Imidazole Catalyses in Aqueous Systems. V. The Enzyme-Like Catalysis in the Hydrolysis of a Phenyl Ester by Copolymers Containing a Benzimidazole Group. Rate Acceleration by Bound Phenols and the Mode of Side Chain Aggregation in Polymer Catalysts

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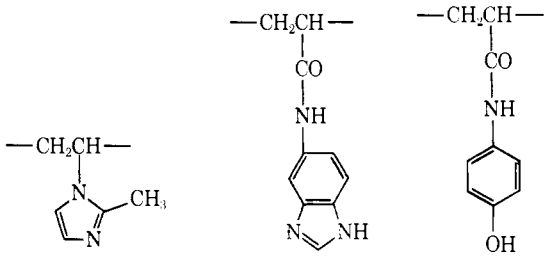
Received July 9, 1970

Abstract: Hydrolyses of *p*-acetoxybenzoic acid catalyzed by water-soluble copolymers of *N*-(5-benzimidazolyl)-acrylamide (BI) were studied at 30° in the neutral pH region in 0.1–1.0 *M* aqueous KCl by employing a pH stat. The catalytic hydrolysis conformed to Michaelis–Menten kinetics as in the enzymatic reaction and formation of the catalyst–substrate complex was ascribable to the hydrophobic interaction. In the catalytic hydrolysis with copolymers of BI and vinylpyrrolidone (VP) were observed rate accelerations due to accumulation of the *p*-hydroxybenzoate anion in the reaction system. Other undissociated phenols similarly accelerated the catalytic rate. These results were explained by the cooperative action of the BI unit and the phenolic compound bound onto the VP unit. In the catalytic hydrolysis with copolymers of BI and acrylamide (AA), the cooperative esterolytic action of the BI unit was not noted in spite of the intramolecular aggregation of the BI unit observed. Terpolymers containing the BI unit and *N*-(*p*-hydroxyphenyl)acrylamide (AP) unit did not show a cooperative catalytic action and the AP unit simply increased hydrophobicity of the catalytic site. The intramolecular aggregation phenomena of these polymer catalysts were discussed on the basis of the hydrophobic nature of monomer units. The modes of aggregation suggested are consistent with the viscometric, potentiometric, and catalytic behavior of the polymer catalysts. The catalytic hydrolysis with 5(6)-acetamidobenzimidazole, a model compound of the BI unit, followed simple second-order kinetics.

Hydrophobic interaction has been recognized to make significant contributions to the structure and activity of enzymes. Therefore, catalytic action of water-soluble polymers with hydrophobic character has been investigated increasingly in recent years as a model of an enzyme system.[1–8] We showed previously that some water-soluble copolymers containing 1-vinyl-2-methylimidazole (MVI) units catalyzed hydrolysis of a phenyl ester according to Michaelis–Menten kinetics as in enzyme reactions. Hydrophobic forces were shown to be responsible for substrate binding,[7] and neutral and charged molecules inhibited the catalysis competitively.[8]

In the present study we selected the benzimidazole group for the catalytic site and prepared water-soluble copolymers from *N*-(5-benzimidazolyl)acrylamide and comonomers. The benzimidazole unit is more hydrophobic than the *N*-methylimidazole group used in the previous study. Therefore it was interesting to study how the increased hydrophobicity of the catalytic group would affect the enzyme-like catalytic behavior of the copolymer.



MVI unit BI unit AP unit

On the other hand, it appears established that the histidine and serine residues are involved in the active site of α-chymotrypsin,[9] and several attempts have been made to attain enhanced catalytic efficiencies in the ester hydrolysis by combinations of the imidazole and hydroxyl functions within a catalyst molecule.[10,11] Thus *N*-(*p*-hydroxyphenyl)acrylamide was copolymerized and the catalytic action of water-soluble copolymers containing the imidazole and hydroxyl functions was investigated. The data obtained are discussed in relation to the rate acceleration phenomena due to bound phenols observed in the vinylpyrrolidone copolymer. *p*-Acetoxybenzoic acid was used as substrate.

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Table I. Preparation of Polymer Catalysts[a]

Copolymer	Monomer, M				Polymerization time, min	Conversion, %	Polymer unit, mol %	
	BI	AP	VP	AA			BI	AP
BI-VP-1	0.034		3.00		30	19.9	2.90	
BI-VP-2	0.020		2.00		30	16.5	1.22	
	0.030		3.00		40	21.8		
BI-VP-3	0.017		3.00		30	20.4	0.91	
	0.017		3.00		30	19.2		
BI-AA-1	0.04			2.00	75	40.0	1.29	
BI-AA-2	0.10			1.00	100	13.8	7.83	
	0.10			1.00	80	16.1		
BI-AP-AA-1	0.02	0.10		1.00	80	15.0	1.4	7.0
BI-AP-AA-2	0.05	0.05		1.00	80	24.4	3.2	3.4
BI-AP-AA-3	0.05	0.02		1.00	80	30.8	3.5	*Ca.* 1.5
AP-AA		0.06		2.00	100	56.0		2.5

[a] 70°; methanol solvent; azobisisobutyronitrile, 0.125 mol % of VP or AA monomer.

