Imidazole Catalyses in Aqueous Systems. I. The Enzyme-like Catalysis in the Hydrolysis of a Phenyl Ester by Imidazole-Containing Copolymers[1]

Toyoki Kunitake, Fumiko Shimada, and Chuji Aso

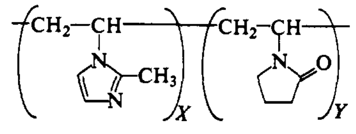
Contribution No. 160 from the Department of Organic Synthesis, Faculty of Engineering, Kyushu University, Fukuoka, Japan. Received August 8, 1968

Abstract: Hydrolyses of 3-nitro-4-acetoxybenzoic acid catalyzed by copolymers of 1-vinyl-2-methylimidazole with 1-vinylpyrrolidone (copolymer A) and with acrylamide (copolymer B) were studied at 30° and mostly at pH 8.0 in 1.0 *M* aqueous KCl. The rate of the catalytic hydrolysis could be described by Michaelis–Menten kinetics, showing substrate saturation phenomena at high substrate concentrations, as in enzymatic reactions. Copolymer A of low (<20%) imidazole contents showed the same kinetic pattern, independent of the copolymer composition, and gave K_m (dissociation constants of the catalyst–substrate complex) of 9.3 m*M* and k_3 (first-order rate constant of the pseudo-intramolecular product formation) of 0.038 min^{-1}. Copolymer B gave K_m of 63 m*M* and k_3 of 0.11 min^{-1}. When, however, copolymer A contained higher amounts of the imidazole unit, the kinetic pattern was not the simple Michaelis–Menten type and the over-all catalytic efficiency decreased, suggesting the presence of the catalytic site of a differing nature. The substrate binding was attributed to hydrophobic interaction, since the electrostatic interaction between catalyst and substrate was found negligible. The kinetic characteristics of copolymer A were reasonably explained by assuming the loop formation of the polymer segment surrounding the substrate molecule. Finally, probable reasons for the relatively small k_3 value for copolymer A were discussed, taking the nature of the catalytic site into consideration.

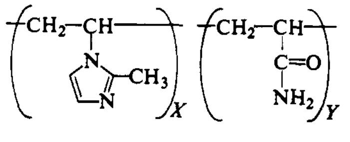
The importance of the hydrophobic force in maintaining the tertiary structure of enzymes including the active site was established from the recent structure determinations of several enzymes by X-ray diffraction. In particular, α-chymotrypsin has been considered to have a hydrophobic binding site,[2,3] and this is compatible with the recent analysis of the three-dimensional structure of this enzyme.[4]

In some of the polymeric systems was clearly noted the influence of hydrophobic bonding on the catalytic action and other properties. Thus, Sakurada, *et al.*,[5] and Yoshikawa, *et al.*,[6] found that hydrophobic bonding was effective in enhancing the rate of the ester hydrolysis catalyzed by polymeric sulfonic acids. Klotz and Stryker found a similar effect with polyethylenimine.[7] Polyvinylpyrrolidone[8] and polymethacrylic acid[9-12] are known to bind organic molecules in aqueous systems due to hydrophobic bonding. Recently Kirsh and coworkers[13] observed the substrate saturation phenomena in the hydrolysis of *p*-nitrophenyl acetate catalyzed by a polyvinylpyridine derivative, and the force responsible for the substrate binding was concluded to be hydrophobic bond. The substrate saturation phenomena were similarly reported by Overberger, *et al.*,[14] using electrostatic forces between a polyvinylimidazole and an anionic ester.

As a model of the active site of α-chymotrypsin, we prepared copolymeric systems which would possess a hydrophobic binding function and an imidazole group as a catalytic function. The catalysts used in the present investigation are copolymers of 1-vinyl-2-methylimidazole and 1-vinylpyrrolidone (copolymer A) and of 1-vinyl-2-methylimidazole and acrylamide (copolymer B).



copolymer A



copolymer B

Experimental Section

Preparation of Copolymers. 1-Vinylpyrrolidone, commercial product, was purified by distillation before use, bp 90–93° (11 mm). 1-Vinyl-2-methylimidazole was provided by Toho Rayon Co., Japan, and distilled before use, bp 93–94° (17 mm). The purified monomers showed single peaks on gas chromatograms. Acrylamide was purified by repeated recrystallization from toluene, mp 83.5–84.5°.

The copolymers of 1-vinyl-2-methylimidazole and 1-vinyl pyrrolidone were obtained by radical polymerization of bulk

---

(1) A preliminary account of this study was given: C. Aso, T. Kunitake, and F. Shimada, *J. Polymer Sci., B*, 6, 467 (1968).

(2) B. S. Hartley in "Structure and Activity of Enzymes," T. W. Goodwin, J. I. Harris, and B. S. Hartley, Ed., Academic Press, New York, N. Y., 1964, p 47.

(3) M. L. Bender, J. V. Killheffer, and F. J. Kézdy, *J. Amer. Chem. Soc.*, **86**, 5331 (1964).

(4) W. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, *Nature*, **214**, 652 (1967).

(5) I. Sakurada, Y. Sakaguchi, T. Ono, and T. Ueda, *Makromol. Chem.*, **91**, 243 (1966).

(6) S. Yoshikawa and O-K. Kim, *Bull. Chem. Soc. Jap.*, **39**, 1515 (1966).

(7) I. M. Klotz and V. H. Stryker, *J. Amer. Chem. Soc.*, 90, 2717 (1968).

(8) P. Molyneux and H. P. Frank, *ibid.*, **83**, 3169, 3175 (1961).

(9) T. M. Birshtein, Ye. V. Anufriyeva, T. N. Nekrosova, O. B. Ptitsyn, and T. V. Sheveleva, *Vysokomol. Soedin.*, **7**, 372 (1965).

(10) J. Eliassaf, *J. Polymer Sci., B*, 3, 767 (1965).

(11) A. M. Liquori, G. Barone, V. Crescenzi, F. Quadrifoglio, and V. Vitagliano, *J. Macromol. Chem.*, **1**, 291 (1966).

(12) G. Barone, V. Crescenzi, A. M. Liquori, and F. Quadrifoglio, *J. Phys. Chem.*, **71**, 2341 (1967).

(13) Yu. E. Kirsh, V. A. Kabanov, and V. A. Kargin, *Dokl. Akad. Nauk USSR*, **177**, 976 (1967); *Vysokomol. Soedin.*, **A10**, 349 (1968).

(14) C. G. Overberger, R. Corett, J. C. Salamone, and S. Yaroslavsky, *Macromolecules*, **1**, 331 (1968).

