).
(10) H. Morawetz, C. G. Overberger, J. C. Salamone, and S. Yaroslavsky, *ibid.*, **90**, 651 (1968); B. Vogel and H. Morawetz, *ibid.*, **90**, 1368 (1968); I. Klotz and V. H. Stryker, *ibid.*, **90**, 2717 (1968).
(11) S. D. Ross and I. Kuntz, *ibid.*, **76**, 3000 (1954); C. G. Swain and L. J. Taylor, *ibid.*, **84**, 2456 (1962); T. Higuchi and L. Lachmann, *J. Amer. Pharm. Assoc.*, 44, 521 (1955); A. K. Colter and S. S. Wang, *J. Amer. Chem. Soc.*, **85**, 114 (1963); A. K. Colter, S. S. Wang, G. H. Megerle, and P. S. Ossip, *ibid.*, **86**, 3106 (1964); F. M. Menger and M. L. Bender, *ibid.*, **88**, 131 (1966); J. A. Mollica and K. A. Connors, *ibid.*, **89**, 308 (1967); F. M. Menger, *ibid.*, **90**, 4387 (1968).
(12) D. Eagland and F. Franks, *Trans. Faraday Soc.*, **61**, 2468 (1965).
(13) J. R. Knowles and C. A. Blyth, *J. Amer. Chem. Soc.*, **93**, 3017 (1971).

Intramolecular and micellar variants on the imidazole–*p*-nitrophenyl ester reactions have been thoroughly studied in connection with the widespread occurrence of histidine at active sites of esterases, and it was therefore of interest to investigate the kinetic consequences of the attachment of an unambiguously monofunctional side chain to this catalytic functional group, and to the corresponding *p*-nitrophenyl ester substrates.

## Experimental Section

All solvents used in preparative work were purified by methods described in Perrin, Armarego, and Perrin,[14] because of the danger of contaminants in kinetic studies where reagent concentrations are as low as $10^{-5}$–$10^{-6}$ *M*. Ethyl bromide was a British Drug Houses AnalaR reagent and was redistilled twice before use. Imidazole, 1-bromobutane, 1-bromohexane, 1-bromooctane, and 1-bromodecane were Puriss reagent grade supplied by Koch-Light Laboratories Ltd. *p*-Nitrophenol, dicyclohexylcarbodiimide, and octanoic acid were all Koch-Light "pure" grades of reagent. *p*-Nitrophenol was recrystallized from deionized water until almost colorless, and dicyclohexylcarbodiimide was distilled *in vacuo*. Other aliphatic carboxylic acids were British Drug Houses AnalaR reagents. Buffer solutions were prepared from AnalaR materials and deionized water. All other chemicals were standard reagent grade materials. Microanalyses were carried out by Drs. Weiler and Strauss of this department. Ultraviolet spectra were recorded on a Unicam SP 800 self-recording spectrometer, and infrared spectra on a Unicam SP 200 instrument. Melting points were determined on a Kofler block and are uncorrected.

Freedom of long chain reagents from near homologs was established by vapor phase chromatography of 2% ethereal solutions of the acids, the esters, and the *N*-*n*-alkylimidazoles. A Perkin-Elmer F 11 gas chromatograph was used, with a stainless steel column DE 302 (1 m × 1 mm) at 120°, packed with HMDS Chromosorb W 80–100 mesh (98.5%) and fluorosilicone oil (1.5%). The carrier gas was nitrogen, at 22 psi.

**Syntheses of *p*-Nitrophenyl Esters.** ***p*-Nitrophenyl acetate** was synthesized by the method of Bodansky and du Vigneaud.[15] It was recrystallized repeatedly from *n*-hexane until white, yield 85%, mp 78.5–79°. *Anal.* Calcd for $C_8H_7NO_4$: C, 53.00; H, 3.86; N, 7.74. Found: C, 52.86; H, 3.79; N, 7.75.

Quantitative hydrolysis of a $10^{-5}$ *M* solution of the ester in 0.02 *M* carbonate buffers gave a purity of 99.8 ± 1% based on the absorbance at 400 nm, assuming a molar extinction coefficient of 18,320.

***p*-Nitrophenyl butyrate,** prepared as above, was purified by column chromatography on silica gel and dry chloroform as eluent, followed by fractional distillation under reduced pressure, bp 98–100° (0.05 mm). A single redistillation yielded ester of 99.2 ± 1% purity. *Anal.* Calcd for $C_{10}H_{11}NO_4$: C, 57.50; H, 5.31; N, 6.72. Found: C, 58.54; H, 6.01; N, 6.44.

***p*-Nitrophenyl hexanoate** was prepared by a slight modification of the above method; otherwise the major product was the *N*-acylurea. In order to favor nucleophilic attack on the *O*-acylurea over rearrangement, a twofold molar excess of *p*-nitrophenol was used at a slightly higher temperature (5°). The ester was purified by fractional distillation under reduced pressure, bp 123–124° (1.0 mm), to yield a material of 99.6 ± 1% purity. *Anal.* Calcd for $C_{12}H_{15}NO_4$: C, 60.9; H, 6.32; N, 5.93. Found: C, 61.03; H, 6.56; N, 6.54.