Experimental Section

5(6)-Acetamidobenzimidazole. 5-Aminobenzimidazole was prepared by reduction of commercial 5-nitrobenzimidazole with tin and dilute hydrochloric acid. Colorless plates (hydrochloride monohydrate), yield 64–82%, mp 108–109° (lit.[12] 108.5–109°), were formed. Sodium acetate (2.1 g, 0.025 mol) was dissolved at 70° in a mixture of 10 ml of acetonitrile and 50 ml of acetic acid. 5-Aminobenzimidazole hydrochloride monohydrate (2.0 g, 0.013 mol) was dissolved with stirring in this mixture at room temperature. Acetic anhydride (1.5 g, 0.015 mol) was added dropwise and stirring was continued for 2 hr. Ethanol was added in order to decompose excess acetic anhydride. A 100-ml sample of a saturated NaCl solution was added and the mixture was extracted with three 50-ml portions of tetrahydrofuran. Solvent was evaporated from the extract and the tan brown residue was recrystallized twice from a 1:1 mixture of 4 *N* hydrochloric acid and acetic acid: colorless needles, mp 240–250° dec, yield 65–70%. *Anal.* Calcd for $C_9H_9N_3O \cdot HCl \cdot H_2O$: C, 47.06; H, 5.27; N, 18.30. Found: C, 47.04; H, 5.24; N, 19.07. Ir (KBr) showed 1660 (carbonyl), 1477 cm^{-1} (imidazole). The purity determined by titration was 98.6%.

***N*-(5-Benzimidazolyl)acrylamide (BI Monomer).** 5-Aminobenzimidazole (2.0 g, 0.013 mol) was dissolved in a mixture of 2 ml of concentrated hydrochloric acid and 3 ml of water, and then added to 2.5 ml of 30% aqueous alkali and 50 ml of dioxane. The solution was cooled in an ice bath and, with stirring, 4.7 ml of 4 *N* aqueous sodium hydroxide and 1.6 g (0.018 mol) of acrylyl chloride (bp 67–70° (760 mm), prepared from benzoyl chloride and acrylic acid[13]) were simultaneously added from separate dropping funnels over 15 min. The reaction mixture was stirred for 1 hr at pH 8–10 at 0–5° and then neutralized to pH 7. Sodium chloride was filtered and solvent was evaporated. The residual solid was dissolved in methanol, added with 2 ml of concentrated hydrochloric acid, and solvent evaporated. This process was repeated once more, and the residue was recrystallized with charcoal treatment from water or from 1:1 aqueous acetic acid, mp 154–156°, yield 62–72%. *Anal.* Calcd for $C_{10}H_9N_3O \cdot HCl \cdot H_2O$: C, 49.70; H, 5.00; N, 17.39. Found: C, 49.66; H, 4.86; N, 16.49. Ir (KBr) showed 1680 (conjugated carbonyl), 1617 (vinyl), 1418 cm^{-1} (imidazole).

***N*-(*p*-Hydroxyphenyl)acrylamide (AP Monomer).** *p*-Aminophenol (10.9 g, 0.1 mol) was dissolved in 40 ml of acetic acid, and 4.5 g (0.05 mol) of acrylyl chloride was added dropwise at 0–10°. After stirring for 1 hr, 200 ml of 1 *N* hydrochloric acid was added and the organic product was extracted with ether. The extract was dried over sodium sulfate and ether was evaporated. Colorless flakes appeared upon cooling the residue, yield 56–76%, mp 194–195° (lit.[14] mp 192–193°).

***p*-Acetamidophenol** was prepared by treating *p*-aminophenol with acetic anhydride in acetic acid, mp 169–171° (lit.[15] mp 169–170.5°).

***N*-Vinylpyrrolidone (VP)** (bp 90–93° (11 mm)) and **acrylamide (AA)** (mp 83.5–84.5°) were obtained by purification of commercial reagents by distillation and recrystallization, respectively.[7] The preparation of the substrate was described previously.[16,17]

Preparation of Polymer Catalysts. Copolymerizations were carried out in methanol at 70° with azobisisobutyronitrile as initiator in sealed ampoules under nitrogen. Monomers and initiator were charged into an ampoule and the mixture was degassed by the freeze–thaw method. The copolymerization with vinylpyrrolidone proceeded homogeneously. The polymer was recovered by pouring the reaction mixture into excess ether, purified by reprecipitation from methanol and ether, and dried *in vacuo*. In the copolymerization with acrylamide the polymers precipitated during polymerization were collected on a glass filter, reprecipitated from water and methanol (or acetone), and dried *in vacuo*. The polymerization results are given in Table I. These copolymers were soluble in 1 *M* aqueous KCl. Increases in the content of BI and/or AP units resulted in formation of water-insoluble polymers.