Table I. Titration Characteristics of the Copolymers[a]

Copolymer	Im unit %	pK_a	n'
A	3.5	7.12	1.07
A	8.2	7.15	1.12
A	15.7	7.09	1.22
A	40.0[b]	6.62	1.43
A	80.4[b]	6.04	1.53
B	2.8	7.16	1.04

[a] pK_a = pH + n' log [(1 − α)/α], 30°, μ = 1.0 *M* KCl. [b] 30°, μ = 1.5 *M* KCl.

monomer mixtures at 70° using azobisisobutyronitrile as initiator. The polymerization mixture was placed in an ampoule, which was sealed under nitrogen. After a given period, the reaction mixture was diluted with methanol, and the copolymer was precipitated by pouring into excess ethyl ether. Conversions were about 50% for the copolymer of 3.5% imidazole (Im) content, and 10–20% for the copolymers of 8 and 15.7% Im contents. The imidazole monomer was incorporated into polymer approximately 2.5 times as fast as the pyrrolidone monomer. Thus, the distribution of the imidazole unit in these copolymers may be assumed homogeneous. The conversions for the copolymer of higher Im contents were 40–60%. The molecular weights of some of the copolymers were measured with a vapor pressure osmometer (Mechrolab, Model 301A). Copolymers of 15.7 and 40.0% Im contents showed molecular weights of 4700 and 2800, respectively.

Copolymerizations of 1-vinyl-2-methylimidazole and acrylamide were carried out in 1:1 aqueous methanol at 60° using azobisisobutyronitrile as initiator. Copolymers precipitated in the polymerization ampoules as they were formed. After given periods, polymers were dissolved by adding some water, precipitated by pouring into methanol, and washed with methanol. Reprecipitation was carried out from water and methanol. Starting from a monomer mixture containing 3.4 mol % imidazole monomer, a copolymer containing 2.8 mol % of the imidazole unit was obtained. All the polymer samples were dried *in vacuo*. The Im contents of the copolymers were determined by titration as described below.

Substrate. 3-Nitro-4-hydroxybenzoic acid, mp 182–184°, which was obtained by nitration of *p*-hydroxybenzoic acid, was acetylated in acetic anhydride–pyridine overnight at room temperature. The reaction mixture was poured into *n*-hexane, and the precipitate formed was separated and dissolved in tetrahydrofuran. A small amount of concentrated hydrochloric acid was added, the solution dried over $CaCl_2$, and the insoluble portion (pyridine hydrochloride) was filtered. The solvent was then evaporated and the pale yellow residue was recrystallized from benzene and from isopropyl ether: mp 154–157° (lit.[15] 152°), yield 16%. Acetylation with acetic anhydride and concentrated sulfuric acid gave less pure products.

Other Materials. Special grade KCl was dried before use. Deionized water was boiled before use to expel dissolved CO_2.

Titration. The copolymer composition was determined by titration of the imidazole group. A pH-Stat system connected with a recorder (TOA Electronics Ltd., Japan, Model HS-1B and Model EPR-2T, respectively) was used for this purpose. A reaction vessel (35-ml beaker) was placed in a water-jacket which was maintained at 30.0 ± 0.05° by circulating water from a constant-temperature bath. A small amount of water was added in the jacket to attain better heat conduction. The reaction vessel was covered with a rubber stopper which was equipped with glass and calomel electrodes, a thermocompensator, and a buret. The content of the vessel was stirred magnetically. NaOH aqueous solutions to be added were standardized by potassium hydrogen phthalate using this titration system. Weighed amounts (*ca.* 150 mg) of the copolymer were dissolved in 1 *M* KCl solution and made acidic (pH 3) by adding a small amount of concentrated hydrochloric acid. Aqueous alkali (0.05–0.1 *N*) was manually delivered from the autoburet in 0.01-ml portions and the pH was recorded. These water-soluble polymers were quite hygroscopic and elemental analyses of the homopolymer indicated that the copolymer contained about 5 wt % of impurities (most probably water). Since the kinetic pattern of the hydrolysis was insensitive to this extent of the variation in the copolymer composition, correction was not made for the copolymer composition determined by titration.

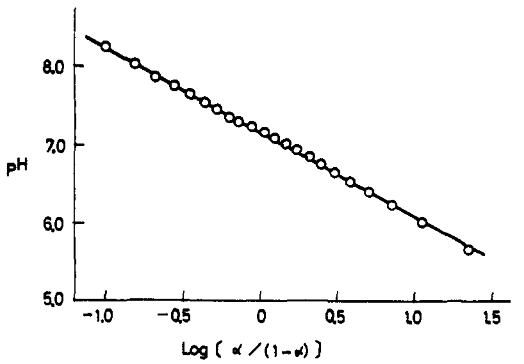
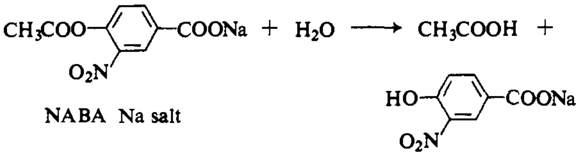


Figure 1. The modified Henderson–Hasselbach relationship of copolymer A (3.5% Im content). Titration condition: total Im concentration; 2.72 m*M*, 30°, 1 *M* KCl. The solid line was calculated by the least-square method.

Hydrolysis. A given amount of the substrate was placed in the reaction vessel and 10 ml of 2.5 *M* aqueous KCl was added. A calculated amount of water was added when necessary. Aqueous alkali was automatically added until the mixture became homogeneous and the predetermined pH was attained. At this point the total volume of the reaction mixture was 25.0 ± 0.3 ml and the ionic strength was 1.0. In case of spontaneous hydrolysis, the alkali consumption with time was recorded thereafter. In catalytic hydrolysis, a given amount of the catalyst solution was added when the rate of alkali consumption reached that of spontaneous hydrolysis. (The total volume was 25 ml including the catalyst solution.) The rate of hydrolysis was determined from the initial rate. As the hydrolysis proceeded, the brownish yellow color of the product (dianion form) developed. Since the substrate had been inevitably hydrolyzed to some extent before the initial rate was determined, the concentration of the substrate was corrected for it from the excess consumption of alkali at the time when the initial rate was measured. The correction was usually 7–8%.[16] The aqueous alkali used was 0.2 *N* at high substrate concentrations. At the substrate concentration of 0.02 *M* or lower, more dilute aqueous alkali was used in order to avoid overtitration. The reaction mixture was kept at a constant pH with fluctuation of ±0.05. Since the pK_a's of the product, 3-nitro-4-hydroxybenzoic acid, are 3.68 and 6.02, it existed almost completely in the dianion form at pH 8.0, where most experiments were run. The ionic strength of the reaction mixture was 1.0 (KCl) plus the substrate concentration, as the carboxyl group of the substrate was first neutralized. Thus, the over-all ionic strength when the initial rate was measured, varied from 1.00 to 1.08, depending on the substrate concentration.