***p*-Nitrophenyl octanoate** was prepared according to ref 15, and was purified by chromatography on silica gel eluting with dry benzene, followed by fractional distillation, bp 130–132° (0.05 mm). The ester was 99.4 ± 1% pure. *Anal.* Calcd for $C_{14}H_{19}NO_4$: C, 63.49; H, 7.16; N, 5.28. Found: C, 63.86; H, 7.88; N, 5.86.

***p*-Nitrophenyl decanoate,** prepared as above, was recrystallized twice from freshly dried ethanol. The ester had mp 35–35.5°, and was 98.4 ± 1% pure. *Anal.* Calcd for $C_{16}H_{23}NO_4$: C, 65.50; H, 7.85; N, 4.78. Found: C, 65.66; H, 7.85; N, 4.79.

(14) D. D. Perrin, W. L. F. Armarego, and D. R. Perrin, "Purification of Laboratory Chemicals," Pergamon, Oxford, 1966.
(15) M. Bodansky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959).

All *p*-nitrophenyl esters showed the expected infrared absorption spectra, with strong bands at 2950–2955 $cm^{-1}$ (aliphatic C–H), 1755–1768 $cm^{-1}$ (ester carbonyl), 1530–1533 and 1195–1218 $cm^{-1}$ (aromatic nitro group).

**Synthesis of *N*-Alkylimidazoles.** Two methods were used. That of Häring[16] is preferred for the lower homologs. The method of Dankova, *et al.*,[17] can also be used for the octyl and decyl compounds, though the higher yield is offset by a longer purification procedure.

***N*-Ethylimidazole** was prepared by the method of ref 16, and was purified by chromatography on silica gel using chloroform as eluent, followed by fractional distillation at reduced pressure, bp 58–60° (0.3 mm). *Anal.* Calcd for $C_5H_8N_2$: C, 62.50; H, 8.33; N, 29.16. Found: C, 61.37; H, 8.45; N, 30.05.

***N*-Butylimidazole** was prepared by the same method as the ethyl homolog, and had bp 79.5–80.5° (0.8 mm). *Anal.* Calcd for $C_7H_{12}N_2$: C, 67.72; H, 9.68; N, 22.60. Found: C, 67.52; H, 9.45; N, 22.80.

***N*-Hexylimidazole** was prepared by the method of ref 17, and was purified by chromatography on alumina with ethyl acetate as eluent, followed by fractional distillation, bp 75–76° (0.1 mm). *Anal.* Calcd for $C_9H_{16}N_2$: C, 70.67; H, 10.67; N, 18.66. Found: C, 70.74; H, 10.24; N, 18.55.

***N*-Octylimidazole** was prepared by the method used for the ethyl homolog. After purification by chromatography on silica gel with ethyl acetate–methanol (3:1, v/v) as eluent, and distillation, it had bp 88–90° (0.1 mm). *Anal.* Calcd for $C_{11}H_{20}N_2$: C, 73.26; H, 11.11; N, 15.55. Found: C, 73.45; H, 11.31; N, 15.71%.

***N*-Decylimidazole** was prepared as above. Two major products were isolated from the reaction mixture on fractional distillation. The first fraction, decanol, bp 52° (0.1 mm), is produced by hydrolysis of bromodecane. Such competition by hydroxyl ion with imidazolyl anion was not observed with the shorter bromoalkanes. The second fraction, bp 110–120° (0.1 mm), was purified by chromatography on silica gel eluting with ethyl acetate. On redistillation, it had bp 108–110° (0.1 mm). *Anal.* Calcd for $C_{13}H_{24}N_2$: C, 75.00; H, 11.53; N, 13.47. Found: C, 74.11; H, 11.40; N, 14.30.

All alkylimidazoles showed the expected infrared spectra, with absorption bands at 3100 (aromatic CH), 2950 (aliphatic CH), 1510–1520, and 1210–1220 $cm^{-1}$ (imidazole ring).

**Kinetic Methods.** The rates of all reactions were followed by recording the change in the absorbance due to the *p*-nitrophenoxide ion at 400 nm, using a Unicam self-recording spectrophotometer SP 800, fitted with an SP 820 constant-wavelength scan unit, an SP 850 constant-temperature cell accessory, and an SP 870 scale expansion unit. This instrument was coupled to an external slave recorder, Sunvic Type 10S. Matched, stoppered 10-mm silica cells were used. In every case, the contents of both cells except for the ester solution (which was added last as a 25-$\mu$l aliquot) were brought to the temperature of the cell block.

The time of mixing and the time lag before recording began never exceeded 10 sec. The half-lives of reaction ranged from 2 min to several hours.