The content of the BI unit in copolymer was determined by titration under the hydrolysis condition. The amount of the AP unit was determined by infrared spectroscopy: infrared spectra (KBr disk) of mixtures of *p*-acetamidophenol (model compound of the AP unit) and phthalimide were measured, and the intensity ratio of the aromatic out-of-plane bending vibrations (at 837 cm^{-1} with *p*-acetamidophenol and at 790 cm^{-1} for phthalimide) was plotted against the mole fraction of the mixture. A linear relation was obtained. Corresponding intensity ratios (835 and 790 cm^{-1}) were determined for a mixture of a polymer sample with a known amount of phthalimide, and compared with the calibration curve mentioned above. The molecular extinction coefficients of the AP unit at 835 cm^{-1} and of *p*-acetamidophenol at 837 cm^{-1} were assumed to be identical. The copolymer compositions are included in Table I.

Titration and hydrolysis procedures are the same as described previously.[7,17] The rate of catalytic hydrolysis $v_{cat.}$ was obtained by subtracting the alkali consumption due to spontaneous hydrolysis from the total rate of hydrolysis. The amount of alkali consumption due to hydrolysis per mole of substrate was estimated from pK_a values of the product under the respective hydrolysis condition. The rate of spontaneous hydrolysis was small for this substrate.

Results

Titration and Viscosity Characteristics of Polymer Catalysts. Titration and viscometric behavior of the polymer catalyst are summarized in Table II. Titration data of polyelectrolytes do not follow necessarily the simple mass law relation employed for titrations of small molecules, because of the electrostatic repulsion among charged units.

Thus the logarithm of the intrinsic ionization constant pK_{int} of an isolated site can be estimated by plot-

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Table II. Titration and Viscosity Characteristics

Catalyst	pK_a[a]	n'	pK_{int}[a]	[η],[b] dl/g — 1.0 M KCl	[η],[b] dl/g — 8 M aqueous urea
AcBI	5.41				
BI-VP-1	5.60	1.01	5.61		
BI-VP-2	5.59	1.03	5.62		
BI-VP-3	5.57	0.98	5.55		
BI-AA-1	5.23	1.24	5.58	0.578	0.796
BI-AA-2	5.12	1.56	5.66	0.122	0.215
BI-AP-AA-1	5.25	1.01	5.27	0.229	0.679
BI-AP-AA-2	5.30	1.06	5.35		
BI-AP-AA-3	5.26	1.10	5.49	0.300	0.583

[a] 30°, 1.0 M KCl, experimental error, ±0.03. [b] 30°; a modified Ubbelohde viscometer was used.

ting the logarithm of the apparent ionization constant pK_{app} against degree of neutralization α in eq 1 and

$$pK_{app} \equiv pH + \log[(1 - \alpha)/\alpha] = pK_{int} + 0.43\Delta G_{el}/RT \quad (1)$$

extrapolating to $\alpha = 1$, *i.e.*, to the uncharged state,[18] where ΔG_{el} is the required electrostatic energy for the removal of an equivalent of protons at a given degree of ionization. The plots were considered to be linear, although some curvature was observed in a few cases at $\alpha < 0.5$. Thus it was concluded that noticeable conformational changes did not occur during titration, unlike in the case of poly(methacrylic acid) and poly(α-L-glutamic acid).[18] Similar results were obtained for copolymers of vinylimidazole and vinylpyrrolidone.[7,19]

The titration data were also plotted according to the modified Henderson–Hasselbach equation.[20] The pK_a

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

value corresponds to pH at the half-neutralization. The deviation of n' from unity is a qualitative measure of electrostatic interaction. These values were calculated from the linear plots obtained.

The titration data of Table II suggest several interesting features of the behavior of imidazole groups embedded in polymer. There is no electrostatic effect observed in the BI–VP copolymer ($n' \approx 1$). In contrast, the BI–AA copolymer showed considerable electrostatic effect, and the n' value increased from 1.24 to 1.56, as the content of the BI unit increased from 1.29 to 7.83 mol %. These n' values are unusually large if one takes into account low contents of the BI unit. Incorporation of the AP unit into the BI–AA copolymer decreased the electrostatic effect among the BI units, as is apparent from diminished n' values ($n' < 1.1$). The implication of the viscosity data will be discussed later.

Catalytic Hydrolysis with 5(6)-Acetamidobenzimidazole (AcBI). The initial rate of the catalytic hydrolysis of *p*-acetoxybenzoic acid with AcBI catalyst was proportional to the substrate concentration (0.01–0.07 M). The second-order rate constant (k') was 0.012 $min^{-1} M^{-1}$ (30°, pH 8.0, 1.0 M KCl).