NABA Na salt

(1)

Results

Titration of the Imidazole Unit. Plots of log [(1 − α)/α] *vs.* pH for copolymer A of 3.5% Im content appear slightly deviated from linearity (Figure 1). It is not clear whether or not this deviation indicated the conformational transition as in aqueous polymethacrylic acid.[17] The

---

[15] C. G. Overberger, T. St. Pierre, N. Vorchheimer, J. Lee, and S. Yaroslavsky, *J. Amer. Chem. Soc.*, 87, 296 (1965).

[16] Since the product dianion conceivably acts as an inhibitor, the additional lowering of the initial rate of hydrolysis may occur. In the absence of the detailed analysis of the inhibitory action of the product, we assume that the binding constant of the product is approximately the same as that of the substrate. Then, the initial rate would be higher by about 7–8% than was actually determined. This probable uncertainty in v_{cat} was included in the estimation of the experimental error.

[17] J. C. Leyte and M. Mandel, *J. Polymer Sci., A*, 2, 1879 (1964).

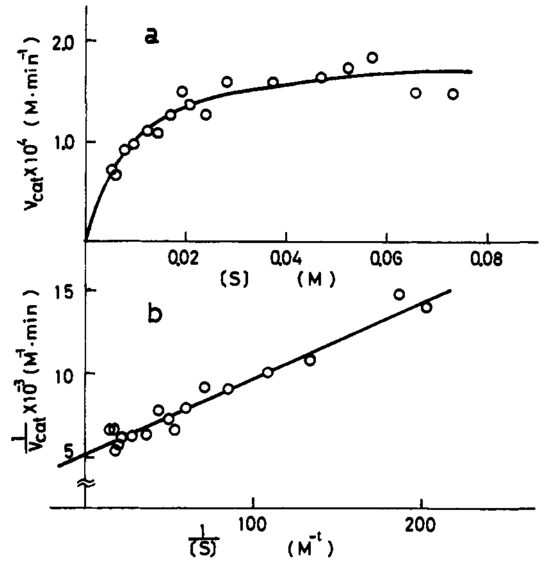


Figure 2. Catalytic hydrolysis: pH 8.0, 30°, 1.0 *M* KCl, catalyst copolymer A (3.5% Im content), total Im concentration 6.3 m*M*. The curve was calculated using K_m and k_3 obtained. (b) The corresponding Lineweaver–Burk plot. The solid line was obtained by the least-square method.

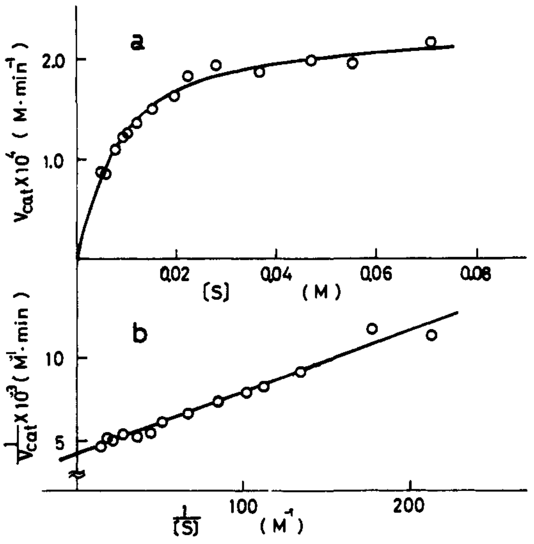


Figure 3. (a) Catalytic hydrolysis: pH 8.0, 30°, 1.0 *M* KCl, catalyst copolymer A (7.5% and 8.2% Im content), total Im concentration 6.7 m*M*. Two kinds of copolymer A were used as catalyst. This extent of the difference in the Im content does not matter kinetically, since [Im] was kept constant. The solid curve was calculated from K_m and k_3 obtained. (b) The corresponding Lineweaver–Burk plot. The solid line was determined by the least-square method.

linearities of this relationship were more satisfactory for other copolymers. Thus the modified Henderson–Hasselbach equation[18] was considered applicable to the titration behavior

$$pK_a = pH + n' \log[(1 - \alpha)/\alpha] \tag{2}$$

where α = fraction of the neutral imidazole unit. The intercept and the slope were determined by the least-square method, and the titration data thus obtained are summarized in Table I. Up to the Im content of 15.7%, copolymer A gave the pK_a values of 7.12 ± 0.03. Copolymer B showed a close value (7.16). The n' values for these copolymers were close to unity. Thus, the electrostatic effect in the titration of these polycations is considered to be quite small. With higher Im contents, pK_a decreased considerably and n' deviated from unity, indicating the enhanced electrostatic effect in the titration.

Spontaneous Hydrolysis. The substrate hydrolyzed spontaneously in the absence of the catalyst. At above pH 8.5, the spontaneous hydrolysis was too fast to obtain reliable data of the catalytic hydrolysis. The rate of the spontaneous hydrolysis was proportional to the substrate concentration at pH 8.0. Polyvinylpyrrolidone added (0.01–0.16 *M* of the monomer unit) had no effect on the spontaneous hydrolysis. The rate constant at pH 8.0 (30°, 1.0 *M* KCl) was $(2.5 \pm 0.2) \times 10^{-3}\ \text{min}^{-1}$.

Catalytic Hydrolysis by Copolymer A. Hydrolysis in the presence of the catalyst is separated into two terms—spontaneous and catalytic hydrolyses. In Figure 2a are

$$v = v_{cat} + v_{spont} \tag{3}$$

plotted the initial rate *vs.* substrate concentration when copolymer A of the 3.5 mol % Im content was used as the catalyst. The concentration of the imidazole unit was 6.27 m*M*. While the hydrolysis rate increased with the substrate concentration until about 0.03 *M*, a saturation phenomenon was observed at higher substrate concentrations. This kinetic behavior is similar to those of enzymatic reactions.