Constancy of temperature during the mixing and reaction was checked using a copper–constantan thermocouple, and was accurate to ±0.1° from 0 to 38°.

After each run, the pH of the solution was measured at the relevant temperature on a Radiometer pH meter TTTlc fitted with a scale expander pHA 630, which had previously been standardized against British Drug Houses standard buffer solutions.

The initial reaction rates, in terms of change in optical density per minute, were taken from the recorder traces and used to calculate apparent bimolecular rate constants for uncatalyzed and catalyzed hydrolysis rates. Twenty different catalyst concentrations were used in the determination of each rate constant, each of which was run in duplicate. Reactions were performed in (i) 0.02 *M* sodium carbonate–bicarbonate buffer solutions at 25°, (ii) 0.02 *M* sodium carbonate–bicarbonate buffer solution–4 *M* urea solution at 25°, (iii) 0.02 *M* sodium carbonate–bicarbonate buffer solutions in the temperature range 10–39°. The data from (iii) were used to calculate activation parameters for certain reactions. For reactions below room temperature, a Rheinische freezing machine was run against the warm thermostat, allowing at least 2 hr for equilibration. All rate constants were computed by the least-squares method using a Hewlett-Packard 9100 calculator–printer.

(16) M. Häring, *Helv. Chim. Acta*, 42, 1845 (1959).

(17) T. F. Dankova, E. I. Genkin, and N. A. Preobrazhenskii, *J. Gen. Chem. USSR*, **15**, 189 (1945); see also *Chem. Abstr.*, 40, 1800 (1946).

## Results

The pH–rate profiles for the hydrolysis of the esters studied showed that the rates of hydrolysis are given by

$$-\mathrm{d[ester]}/\mathrm{d}t = k_{OH^-}[OH^-][\mathrm{ester}] + k_{H_2O}[\mathrm{ester}][H_2O]$$

where $k_{OH^-}$ and $k_{H_2O}$ are apparent second-order rate constants which may include an undetermined buffer term (see ref 13). Though the initial uncatalyzed hydrolysis rates of the long chain esters *p*-nitrophenyl decanoate and octanoate obeyed this relationship when the pH of the solution was varied, the time course for the release of *p*-nitrophenol was anomalous. Thus the initial rate and the rate after 25% reaction did not give the same rate constant, the initial release of phenol being faster than expected. Precisely the same features were observed after multiple recrystallization of the decanoate ester, so the anomaly is unlikely to be caused by contamination of ester with a higher homolog. Such contamination was also rendered unlikely by the analysis of the esters by glc. Repurification of all reagents and buffer materials did not affect the position or magnitude of the anomalous part of the progress curve. Denaturing agents such as urea removed the anomalous characteristics, and the expected first-order progress curve was observed. It therefore seems likely that the unexpectedly rapid fall-off in reaction rate is caused by the unproductive aggregation of substrate and products. This possibility of product inhibition was not further studied since the hydrolysis reaction is merely a small (2–7%) fraction of the catalyzed reaction rate.

For the *N*-alkylimidazole-catalyzed hydrolysis of the *p*-nitrophenyl esters, the pH–rate profiles showed that the catalyzed reaction rate depends on the free base form of alkylimidazole. For reactions between short and between long chain reactants, apparent $pK_a$ values were in the region of 7.1 ± 0.2, and all subsequent experiments were therefore performed at pH values greater than 9.0, at which the concentration of catalyst conjugate acid is negligible. After subtraction of the rate in the absence of the catalyst, all reactions were first order in ester and in imidazole. The only deviations from the expected behavior occurred at high concentrations of the long-chain reagents. The rate constants were therefore measured at catalyst concentrations less than 0.10 m*M*, in which region the rate is given by $-\mathrm{d[ester]}/\mathrm{d}t = k_c[\mathrm{ester}][N\text{-alkylimidazole}]$ + hydrolysis terms, where $k_c$ is the apparent second-order rate constant determined under pseudo-first-order reaction conditions. A typical experimental plot is given in Figure 1.

**Rate Constants and Ratios.** Apparent second-order constants for the hydrolysis of each of the five esters catalyzed by each of the five *N*-alkylimidazoles are shown in Table I. Major trends are immediately obvious, but become much more so when the rate constants are expressed relative to the $C_2$ compound, or are plotted on a logarithmic scale against chain length (see Figures 2 and 3).