Catalytic Hydrolysis with BI–VP Copolymers. In the catalytic hydrolysis with BI–VP copolymers, typical saturation phenomena of the initial rate were observed with increasing substrate concentration. An example is given in Figure 1. Thus it was concluded that the copolymer catalyzed hydrolysis of the

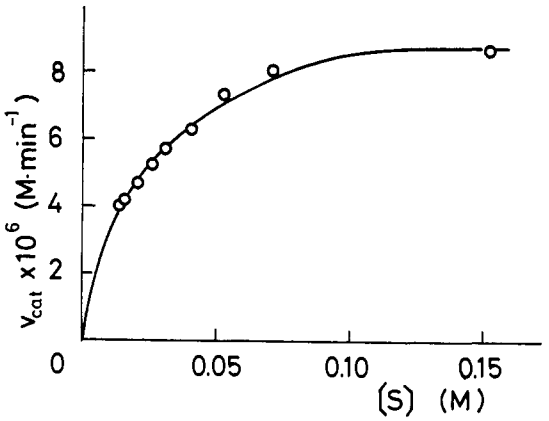
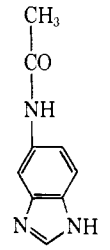


Figure 1. Catalytic hydrolysis: catalyst, BI–VP-3; total imidazole concentration, 1.10 mM; pH 8.0; 30°; 1.0 M KCl.



AcBI

substrate according to Michaelis–Menten kinetics (eq 3 and 4), as in the previous system,[7] where C and

$$\text{catalyst} + \text{substrate} \underset{K_m}{\rightleftharpoons} \text{catalyst} \cdot \text{substrate} \xrightarrow{k_{cat.}} \text{catalyst} + \text{product} \quad (3)$$

$$v_{cat.} = \frac{k_{cat.}[C][S]}{K_m + [S]} \quad (4)$$

S denote catalyst and substrate, respectively.

The kinetic constants, K_m and $k_{cat.}$, were determined from the linear Lineweaver–Burk plot between $1/v_{cat.}$ and $1/[S]$.[21] The Michaelis constant K_m may be assumed to represent a true dissociation constant of catalyst and substrate.[7] The results are given in Table III.

Table III. Catalytic Hydrolysis[a]

Catalyst	[C],[b] mM	K_m,[c] mM	$k_{cat.}$,[c] $min^{-1} \times 10^3$	$k_{cat.}/K_m$, M^{-1} min^{-1}
AcBI	7.57			($k' = 0.012$)
BI-VP-1	0.94	24	9.7	0.40
BI-VP-2	1.20	23	9.2	0.39
BI-VP-3	1.10	23	9.2	0.39
BI-AA-1	1.06	53	19	0.36
BI-AA-2	1.09	77	18	0.23
BI-AP-AA-1	1.70	23	4.3	0.18
BI-AP-AA-2	1.50	31	5.0	0.16
BI-AP-AA-3	2.06	40	5.3	0.11
AP-AA[d]				0.00

[a] Reaction condition: pH 8.0; 30°; 1.0 M KCl. [b] Total imidazole concentration. [c] Experimental error, <5%. [d] No catalytic activity observed.

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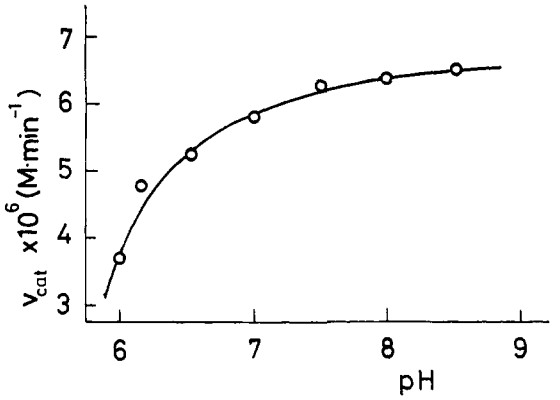

Figure 2. Dependence of the catalytic rate on pH: catalyst, BI-VP-3; total imidazole concentration, 1.10 mM; substrate, 40 mM; 30°; 1.0 M KCl.

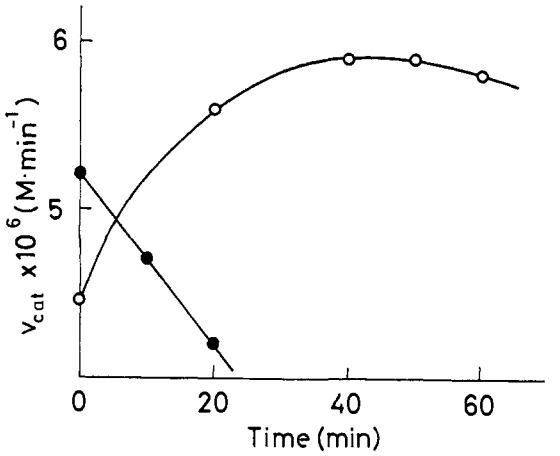

Figure 3. Variation of the catalytic rate with time: catalyst, BI-VP-3; total imidazole concentration, 1.10 mM; substrate, 40 mM; pH 8.0; 30°; 0.10 M KCl; ○, no additive; ●, 0.38 mM p-hydroxybenzoic acid added.