The enzymatic reaction, in its simplest form, is considered to proceed *via* complex formation between the enzyme and the substrate as shown in eq 4 (Michaelis–Menten kinetics)[19]

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \tag{4}$$

where E, S, and P are enzyme, substrate, and product, respectively. ES is known as the Michaelis complex. When $k_2 \gg k_3$, the Michaelis constant

$$K_m = \frac{k_2 + k_3}{k_1} \tag{5}$$

is reduced to the dissociation constant of the complex.

$$K_s = k_2/k_1 \tag{6}$$

As described below, k_3's of the present catalytic system are rather small. Therefore, the Michaelis constant K_m may be considered to represent the true dissociation constant K_s in the following discussion. When [E] ≪ [S], the rate of the product formation is expressed as

$$v = \frac{k_3[E][S]}{K_m + [S]} \tag{7}$$

k_3 and K_m are determined from the Lineweaver–Burk plot[20] between $1/v$ and $1/[S]$ (eq 8).

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(\frac{1}{[S]}\right) + \frac{1}{V_{max}} \tag{8}$$

---

(18) A. Katchalsky and P. Spitnik, *J. Polymer Sci.*, **2**, 432 (1947).

(19) M. Dixon and E. C. Webb, "Enzymes," Academic Press, New York, N. Y., 1958, p 62.

(20) H. Lineweaver and D. Burk, *J. Amer. Chem. Soc.*, **56**, 658 (1934).

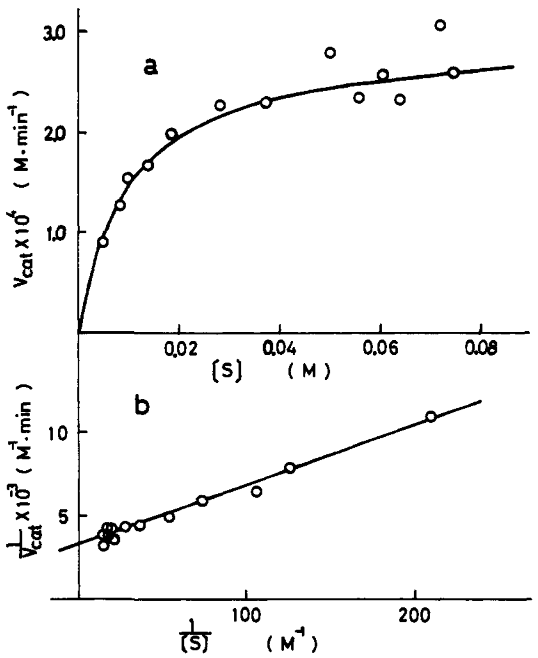


Figure 4. (a) Catalytic hydrolysis: pH 8.0, 30°, 1.0 M KCl, catalyst copolymer A (15.7% Im content), total Im concentration 9.8 mM. The solid curve was calculated from K_m and k_3 obtained. (b) The corresponding Lineweaver–Burk plot. The solid line was determined by the least-square method.

$$V_{max} = k_3[E] \tag{9}$$

A similar treatment can be made for the hydrolysis data obtained here, considering that the concentration of the imidazole unit corresponds to [E]. The Lineweaver–Burk plot of the data of Figure 2a is shown in Figure 2b. The linearity obtained indicates that the Michaelis–Menten kinetics is a suitable expression for the catalytic behavior of the imidazole group in this copolymer. K_m and k_3 determined from the plots of Figure 2b were 8.7 ± 1.5 mM and 0.036 ± 0.008 min^{-1},[21] respectively.

The effects of the substrate concentration on v_{cat} are given in Figures 3a and 4a for copolymer A of the Im content of *ca.* 8 mol % and 15.7 mol %, respectively. In both cases the substrate saturation phenomena are apparent. The corresponding Lineweaver–Burk plots are shown in Figures 3b and 4b. K_m were 8.8 ± 1.5 mM (Im content: 8 mol %) and 10.9 ± 1.5 mM (Im content: 15.7 mol %) and k_3 were 0.042 ± 0.008 min^{-1} (Im content: 8 mol %) and 0.036 ± 0.008 min^{-1} (Im content: 15.7 mol %).

The hydrolysis results shown above indicate that the catalytic activity of the imidazole group was not affected by the copolymer composition up to the Im content of 15.7 mol %. However, this conclusion could not be extrapolated to the hydrolysis by the copolymers of higher Im contents. Figure 5 shows the plots of v_{cat} *vs.* substrate concentration obtained with a copolymer containing 40.0 mol % Im unit. v_{cat} did not show a saturation phenomenon and increased (though not linearly) with the substrate concentration. The corresponding Lineweaver–Burk plots were not linear. Apparently the imidazole unit in this copolymer gives a different catalytic behavior from those mentioned above. A similar result was obtained as shown in Figure 6, when a copolymer of the Im content of 80.4 mol % was used as catalyst. v_{cat} did not saturate completely, but showed a small upward trend with the increasing substrate concentration. Furthermore, v_{cat} is smaller over the whole range of the substrate concentration as compared with v_{cat} for the copolymer of lower Im contents. Thus, the latter two cases could not be analyzed using the simple Michaelis–Menten kinetics (eq 7). The homopolymer of 1-vinyl-2-methylimidazole was not used as the catalyst, because it was insoluble under the experimental conditions used.

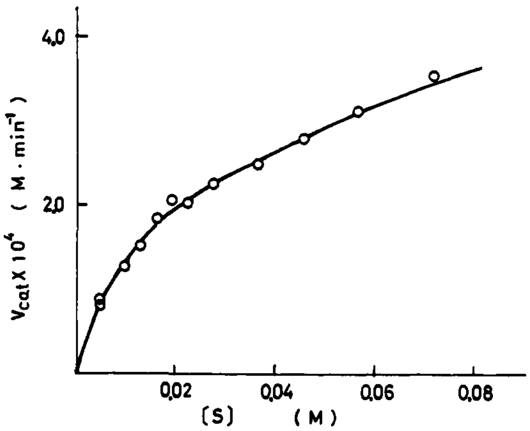


Figure 5. Catalytic hydrolysis: pH 8.0, 30°, 1.0 M KCl, catalyst copolymer A (40.0% Im content), total Im concentration 11.5 mM.

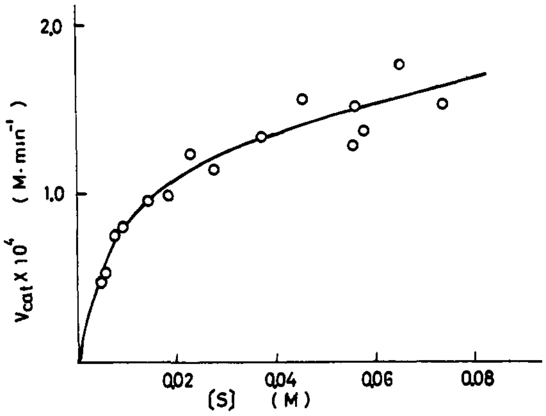


Figure 6. Catalytic hydrolysis: pH 8.0, 30°, 1.0 M KCl, catalyst copolymer A (80.4% Im content), total Im concentration 10 mM.