**The Effect of Denaturing Agents.** The reaction rates in solutions of denaturing agents such as 50% (v/v) dioxane–water mixtures, and 8 *M* urea solutions, were reduced (relative to the uncatalyzed rates) to

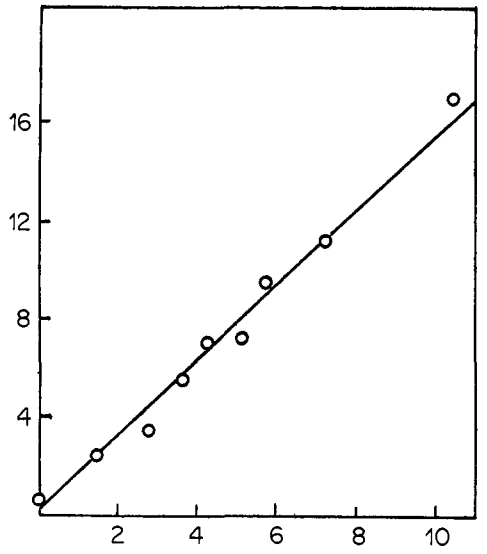


Figure 1. The rate of *N*-decylimidazole-catalyzed hydrolysis of *p*-nitrophenyl decanoate at 25°, 0.02 *M* carbonate buffer, 0.99% (v/v) in acetone: ester concentration, 11.3 $\mu M$; ordinate, $10^3 \times$ absorbance change/min; abscissa, $10^5 \times$ *N*-decylimidazole concentration (*M*).

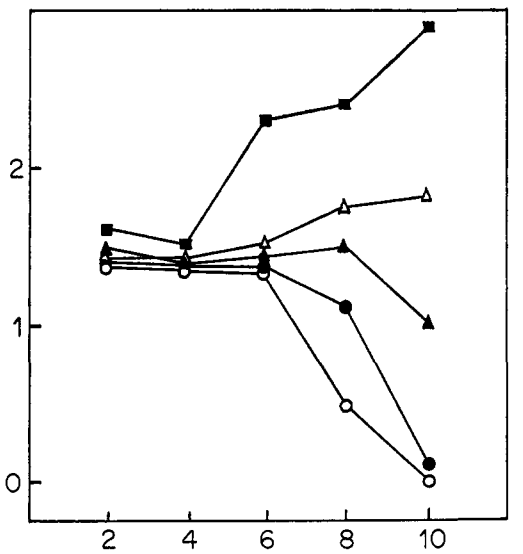


Figure 2. Vertical trends in Table I. Log $k_c$ values *vs.* chain length of the *p*-nitrophenyl esters: *N*-ethylimidazole (○), *N*-butylimidazole (●), *N*-hexylimidazole (▲), *N*-octylimidazole (△), *N*-decylimidazole (■); ordinate, log $k_c$; abscissa, chain length of ester.

the point where the desired rate ratios were inaccurate. However, reactions in 4 *M* aqueous urea gave measurable catalyzed rates, and Table II shows the apparent second-order rate constants obtained under these conditions.

**Table I.** Apparent Second-Order Rate Constants ($M^{-1}$ $min^{-1}$) for the Hydrolysis of *p*-Nitrophenyl Carboxylates in the Presence of *N*-Alkylimidazoles[a,b]

| *p*-Nitrophenyl ester | $OH^-$ | *N*-Alkylimidazoles | | | | |
|---|---|---|---|---|---|---|
| | | Ethyl[h] | Butyl[i] | Hexyl[j] | Octyl[k] | Decyl[l] |
| Acetate[c] | 724 | 23.8 | 24.2 | 25.7* | 27.2 | 36.8 |
| Butyrate[d] | 404 | 21.2 | 21.4 | 21.8 | 23.4 | 26.8* |
| Hexanoate[e] | 380 | 19.3 | 23.3 | 24.1 | 29.4 | 173 |
| Octanoate[f] | 194 | 2.7 | 11.8 | 28.6 | 45.3 | 332 |
| Decanoate[g] | 28.9 | 0.82* | 1.12 | 8.84 | 56.0 | 700 |

[a] 25°, in 0.02 *M* sodium carbonate–bicarbonate buffers containing 0.99% (v/v) acetone. [b] Experimenal errors were estimated by repeat determinations at ±1.5–3%, except for the rate constants marked with an asterisk. These are less accurate because of the relatively high rates of uncatalyzed hydrolysis, and are subject to errors of the order of ±5%. [c] 21.8 $\mu M$. [d] 33.5 $\mu M$. [e] 9.65 $\mu M$. [f] 11.6 $\mu M$. [g] 5.0–9.0 $\mu M$. [h] 0.148–4.82 m*M*. [i] 0.614–4.41 m*M*. [j] 0.100–0.918 m*M*. [k] 0.406–2.02 m*M*. [l] 0.0142–0.107 m*M*.

**Table II.** Apparent Second-Order Rate Constants ($M^{-1}$ $min^{-1}$) for the Hydrolysis of *p*-Nitrophenyl Carboxylates Catalyzed by *N*-Alkylimidazoles in 4 *M* Urea[a,b]

| *p*-Nitrophenyl esters | *N*-Alkylimidazoles | |
|---|---|---|
| | Ethyl | Decyl |
| Acetate | 20.1 (23.8) | 27.9 (32.9) |
| Octanoate | 11.3 (2.69) | 164 (332) |
| Decanoate | 3.61 (0.82) | 340 (700) |

[a] 25°, in 4 *M* urea–0.02 *M* sodium carbonate–bicarbonate buffers containing 0.99% (v/v) acetone. Concentrations of reagents are the same as those used in the absence of urea (see Table I). [b] Analogous figures for reaction in the absence of urea are shown in parentheses.