The small variation in the BI–VP composition did not affect the kinetic behavior of the catalyst, and the kinetic parameters for these catalysts were constant within the experimental error.

The catalytic rate decreased with decrease in ionic strength of the medium. For instance, $v_{cat.}$ decreased from 6.4×10^{-6} to 4.1×10^{-6} M min^{-1} ([C] = 1.10 mM, [S] = 0.040 M, pH 8.0, 30°), when the ionic strength (KCl) was decreased from 1.0 to 0.1. $v_{cat.}$ is dependent of K_m and $k_{cat.}$ terms as given in eq 4. Since the reaction of the neutral imidazole group with a substrate molecule ($k_{cat.}$ term) will not be affected directly by the variation of ionic strength, the observed influence of ionic strength may be ascribed to the K_m term. In fact, substrate binding based on hydrophobic forces is expected to increase with increasing ionic strength (*cf.* salting-out of organic substances).

In Figure 2 is shown the variation of $v_{cat.}$ with pH of the reaction system. The total catalyst concentration was kept constant. $v_{cat.}$ increased with pH at the low pH region but reached a limiting value at high pH. The corresponding plot of the neutral benzimidazole fraction *vs.* $v_{cat.}$ was linear. Thus, only the unprotonated benzimidazole unit was effective as catalyst and the presence of the protonated BI unit showed little influence on the catalytic action of the neutral benzimidazole unit toward the anionic substrate.

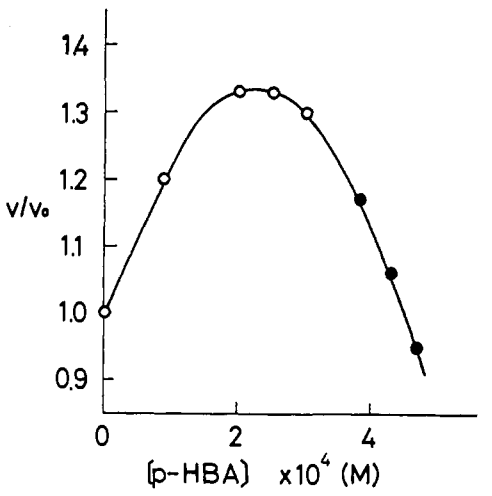

Figure 4. Variation of the relative catalytic rate with the concentration of p-hydroxybenzoic acid in the reaction system: v_0, the catalytic rate in the absence of p-hydroxybenzoic acid. The reaction condition is the same as that of Figure 3.

Acceleration of Catalytic Hydrolysis. In the above experiments, the kinetic behavior was determined from the initial rate of hydrolysis in order to avoid the complex kinetic treatment due to the presence of the product. In most cases, the rate of alkali consumption was constant or decreased slowly with time at small extents of reaction. In the hydrolysis with BI–VP copolymers, however, the rate of alkali consumption initially increased with time, passed through a maximum, and then decreased. This is clearly shown in the variation of $v_{cat.}$ with reaction time (Figure 3). The maximum corresponded to formation of 0.23 mM hydrolysis products. $v_{cat.}$ in this case was determined from the tangent of the alkali consumption curve at the respective reaction time. Since the product concentration was the only variable with time in this system, sodium acetate and sodium p-hydroxybenzoate were added separately to the reaction system. When 0.5 mM sodium acetate was added, the variation of $v_{cat.}$ with time followed the same course (the same initial rate was observed, and maximum $v_{cat.}$ occurred with 0.25 mM product concentration). On the other hand, addition of 0.38 mM sodium p-hydroxybenzoate increased the initial rate by some 16% and the maximum disappeared (Figure 3). This rate variation was replotted in Figure 4 against the concentration of p-hydroxybenzoic acid in the reaction system. The two separate curves in Figure 3 merged into a smooth single curve in Figure 4. Thus it is suggested that the peculiar behavior of $v_{cat.}$ with time was induced by p-hydroxybenzoate ion accumulated in the system. The product concentrations at the maximum rate $[P]_{max}$ are shown in Table IV for the

Table IV. Product Concentration at the Maximum Rate

Polymer	BI unit in polymer, mol %	[P] at v_{max}, $M \times 10^4$	[P] at v_{max}/[C]
BI-VP-1	2.90	4.5	0.48
BI-VP-2	1.22	2.6	0.22
BI-VP-3	0.91	2.4	0.22