Effect of pH on the Catalytic Hydrolysis. The dependence of v_{cat} on the pH of the reaction medium was investigated at a given substrate concentration. Copolymer A used contained 3.5 mol % Im unit. The pH of the system was varied from 6.8 to 8.0. The amount of the protonated imidazole unit was calculated from the titration curve, and the fraction of the protonated imidazole unit varied from 13 to 67%. Since the product was not completely in the dianion form at lower pH, a correction was made in calculating v_{cat} from the alkali consumption. v_{cat} increased proportionally with the fraction of the neutral imidazole unit. The pseudo-first-order rate

---

(21) Some of the imidazole unit are protonated at pH 8.0. Thus the concentration of the neutral imidazole unit was calculated from the titration curve, and k_3's were corrected for the protonated species.

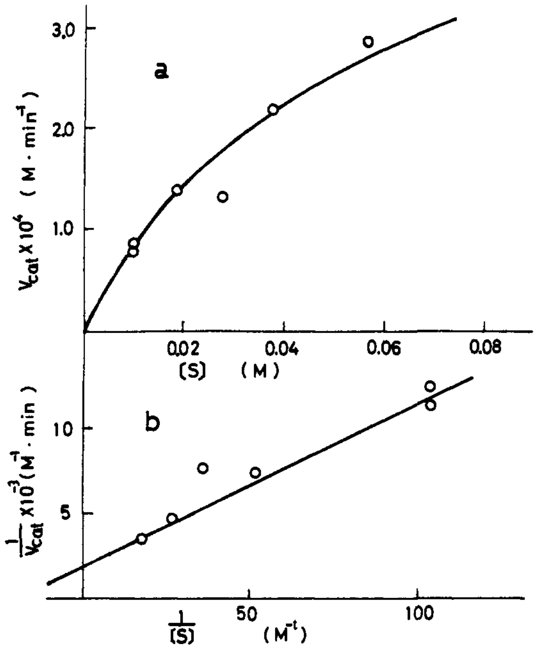


Figure 7. (a) Catalytic hydrolysis: pH 8.0, 30°, 1.0 *M* KCl, catalyst copolymer B (2.8% Im content), total Im concentration 5.0 m*M*. The solid curve was calculated from K_m and k_3 obtained. (b) The corresponding Lineweaver–Burk plot. The solid line was determined by the least-square method.

Table II. Kinetic Constants of Catalytic Hydrolyses

Copolymer	Im unit %	K_m, m*M*	k_3, min$^{-1\,b}$
A	3.5	8.7[a]	0.036
A	7.5, 8.2	8.8[a]	0.042
A	15.7	10.9[a]	0.036
B	2.8	52[c]	0.109[d]
B	2.8	79[c]	0.104[d]

[a] Estimated error, ±1.5 m*M*. [b] Estimated error, ±0.008 min^{-1}. [c] Average, 66 m*M*. [d] Average, 0.11 min^{-1}.

constant was 0.021 min^{-1} at 0.01 *M* substrate concentration and 6.3 m*M* total imidazole concentration.

Catalytic Hydrolysis by Copolymer B. In order to investigate the role played by the vinylpyrrolidone unit in the enzyme-like catalysis of copolymer A, the hydrolysis was carried out using copolymer B as the catalyst. Again, the catalytic hydrolysis did not follow the common second-order kinetics. Although the substrate saturation phenomenon is not clear from Figure 7a, the corresponding Lineweaver–Burk plot gave a straight line (Figure 7b). K_m and k_3 were obtained as 52 m*M* and 0.109 min^{-1}, respectively. A separate series of experiments with the same system gave 79 m*M* (K_m) and 0.104 min^{-1} (k_3). The kinetic data are summarized in Table II.

Discussion

The Michaelis–Menten Kinetics. Data given in the previous section indicate that the catalytic hydrolysis did not proceed by the common second-order kinetics. The dependence of v_{cat} on the substrate concentration was analyzed satisfactorily in terms of the Michaelis–Menten kinetics as in eq 10. However, we cannot deny completely the existence of the bimolecular process from the available data.

$$\underset{\text{C}}{\text{catalyst}} + \underset{\text{S}}{\text{substrate}} \underset{K_m}{\rightleftharpoons} \underset{\text{CS}}{\text{catalyst}\cdot\text{substrate}} \xrightarrow{k_3} \text{catalyst} + \underset{\text{P}}{\text{product}} \qquad (10)$$

$$v_{cat} = \frac{k_3[\text{C}][\text{S}]}{K_m + [\text{S}]} \qquad (11)$$

Consider that a given catalytic site can act in two ways—enzyme-like and bimolecular catalyses—as in eq 10 and 12.

$$\text{catalyst} + \text{substrate} \xrightarrow{k'} \text{catalyst} + \text{product} \qquad (12)$$

$$v_{cat} = k'[\text{C}][\text{S}] \qquad (13)$$

Then, the over-all catalytic rate

$$v_{cat} = k_3[\text{CS}] + k'[\text{C}]_{\text{uncomplexed}}[\text{S}] \qquad (14)$$

$$= (k_3 + k'K_m)\frac{[\text{C}]_{\text{total}}[\text{S}]}{K_m + [\text{S}]} \qquad (15)$$

Thus, the substrate saturation phenomena may be observed even in the presence of the bimolecular process, as long as the complexation occurs. Two extreme cases are expected from eq 15: case I, when $k' = 0$, the truly enzyme-like kinetic behavior is obtained, as given by eq 10 and 11; case II, when $k_3 = 0$, the substrate saturation phenomena would indicate that the substrate molecule which is complexed with the polymer is not susceptible to hydrolysis and that only the free substrate molecule can be catalytically hydrolyzed by the imidazole group *via* the common bimolecular kinetics (eq 12). This case was recently suggested for the methanolysis of tetrachlorophthalic anhydride catalyzed by dimethylaniline.[22]

In the intermediate case, both of the enzyme-like and bimolecular processes will coexist, and the relative importance of these processes is determined from a comparison of k_3/K_m and k'.

Since polyvinylpyrrolidone (0.01–0.16 *M* of the monomer unit) had no effect on the rate of the spontaneous hydrolysis when added to the reaction mixture, it is improbable that the substrate is protected against hydrolysis upon complexation with the polymer.