**The Effect of Temperature.** In order to obtain activation parameters for the unapproximated and approximated processes, the reactions between *p*-nitrophenyl acetate and *N*-ethylimidazole and between *p*-nitrophenyl decanoate and *N*-decylimidazole were investigated between 10 and 39°. The Arrhenius plots for these reactions are shown in Figures 4 and 5, and the derived activation parameters in Table III. These

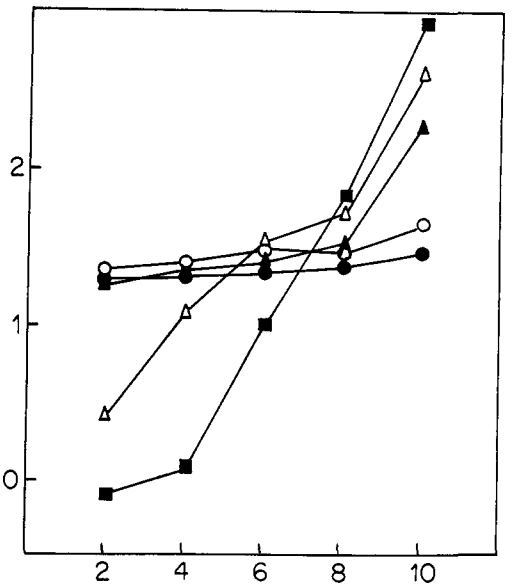


Figure 3. Horizontal trends in Table I. Log $k_c$ values *vs.* chain length of the *N*-alkylimidazoles: acetate (○), butyrate (●), hexanoate (▲), octanoate (△), decanoate (■); ordinate, log $k_c$; abscissa, chain length of *N*-alkylimidazole.

**Table III.** Activation Parameters for *N*-Alkylimidazole-Catalyzed Hydrolyses of *p*-Nitrophenyl Esters at 25°

| Reaction | $\Delta G^{\ddagger}$, kcal/mol | $\Delta H^{\ddagger}$, kcal/mol | $T\Delta S^{\ddagger}$, kcal/mol | $\Delta S^{\ddagger}$, eu |
|---|---|---|---|---|
| Acetate–*N*-ethylimidazole | 17.9 | 8.0 | −9.9 | −33 |
| Decanoate–*N*-decylimidazole | 15.9 | 9.1 | −6.8 | −23 |

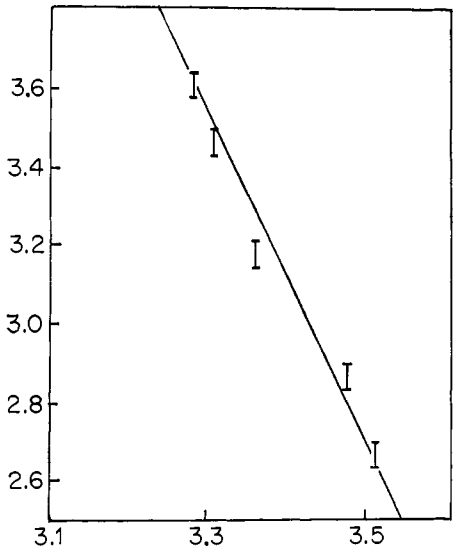


Figure 4. Arrhenius plot for the reaction of *N*-ethylimidazole with *p*-nitrophenyl acetate: ordinate, ln $k_c$; abscissa, $10^3/T$ (°K$^{-1}$).

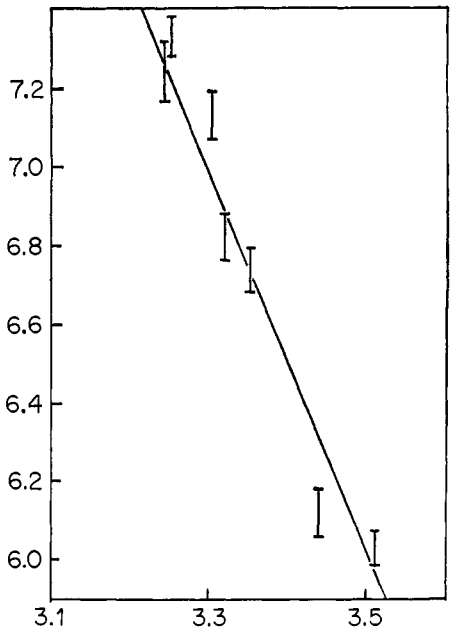


Figure 5. Arrhenius plot for the reaction of *N*-decylimidazole with *p*-nitrophenyl decanoate: ordinate, ln $k_c$; abscissa; $10^3/T$ (°K$^{-1}$).

values are estimates only but experimental problems (solubility in the long chain pair, and rapidly increasing blank rate in the shorter chain pair) precluded comparison over a larger temperature range.