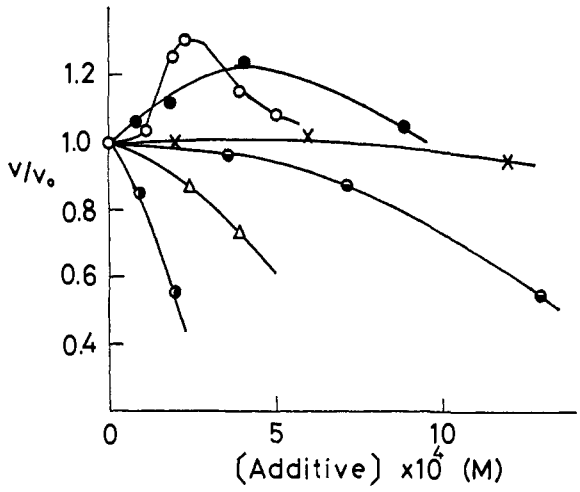

Figure 5. Influence of additives on the initial rate of the catalytic hydrolysis: catalyst, BI-VP-3; total imidazode concentration, 1.10 m*M*; substrate, 40 m*M*; pH 8.0; 30°; 0.10 *M* KCl; ○, phenol; ●, *p*-hydroxybenzoic acid; ×, cyclohexanol; ◒, benzoic acid; △, nitrobenzene; ◑, 2,4-dinitrophenol.

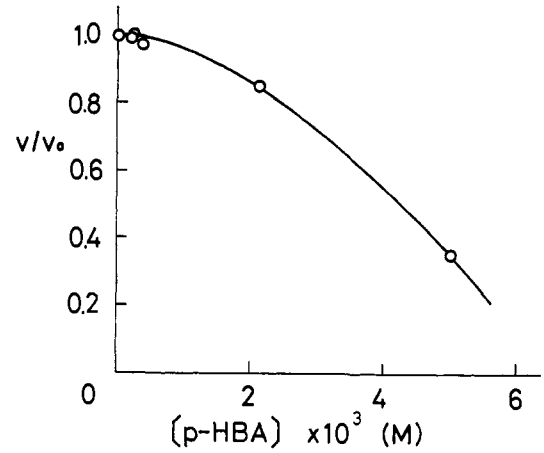

Figure 6. Variation of the relative rate of the catalytic hydrolysis with the concentration of *p*-hydroxybenzoic acid: catalyst, BI-AA-1; total imidazole concentration, 1.06 m*M*; substrate, 40 m*M*; pH 8.0; 30°; 0.10 *M* KCl; v_0, $v_{cat.}$ in the absence of *p*-hydroxybenzoic acid.

three BI-VP copolymers. Interestingly, $[P]_{max}$ increased with the content of the BI unit in copolymer, indicating that the rate acceleration is sensitive to the polymer composition.

In order to define the role of *p*-hydroxybenzoate ion more clearly, the influence of several additives on the initial rate was studied as shown in Figure 5. Addition of phenol and *p*-hydroxybenzoic acid gave rise to rate maxima at certain concentrations, whereas cylohexanol, benzoic acid, nitrobenzene, and 2,4-dinitrophenol simply decreased $v_{cat.}$. 2,4-Dinitrophenol (pK_a = 4.1) is completely dissociated at pH 8.0. Thus it appears that the influence of the additives is closely related to the presence or absence of the undissociated phenol group under the hydrolysis condition. When additive molecules did not contain the undissociated phenol group, they simply decreased the initial $v_{cat.}$. On the other hand, the influence was more complex, when additives contained the undissociated phenol group. The $v_{cat.}$-time curve possessed a maximum when the concentration of these additives was smaller than that at which the rate ratio (v/v_0) was maximal. At greater concentrations of these additives, $v_{cat.}$ decreased with time without showing a rate maximum. For example, the $v_{cat.}$-time curve showed a small rate maximum with 0.4 m*M* added phenol, but $v_{cat.}$ decreased with time with 0.88 m*M* added phenol (*cf.* Figure 5). The same situation is clearly shown for *p*-hydroxybenzoic acid in Figures 3 and 4.

Concerning the additives which did not contain the undissociated phenol group, the *initial* rate decreased with increasing concentrations of additives. However, the rate maximum appeared in the $v_{cat.}$-time curve at the reaction time when 0.20 ± 0.05 m*M* *p*-hydroxybenzoic acid was accumulated in the system. Therefore, in spite of the overall decrease in $v_{cat.}$ due to the presence of inhibitory additives, the rate maximum was induced by accumulation of the certain amount of the product.