Another experiment which can distinguish the above two cases would be the effect of urea addition. Concentrated aqueous solution of urea is known to destroy the hydrophobic bonding.[23] Since the force responsible for the substrate binding was found to be hydrophobic bonding (see below), the catalyst–substrate complex will be destroyed to a certain extent in 6 *M* aqueous urea. For case II, the addition of urea should increase v_{cat}, because the amount of the free substrate will increase. On the contrary, v_{cat} would decrease upon addition of urea if the enzyme-like mechanism is operating (case I), as the rate enhancement due to complexation will be lost.

When the catalytic hydrolyses were carried out in 6 *M* aqueous urea,[24] v_{cat} decreased at particularly small

---

(22) F. M. Menger, *J. Amer. Chem. Soc.*, 90, 4387 (1968).

(23) W. Bruning and A. Holtzer, *ibid.*, 83, 4865 (1961).

(24) F. Shimada, unpublished results; catalyst: copolymer A of 40% Im content, pH 8.0, 30°, 1.0 *M* KCl.

substrate concentrations and was approximately first-order with respect to the substrate concentration ($k' \cong 0.4\ M^{-1}\ \text{min}^{-1}$). In contrast, v_{spont} was affected little by the presence of urea ($k_{\text{spont}} = 2.3 \times 10^{-3}\ \text{min}^{-1}$ in 6 M urea). These results undoubtedly indicate that the enzyme-like process (eq 10) is more important than the bimolecular process (eq 12) in the present system, and the rate acceleration due to complexation can be estimated to be approximately tenfold from a comparison of k_3/K_m ($\cong 4\ M^{-1}\ \text{min}^{-1}$) and k'($\sim 0.4\ M^{-1}\ \text{min}^{-1}$).

Absence of Electrostatic Interaction in Substrate Binding. Under the hydrolysis condition, the imidazole group in the copolymer is protonated to a certain extent. Recently, the hydrolysis of phenyl esters with imidazole- and pyridine-containing polymers were studied, and rate enhancements were observed between catalysts and substrates having opposite charges.[15,25,26] In particular, substrate saturation curves were obtained in the system of partially protonated polyvinylimidazoles and negatively charged substrates.[14,27] Thus, there arises a possibility that in the present system the electrostatic interaction contributes to the binding between the partially protonated polymer and the anionic substrate. However, this possibility appears remote from the following considerations. The copolymer A of 3.5 mol % Im content, for example, possesses 13% of the imidazole group protonated at pH 8.0 (*cf.* Table I). Therefore, the amount of the protonated unit per monomer unit is only 0.46 mol %, which is apparently too small for the effective substrate binding. In addition, v_{cat} at 0.01 M substrate concentration increased proportionally with the fraction of the neutral imidazole unit. This result indicates that the catalytic activity depended only on the concentration of the neutral imidazole unit and not on the concentration of the positive group, thus denying the rate enhancement is due to an electrostatic interaction. It is possible, however, that the high ionic strength used (1.0 M KCl) dampened the electrostatic interaction, if any.[28]

The fraction of the charged monomer unit at pH 8.0 increases slightly with the Im content in the copolymer. However, the electrostatic interaction seems to be negligible at least for the copolymer A of up to 15.7 mol % Im content, because these copolymers gave the single K_m and k_3 values. With copolymer A of higher Im contents (40 and 80 mol %), the amount of the charged monomer unit increases to 2–4% at pH 8.0. Since the kinetic patterns for these copolymers are different from those of copolymer A of lower Im contents, it was not ascertained, though likely, that the electrostatic interaction did not contribute to substrate binding.

The contribution of the electrostatic interaction to substrate binding will not be significant for copolymer B as well, because the protonated imidazole unit was only 0.38% per monomer unit at pH 8.0.

The Hydrophobic Mechanism of Substrate Binding. The substrate binding was not unexpected particularly for copolymer A, since polyvinylpyrrolidone was known to bind many organic molecules in aqueous systems. The interaction of polyvinylpyrrolidone with cosolutes was studied by Molyneux and Frank[8] in detail. From the binding tendencies of many aromatic cosolutes with the polymer, they concluded that the nature of the interaction responsible for binding was mainly hydrophobic bonding. The minimum number of monomer units which can accommodate one cosolute molecule (or ion) was found to be essentially constant (10 ± 3), irrespective of the size of the cosolutes.

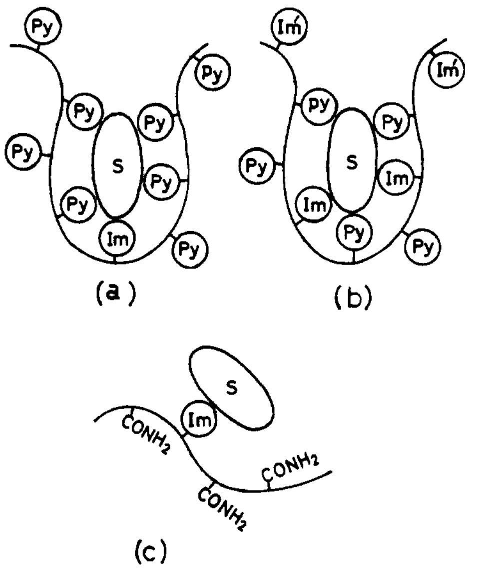


Figure 8. Schematic representations of the catalyst–substrate complex: (a) catalyst, copolymer A of low Im contents, substrate (S) is surrounded by one Im unit and by several pyrrolidone (Py) units; (b) catalyst, copolymer A of high Im contents, substrate is surrounded by more than one Im unit and by several Py units [the imidazole group which is present outside the loop (Im′) behaves line the Im unit in copolymer B]; (c) catalyst, copolymer B, substrate is in contact only with the Im unit, the catalytic loop is not formed.

Though they did not give any proper explanation, this rather surprising result may be reasonably explained by assuming that the binding site is a loop made of the polymer segment. The hydrophobic interaction of the bound cosolute with polymer would be maximal by being surrounded by the polymer segments which have hydrophobic side chains, and the number of the monomer unit which are necessary to constitute a loop can be considered to be 10 ± 3. That small molecules such as nitrobenzene or phenol require ten monomer units as a binding site would be difficult to explain without considering a loop of the polymer segment. Contraction of the polymer chain on substrate binding[8] is similarly compatible with the concept of the loop formation.