## Discussion

**The Reaction Pathway.** Jencks and Carriuolo[18] have investigated the hydrolysis of a variety of activated esters in various buffers capable of general base or of nucleophilic catalysis. While acyl-activated esters are subject to general base catalysis, alkyl-activated esters (*e.g.*, *p*-nitrophenyl esters) undergo nucleophilic catalysis as evident from the detection of acyl-nucleophile intermediates and from insignificant solvent isotope effects. For *p*-nitrophenyl acetate, the rate of imidazole-catalyzed hydrolysis is proportional to the concentration of free base,[19] and *N*-acetylimidazole can be detected spectroscopically.[20] The nucleophilic mechanism is favored[21] in cases of intramolecular catalysis by imidazole itself, and it has also been proposed for the imidazole-catalyzed hydrolysis of a *p*-nitrophenyl ester in a micelle.[22] General base catalysis by imidazole has been ruled out for intermolecular catalysis involving small molecules[20] and for reactions in micelles.[23] It therefore seems reasonable to assume that the nucleophilic pathway is followed by all types of imidazole-catalyzed hydrolysis of *p*-nitrophenyl esters, including the one studied in the present work. The nucleophilic pathway here involves the attack by *N*-alkylimidazole on the ester to give the *N*-alkyl,*N'*-acylimidazolium ion intermediate, which is rapidly hydrolyzed to the acid and *N*-alkylimidazole. This hydrolysis step is analogous to the acid-catalyzed decomposition of acylimidazolium ions, and there is no doubt that the first step of the reaction, formation of acylimidazolium ion, is rate determining. This fact simplifies the interpretation of effects on the catalytic rate constants, as will be seen later.

**Rate Constants. Vertical Trends.** From Table I, the **hydroxyl ion** rate constants show the expected effect of steric hindrance on going from the $C_2$ (acetate) to the $C_{10}$ (decanoate) ester. A sharp drop is apparent between the octanoate and decanoate esters, which may be a result of a direct steric shielding of the susceptible carbonyl group by the coiling-up of the long chain ester.

The ***N*-ethylimidazole** rate constants show the same trend as the $k_{OH^-}$ values, with a similar marked fall at the $C_8$ and $C_{10}$ esters.

The trend in the ***N*-hexylimidazole** rate constants is different from the *N*-ethyl- and *N*-butylimidazoles. Although the nucleophile is presumably more sterically hindered than the lower homologs, the rate of hydrolysis of the $C_8$ ester is very close to that of the $C_2$ ester, and even for the $C_{10}$ ester the rate constant only falls slightly (to 8.84: *cf.* 0.82 for *N*-ethylimidazole). These trends are readily seen in Figure 2.

If it is the case that a hydrophobic approximating force outweighs coiling-up in water, increasingly the catalyst chain length may overcome steric hindrance to the point at which the longer chain esters are *more* reactive than the relatively unhindered shorter chain esters.

The relative rate constants for ***N*-octylimidazole** are all larger than for the $C_6$ catalyst. More significantly, the esters longer than $C_4$ become progressively more reactive as the chain length is increased.

The trend for ***N*-decylimidazole** on increasing the chain length of the ester shows a large increase in rates. The expected coiling-up in water of *p*-nitrophenyl decanoate which would normally reduce the rate of catalyzed hydrolysis is relatively insignificant when the catalyst possesses an equivalent hydrophobic side chain. The effect on the absolute value of the rate

(18) W. P. Jencks and J. Carriuolo, *J. Amer. Chem. Soc.*, 83, 1743 (1961).
(19) T. C. Bruice and G. L. Schmir, *ibid.*, 80, 148 (1958).
(20) M. L. Bender and B. W. Turnquest, *ibid.*, 79, 1656 (1957).
(21) W. P. Jencks and J. Carriuolo, *ibid.*, 83, 1743 (1961).
(22) T. E. Wagner, C-J. Hsu, R. G. Shorenstein, and C. S. Pratt, *ibid.*, 90, 6199 (1968).
(23) C. Gitler and A. Ochoa-Solano, *ibid.*, 90, 5004 (1968).

constant is large (700 $M^{-1}$ $min^{-1}$ compared with 36.8 $M^{-1}$ $min^{-1}$, see Table I), and a hydrophobic proximity effect is the most reasonable explanation in view of the variation of the rate enhancement with increasing ester chain length, as illustrated in Figure 2.