Catalytic Hydrolysis with AA Copolymers. The kinetic results for the catalytic hydrolysis with AA copolymers are given also in Table III. The substrate saturation phenomena were less pronounced with the BI-AA copolymers relative to those of the BI-VP copolymers, as is apparent from the difference in K_m values. The intracomplex rate constants $k_{cat.}$ were greater than those of the BI-VP copolymers (19×10^{-3} min^{-1} *vs.* 9.5×10^{-3} min^{-1}). When the AP unit was additionally incorporated into the BI-AA polymer, the binding capacity of the catalyst increased to some extent, but $k_{cat.}$ decreased by a factor of approximately 4. The K_m and $k_{cat.}$ values decreased with increasing contents of the AP unit in the terpolymer. This result shows that the AP unit in the polymer catalyst acted to reinforce the binding site. The phenol group did not show the esterolytic activity by itself, as reflected by the lack of the catalytic activity with the AP-AA copolymer.

The AA copolymers did not give rate maxima in their $v_{cat.}$-time curves, unlike the BI-VP copolymers. Figure 6 shows the influence of added *p*-hydroxybenzoic acid on the initial rate with a BI-AA copolymer (BI-AA-1). There were no rate maxima observed. Thus the rate acceleration due to bound phenols could not be found for the BI-AA copolymer.

Discussion

Intramolecular Aggregation of Polymer Catalysts. It is well known that the tertiary structure of globular proteins is largely maintained by hydrophobic forces.[22] Similarly, synthetic vinyl polymers are expected to show intramolecular aggregation phenomena if hydrophobic groups are properly located along the polymer chain. The aggregation may be likened to formation of the tertiary structure, though in a very crude sense, and it is expected that a new catalytic site of different nature be formed. For example, aqueous poly(methacrylic acid) is known to take a compact conformation in the undissociated state, and unfolding of the polymer chain occurs upon neutralization.[23-27] Eizner, *et al.*,[28,29] dis-

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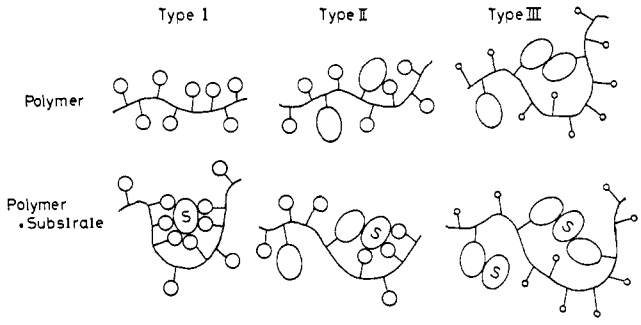

Figure 7. Schematic representations of the mode of intramolecular aggregation and substrate binding: S, substrate. For explanation, see text.

cussed the conformational transition of macromolecules possessing both lyophilic and lyophobic groups. Nakagawa and Inoue[30] developed a statistical theory for the solution state of polysoap and discussed the transition from polyelectrolyte to polysoap, *i.e.*, intramolecular micellization.

The mode of intramolecular aggregation is probably influenced by the distribution and the size of hydrophobic groups. Figure 7 shows schematic representations of typical modes of the intramolecular aggregation of the side chains employed in our hydrolysis studies. The polymer was assumed to be uncharged for the sake of simplicity, and the hydrophobicity of the side chain is shown by circles and ellipses. The type I polymer possesses side chains of intermediate hydrophobicity. The VP and MVI units belong to this class. The type II polymer consists of side chains of intermediate and large hydrophobicity, and the type III polymer consists of side chains of large and small hydrophobicity. The BI, AP, and phenylimidazole (PI, *cf.* ref 31) units may be represented by ellipses (large hydrophobicity), and hydrophobicity of the AA unit is considered to be small (small circle in Figure 7).

Aqueous poly(vinylpyrrolidone) is known to bind various organic substances,[32-34] and we proposed in a previous publication that small organic molecules are surrounded by the polymer segment in the bound state.[7] This loop hypothesis is supported by contraction of polymer upon binding of small molecules and by the constancy (10 ± 3) of the number of the monomer unit required to constitute one binding site.[34] Concentrated aqueous urea is known to unfold globular proteins by destruction of hydrophobic bonds.[35,36] Similarly the intramolecular aggregation of side chains due to hydrophobic forces would be destroyed in concentrated urea solutions. Since the intrinsic viscosity of an aqueous solution of poly(vinylpyrrolidone) did not increase by addition of 8 *M* urea,[37,38] this polymer does not appear to be intramolecularly associated in the absence of substrate molecules. Therefore, the formation of the catalytic loop may find an analogy in the "induced fit" conformational change[39] of enzyme molecules.

In the type II polymer a large hydrophobic group is conceived to be associated with the neighboring intermediately hydrophobic group because of the positional advantage. The direct aggregation of the large hydrophobic groups appears unlikely in this type. The local aggregation of the side chains (Figure 7, type II) is not expected to lead to appreciable contraction of the polymer coil. A substrate molecule will interact with both types of the side chain, although the contribution of the intermediate group to substrate binding is probably smaller than that in the type I polymer.