The effect of the composition of the polymer catalyst on its kinetic behavior can be most satisfactorily interpreted in terms of the loop hypothesis. The structures of the catalyst–substrate complexes are schematically represented in Figure 8. Since the catalytic site must contain at least one imidazole unit, it will be a loop made of one imidazole group and several pyrrolidone units. A loop of this type is possible only for copolymer A of low Im contents (Figure 8a). If the loop consists of 10 ± 3 monomer units, the same kinetic behavior will be expected

---

(25) R. L. Letsinger and T. J. Savereide, *J. Amer. Chem. Soc.*, **84**, 114, 3122 (1962).

(26) C. G. Overberger, R. Sitaramaiah, T. St. Pierre, and S. Yaroslavsky, *ibid.*, **87**, 3270 (1965).

(27) R. L. Letsinger and I. S. Klaus, *ibid.*, **87**, 3380 (1965).

(28) The present results do not exclude the possibility that the copolymers used exert the electrostatic attraction for anionic substrates under different hydrolysis conditions. Especially, the homopolymer of 1-vinyl-2-methylimidazole may well show the electrostatic rate acceleration under suitable conditions (lower pH, less polar medium, smaller ionic strength etc.,) as in other polymeric imidazole systems.[14]

for the copolymers which contain less than 10 to 15 mol % of the imidazole unit. The nature of the loop will be different for copolymer A of higher Im contents, because more than one imidazole units have to be incorporated on average within a given loop (Figure 8b). In fact, the same kinetic pattern (constant k_3 and constant K_m) was observed for copolymer A of up to 15.7 mol % Im content, and different kinetic behaviors were obtained for copolymer A of higher Im contents (40.0 mol % and 80.4 mol %). As will be discussed later in this paper, the latter kinetic patterns can be expressed as an overlapping of various Michaelis–Menten kinetics.

Our initial assumption on the substrate binding by copolymer A was that only the pyrrolidone unit was responsible for binding.[1] However, the hydrolysis catalyzed by a copolymer of 1-vinyl-2-methylimidazole and acrylamide (copolymer B) could also be described by the Michaelis–Menten kinetics as shown in Figure 7a. Since aqueous polyacrylamide is thought to be incapable of binding organic molecules hydrophobically,[10] the binding as well as catalytic functions may be attributed to the imidazole unit. As the homopolymer of 1-vinyl-2-methylimidazole was insoluble in water at pH 8, the imidazole unit will be capable of hydrophobic interaction with the substrate molecule, although insolubility in water does not necessarily correspond to the hydrophobic binding force.

The difference in the binding capacity between copolymer A of low Im contents and copolymer B (K_m: 9.5 m*M* *vs.* 66 m*M*) is considered to arise from the presence of the vinylpyrrolidone unit in the former but not in the latter. In copolymer A of low Im contents, two kinds of the monomer units work cooperatively within a loop for binding the substrate.[29] On the other hand, only the imidazole unit is effective for binding in copolymer B and formation of a loop may not be conceived (Figure 8c). In other words, the assumption of the loop formation is necessary to explain the enhanced binding capacity of copolymer A.

Influence of the Copolymer Composition on the Kinetic Pattern. The simplest enzyme kinetics (eq 4) deal with the single active site. On the other hand, when a copolymer such as used in the present investigation shows enzyme-like kinetic behavior, the sites of catalysis are not necessarily uniform. Therefore, its kinetics and the corresponding catalytic rate will be expressed as in eq 16 and 17, respectively

$$\mathrm{C}_i + \mathrm{S} \underset{K_{mi}}{\rightleftarrows} \mathrm{C}_i\mathrm{S} \longrightarrow \mathrm{C}_i + \mathrm{P} \qquad (16)$$

$$v_{\mathrm{total}} = \sum_{i=1}^{n} v_i = \sum \frac{k_{3i}[\mathrm{C}_i][\mathrm{S}]}{K_{mi} + [\mathrm{S}]}$$

$$= [\mathrm{C}][\mathrm{S}]\sum \frac{\alpha_i k_{3i}}{K_{mi} + [\mathrm{S}]} \qquad (17)$$

where C_i is the *i*th catalytic site and α_i is the fraction of the *i*th catalytic site ($\sum \alpha_i = 1$). If $K_{mi} = K_m$

$$v_{\mathrm{total}} = \frac{[\mathrm{C}][\mathrm{S}]\sum \alpha_i k_{3i}}{K_m + [\mathrm{S}]} \qquad (18)$$

$$= \frac{\bar{k}_3[\mathrm{C}][\mathrm{S}]}{K_m + [\mathrm{S}]} \qquad (19)$$

where $\bar{k}_3 = \sum \alpha_i k_{3i}$. Thus, when the Michaelis constant (K_m) is the same for all sites, the expression of the over-all catalytic rate for *n* sites (eq 17) is reduced to that for the single site (eq 11). The Lineweaver–Burk plot of eq 19 is linear, and a single set of K_m and k_3 are obtainable.

As already mentioned, the Lineweaver–Burk plots were linear and K_m and k_3, respectively, agreed within the experimental uncertainty among copolymer A of up to 15.7 mol % Im content. These results strongly indicate that the catalytic sites of copolymer A of lower Im contents are uniform concerning the binding capacity and the over-all rate of the pseudo-intramolecular process.

The next question to be asked may be whether or not k_3 is constant. The stereochemistry of the main chain of the copolymers is safely assumed to be atactic, as the copolymers were prepared by radical polymerization at a high temperature. The interaction of the bound substrate with the imidazole group may be dependent on the conformation of the loop (for copolymer A) which, in turn, will depend on the stereochemistry of the main chain. Therefore, the k_3 value most probably would not be constant, but rather an average value ($\bar{k}_3$). This problem may be elucidated by using polymer catalysts of the known tacticity.

The kinetic behavior of copolymer B conforms to the simple Michaelis–Menten kinetics (eq 10 and 11). Since binding was assumed to occur at the imidazole group without formation of a loop, the catalytic sites in copolymer B can be considered homogeneous. The low Im content of this copolymer should help the catalytic sites to act independently.