**Horizontal Trends.** All alkylimidazoles are more reactive toward every ester than is the smallest, *N*-ethylimidazole. The relative rate constants of ***p*-nitrophenyl acetate** show a small steady increase with increasing chain length. This effect, which opposes that expected on steric grounds, may be interpreted as due to hydrophobic interactions involving the aromatic ring of the ester, and the long chain of the catalyst. The short ester can "uncoil" the long catalyst through its nitrophenyl group, whereas the short catalyst cannot similarly overcome steric hindrance in the long ester. A similar effect was reported for aminolysis by decylamine of *p*-nitrophenyl acetate, compared with the decanoate–ethylamine reaction.[13]

For ***p*-nitrophenyl decanoate**, the relative rate constants extend from 1 to 855 for *N*-ethyl- to *N*-decylimidazole. Thus the catalyst which might be expected to exhibit the slowest rate because of steric hindrance in fact is characterized by a rate constant almost three orders of magnitude greater than that of *N*-ethylimidazole. The movement of the relative rate constants is shown in Figure 3.

**General Discussion.** One of the more difficult questions which have to be faced in studies of this kind is how to define the kinetic effect of the approximation. The rate enhancement for *N*-decylimidazole relative to *N*-ethylimidazole for *p*-nitrophenyl decanoate is 855 (Table I). Yet can this be ascribed to the proximity effect, since steric factors contribute to both reactions? One may argue either that the reaction between the long chain reactants is the most likely to suffer from steric hindrance (which makes 855 a lower limit for the enhancement), or that the long chain reactants suffer less from steric hindrance since they uncoil against each other prior to reaction. Alternatively, one could take the ratio $k_{decanoate}/k_{acetate}$ for *N*-decylimidazole, which is 19 (Table I), though this is surely an artificially low estimate of the enhancement since we have argued above that the short ester interacts hydrophobically *via* its *p*-nitrophenyl group with the long catalyst. A third approach can be taken on the basis that the intrinsic activation free energy (for the reaction itself) is independent of the length of alkyl side chains. In this case we can compare the rate of decanoate ester and decylimidazole, with acetate ester and ethylimidazole. This ratio is 700/23.8 = 30, and provides a minimal estimate of approximation. That is, *despite the very considerable steric hindrance expected in a reaction involving two $C_{10}$ reactants, this process is still 30 times faster than the corresponding reaction involving the $C_2$ components.* Finally, if the steric disadvantage of the reaction between $C_{10}$ reactants is accounted for by comparing $k_{decyl}/k_{ethyl}$ for long and short chain esters [*i.e.*, (700/0.82)/(36.8/23.8) from Table I], or its equivalent ($k_{decanoate}/k_{acetate}$ for long and short chain catalysts), we obtain what is probably the *most realistic value for the rate enhancement—550.*

How does this rate enhancement arise? For the long chain reactants, either the free energy of the ground state must be effectively raised, or the transition state for the rate-determining step (which is that of the formation of *N*-alkyl,*N'*-acylimidazolium ion) must be lowered. The latter is unlikely for the following reasons. (a) The basicities of the short and long chain imidazoles are so close that it is very unlikely that variations in intrinsic nucleophilicity could account for the increased reactivity of the long chain catalyst. In any case, this is not apparent for short chain esters. (The possibility that reactant complexation results in catalyst desolvation and a consequent reactivity increase cannot be ruled out, but see below.) (b) Steric effects can only disfavor the reaction of long chain species, yet these rates are the fastest. (c) A lowering of the effective microscopic dielectric constant (arising from the coiling round of the alkyl side chains) should slow down the reaction, the rate-determining step of which involves charge separation on going to the transition state.

The explanation of the enhancement must lie, therefore, in the effective raising of the ground-state free energies for the reaction of long chain species. As discussed earlier,[13] at concentrations well below the critical micelle concentrations, molecules with long alkyl side chains associate in aqueous solution. So the rate enhancement most probably arises from the association of the two reactants prior to (and during) the covalency changes which constitute the catalytic process.

A prereaction complex could not be detected by kinetic methods since the reaction was first order in each component, but the apparent second-order rate constants for the reactions of long chain species can be viewed as the product of an indeterminable association constant, and a first-order rate constant for the intracomplex reaction. This is equivalent to $k_{cat}/K_m$ for enzymic reactions.

By way of confirmation of the coiling up and association of reactants by hydrophobic forces, the rate data in 4 *M* urea (Table II) show the following. (a) Whereas the reaction between short chain reactants is slightly slower in urea, the rates of reaction of the long chain esters with *N*-ethylimidazole are four to five times *faster* in urea. This is consistent with the view that these esters in water coil up alone or to form oligomers, resulting in steric hindrance to the small nucleophiles, hydroxyl ion, and *N*-ethylimidazole. (b) By contrast, the reaction between *N*-decylimidazole and the decanoate ester is *slower* in urea solution, which is most readily interpreted in terms of a destabilizing effect on the intermolecular interaction on which the rate enhancement depends. The rate enhancement for the reaction of long chain species (550) is severely depressed (to 68, see Table II).