Increase in the Im content in copolymer A resulted in the failure of the simple Michaelis–Menten kinetics. Furthermore, the over-all catalytic efficiency of the imidazole group decreased. These results suggest that the nature of the catalytic site changed from uniform to complex at about 20 mol % Im content. The over-all catalytic activity at higher Im contents may be understood as an overlapping of the action by various catalytic sites (eq 17), of which two extremes are probably those of copolymer A of lower Im contents and of copolymer B. That is, on increasing the Im content, there will be contained more than one imidazole units in a loop (Im in Figure 8b). On further increase, some of the imidazole unit will become difficult to be included within the loop and may exist outside the loop (Im′ in Figure 8b). The latter imidazole unit may behave the same way as in copolymer B. Thus, the rate data shown in Figures 5 and 6 may be approximated as a combination of the two extreme situations

$$v_{\mathrm{cat}} = \frac{k_3\alpha[\mathrm{C}][\mathrm{S}]}{K_m + [\mathrm{S}]} + \frac{k_3'(1 - \alpha)[\mathrm{C}][\mathrm{S}]}{K_m' + [\mathrm{S}]} \qquad (20)$$

where the second term refers to the catalytic site of the type of copolymer B and the first term to the rest of the catalytic sites, $(1 - \alpha)$ is the fraction of the catalytic site of the copolymer B type.

Putting K_m' and k_3' values equal to those for copolymer B and knowing the total concentration of the imidazole unit, K_m, k_3, and α can be obtained from Figures 5 and 6 by curve fitting. Table III gives the results. Although these data are obtained using rather crude approximations,

---

[29] Increase in the number of the binding site does not raise the binding constant. That is, the number of sites and the binding constant are independent parameters in the Langmuir isotherm which was used for analyzing the binding data.[30]

[30] I. M. Klotz, F. M. Walker, and R. B. Pivan, *J. Amer. Chem. Soc.*, **68**, 1486 (1946).

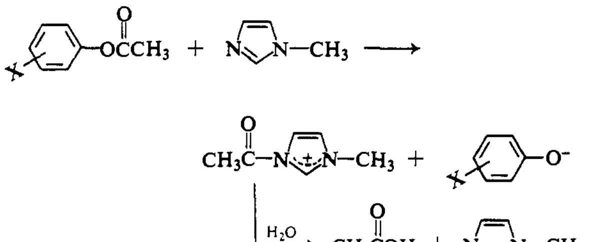
Table III. Kinetic Constants for Copolymer A of Higher Im Contents

Im contents, %	α	K_m, mM	k_3, min^{-1}
40.0	0.71	9.8	0.020
80.4	0.89	5.3	0.011

they seem to reflect the varying nature of the catalytic site with the increase in the Im content as mentioned above. The smaller k_3 values (0.02 and 0.011 min^{-1}) as compared with those of copolymer A of low Im contents (0.038 min^{-1}) and rather high α values imply that more than one imidazole groups are included within a loop, and that their average catalytic efficiency decreases because the relative disposition of the substrate molecule is fixed.

Another possibility to explain the over-all catalytic inefficiency of the imidazole group at higher Im contents is the presence of the tertiary structure in the polymer catalyst. If the polymer coil becomes more compact with increasing Im contents because of the higher hydrophobicity of the imidazole unit, the imidazole group buried in the interior region of the polymer coil will be less accessible to the substrate, giving rise to a decrease in the over-all catalytic activity. Existence of the globular form was proposed for undissociated polymethacrylic acid in water,[9-11] and the influence of the intramolecular aggregation of polymers on the their reactivities was observed in several systems.[31,32]

Pseudo-Intramolecular Process. Jencks and Carriuolo[33] showed by the trapping experiments the intermediacy of the N-acyl derivative in the hydrolysis of phenyl esters catalyzed by N-methylimidazole (eq 21).



(21)

Whether or not the bond-breaking process of the catalyst–substrate complex shown in Figure 8 follows the same route is not certain. Two conceivable catalytic roles of the imidazole group are nucleophilic and general base catalyses. The nucleophilic catalysis will follow the same route as eq 21. The general base catalysis requires the presence of solvent water at the catalytic site. Although the substrate molecule is considered to be bound hydrophobically, there are highly polar N-alkylamide groups at the catalytic site and water molecules may be readily bound within the catalytic loop.

One of the characteristics of the enzyme reaction is the high efficiency of the catalytic action. This is in part attributed to the intramolecular nature of the chemical transformation.[34] Bruice and Benkovic[35] found that the ratio of intramolecular and intermolecular rate constants (in M units) was in the order of 10^3 for the phenyl ester hydrolysis catalyzed by the dimethylamino group. However, the unimolecular rate constants, k_3, obtained in the present investigation was not remarkable, although a quantitative comparison cannot be made because the corresponding bimolecular rate constant could not be determined. Several possibilities may be presented to explain the apparent low efficiency of the pseudo-intramolecular catalysis. One of the possibilities is that the geometry of the catalyst–substrate complex is not particularly favorable for catalysis. Another possibility is that substitution at the 1 and 2 positions of the imidazole group lowered the catalytic activity. In fact, the use of a 4(5)-monosubstituted imidazole as a catalytic site seems to give a much higher catalytic activity.[36] Thirdly, the formation of the charged acylimidazole intermediate in the case of nucleophilic catalysis may not be favored in the predominantly hydrophobic site of catalysis. The greater k_3 value observed for copolymer B (0.1 min^{-1}) as compared with copolymer A of low Im contents (0.038 min^{-1}) suggests that the pseudo-intramolecular rate constant is affected considerably by the bound state of the substrate.

In conclusion, copolymers A and B showed the enzyme-like catalysis in the hydrolysis of a phenyl ester. Recently, we found that the enzyme-like catalysis of copolymer A was inhibited competitively by neutral and charged molecules.[37] This result again speaks against the inactivation of the complexed substrate (case II) and supports the validity of the Michaelis–Menten kinetics applied to the present system. The Michaelis–Menten kinetics were also observed for a phenyl ester hydrolysis catalyzed by a naphthylimidazole derivative (a small molecule) in an aqueous system.[36] Thus, the substrate saturation due to hydrophobic interaction can be a general phenomenon. Investigations are being carried out on the catalytic action of different types of imidazole derivatives in these laboratories.

---

(31) S. Yoshikawa, O-K. Kim, and T. Hori, *Bull. Chem. Soc. Jap.*, **39**, 1937 (1966).

(32) I. Sakurada, Y. Omura, A. Omori, and Y. Sakaguchi, *Kobunshi Kagaku*, **24**, 505 (1967).

(33) W. P. Jencks and J. Carriuolo, *J. Biol. Chem.*, **234**, 1272, 1280 (1959).

(34) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. 1, W. A. Benjamin, Inc., New York, N. Y., 1966, p 119.

(35) T. C. Bruice and S. J. Benkovic, *J. Amer. Chem. Soc.*, **85**, 1 (1963).

(36) C. Aso, T. Kunitake, and S. Shinkai, *Chem. Commun.*, 1483 (1968).

(37) T. Kunitake, F. Shimada, and C. Aso, submitted for publication.