The denaturing effect of urea on proteins is primarily due to the alteration of local water structure around the polymer,[24] and the effect of urea on the rate constants in the present case is entirely consistent with the breaking down of solvent structure by the urea, destabilizing the hydrophobic interactions. (The possibility of urea clathrate formation along the hydrocarbon chains must also be borne in mind.)

The differences in activation parameters for the "short:short" and "long:long" reactions (Table III) show that the "long" reaction is favored in the entropy term by 3.1 kcal/mol, whereas the "short" reaction is

(24) G. C. Hammes and J. C. Swann, *Biochemistry*, 6, 1591 (1967).

favored by 1.1 kcal/mol in the enthalpy term. Caution must be exercized in interpreting these differences, since there may be significant compensation in enthalpy and entropy terms for processes involving changes in water structure such as accompany hydrophobic bond formation.

However, the formation of a hydrophobic interaction *is* predominantly due to an entropy change and the more favorable entropy of activation for the long chain pair may arise in this way. It is probably improper to equate the favorable entropy of hydrophobic bond formation directly with the kinetic consequences of this interaction (*i.e.*, the rate enhancement), but on the basis of estimates of the strength of a hydrophobic interaction in proteins[25] and a model system,[23] which are about 650 cal(methylene unit mol), a hydrophobic interaction between eight extra $-CH_2-$ groups in each member of the long chain pair would give rise to a binding energy of 4.9 kcal/mol. *If* all the binding energy were used to lower the overall activation free energy, and if we accept 550 as the most realistic estimate for the enhancement due to approximation, we are observing an effective interaction energy of approximately 3.7 kcal/mol. In rough agreement with this, Bruice and his coworkers[26] have suggested from a study of the dependence of the activation entropy of a number of acyl reactions on the kinetic order of the process, that the kinetic advantage of turning a bimolecular reaction into a hypothetical unimolecular reaction amounts to about $10^3$ in the rate, corresponding to 4–6 kcal/mol.

In physical terms, however, can we ascribe the whole of the observed rate acceleration to a higher encounter frequency of reactants arising from the enhanced local concentration of catalyst and substrate brought about by their approximation? Koshland[2] has pointed out that the maximum increase in encounter frequency from an increase in local concentration will occur if one reactant is completely surrounded by molecules of the other. Even for reactant water (where the concentration is 55 *M*), this only amounts to eight entropy units, *i.e.*, 2.4 kcal/mol at 25°, and clearly the rate enhancement observed here exceeds that ascribable to a concentration effect. (This agrees with calculations based on the Sackur–Tetrode equation that the loss of independent transition modes when two molecules come together to form a complex is six to ten entropy units.[27]) However, all these estimates are based on spherical model reactants, and the contribution of reactant orientation—even in the present case—may be large enough to account for the observed enhancement.

**Limitations of the System.** This model system illustrates the kinetic effect of complexation prior to reaction, substrate specificity, and "denaturation" by urea. Other features of an enzymic reaction are not apparent. The existence of a catalyst–substrate complex cannot be demonstrated in terms of a kinetically determined value for "$K_m$" because the complex has too high a dissociation constant. Saturation of catalyst with ester cannot be observed because the blank rate becomes too high relative to the catalytic rate. It also proved impossible to saturate the ester with excess catalyst, because at an ester concentration of about 5 $\mu M$ (this ester concentration is the minimum possible for techniques of the sensitivity available), the catalyst becomes insoluble before saturation can be observed.

Inhibition of the reaction by the product anions was not observed, and addition of a second aliquot of ester after completion of reaction did not result in a charged catalytic rate. Since a Michaelis constant for the complex cannot be obtained, inhibition by substrate analogs was not investigated, since $K_i$ values would also be unavailable.

Such limitations are, of course, not unexpected for a model system which relies on hydrophobic forces for its specificity. Saturation phenomena have been observed by other workers, but only for systems involving one (or two) polymeric reactants, or involving multifunctional interactions. We have preferred to study this particularly simple system since the forces contributing to the rate enhancements are readily defined, even though a number of enzyme-like characteristics are consequently unobservable.

## Conclusion

In summary, we see that the existence of an approximating interaction of about 5 kcal/mol between two reactants in a simple bimolecular reaction can lead to a reduction of about 4 kcal/mol in the overall activation free energy, and that the *mere collection* of substrate, cofactors, and catalytic functionalities at an enzyme's active site may lead to very considerable rate accelerations of chemical transformations.

**Acknowledgments.** We are grateful to Dr. J. Albery, Dr. R. J. P. Williams, and Dr. A. C. Day for helpful discussions, and to the Science Research Council for support.

(25) G. Nemethy and H. A. Scheraga, *J. Chem. Phys.*, **36**, 3382, 3401 (1962).

(26) T. C. Bruice and S. J. Benkovic, *J. Amer. Chem. Soc.*, **86**, 418 (1964).

(27) I. Z. Steinberg and H. A. Scheraga, *J. Biol. Chem.*, **238**, 172 (1963).