The electrostatic effect was not observed in the titration of BI–VP copolymers and PI–VP copolymers.[31] In addition, the intrinsic viscosity of an aqueous copolymer of *N*-cyclohexylacrylamide and vinylpyrrolidone did not change upon addition of 8 *M* urea.[38] Although the corresponding viscosity data for the BI–VP polymer are not presently available, these results are consistent with the above-mentioned presumption that direct association of large hydrophobic groups (BI or PI) are not appreciable and aggregation of the local hydrophobic groups is present in an aqueous solution of the type II polymer.

Okubo and Ise[40] recently showed that aqueous poly(acrylamide) did not bind naphthalene or biphenyl in contrast to aqueous poly(vinylpyrrolidone). Thus the binding capacity of the AA unit (shown by small circles) due to hydrophobic forces is considered to be negligible. In the type III polymer, therefore, aggregation would occur among large hydrophobic groups, and the extent of aggregation would increase as its content increases. When the aggregation occurs between side groups far apart along the polymer chain, the polymer coil should contract considerably. The intrinsic viscosity of the BI–AA and PI–AA copolymers[31] increased appreciably in 8 *M* aqueous urea. Moreover, the increment increased with the content of the BI unit in copolymer (Table II). Similarly, a copolymer of *N*-cyclohexylacrylamide (10 mol %) and acrylamide showed an increase in the intrinsic viscosity from 0.78 dl/g (aqueous solution, 30°) to 1.87 dl/g in 8 *M* aqueous urea.[38] These data support the mode of association depicted in Figure 7. The electrostatic effect observed in the BI–AA copolymer, when compared with its absence in the BI–VP copolymer, also suggests the occurrence of the direct interaction between charged BI units. The greater n' value and the greater viscosity increment observed for BI–AA-2 relative to those in BI–AA-1 would indicate that the extent of the intramolecular aggregation increases with increasing BI contents. The intramolecular association of this type offers a very interesting possibility of bifunctional catalysis. By incorporation of the AP unit into the BI–AA system, the electrostatic effect almost disappeared, *i.e.*, $n' \approx 1$. Since the intrinsic viscosity of the terpolymers increased

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appreciably in 8 M aqueous urea (see Table II), the intramolecular aggregation was not lost and aggregation among the BI unit in the BI–AA copolymers was replaced by that between the BI and AP units in the terpolymer. In this type of polymer catalysts a substrate molecule will bind on a single side chain or on a site composed of more than one side chains, and the binding capacity of the polymer catalyst will vary with the content of the hydrophobic group because these two types of the binding site are expected to show different binding capacities.

It is to be noted at this point that the modes of aggregation of Figure 7 were proposed for polymers with relatively short side chains. The size of substrate molecules is similarly assumed to be relatively small. It is readily expected that the mode of aggregation (and binding) would be different when polymer catalysts and/or substrates with long alkyl chains were employed.

Michaelis–Menten Kinetics. It is shown in the Results that all the benzimidazole-containing polymers catalyzed the hydrolysis of *p*-acetoxybenzoic acid according to Michaelis–Menten kinetics. That is, the BI unit in the copolymer, but not AcBI catalyst, possesses sufficient binding capacity toward the substrate molecule under the hydrolysis conditions used. The binding capacity is considered to be intrinsically based on the hydrophobic interaction between catalyst and substrate. However, the binding tendency may as well be strongly influenced by the dipolar interaction.[7] Recently, Connors and coworkers[41] concluded that the molecular complexes of theophylline with cinnamate esters were formed in aqueous solution in such a way as to permit extensive local dipole and induced dipole interactions in the face-to-face orientation. The flat benzimidazole ring appears to be favorable for the face-to-face orientation of the substrate molecule, and this orientation may not necessarily favor the chemical interaction of the imidazole unit with the ester group.

As mentioned previously, Michaelis–Menten kinetics are observed as long as the catalyst–substrate complex is formed, irrespective of whether or not the enzyme-like pathway (eq 3) is more efficient than the bimolecular pathway.[7] The relative importance of these two pathways can be determined by comparing the $k_{cat.}/K_m$ term with the hypothetical second-order rate constant of the catalytic system. AcBI is considered to be a suitable model compound of the isolated BI unit in the polymer catalyst. As is evident from Table III, the catalytic efficiency of AcBI ($k' = 0.012\ M^{-1}\ min^{-1}$) was much smaller than those of the polymer catalysts ($k_{cat.}/K_m = 0.1–0.4\ M^{-1}\ min^{-1}$). Thus it is concluded, for the polymer catalysts used, that the enzyme-like pathway is more efficient than the possible bimolecular pathway.

The kinetic parameters and the titration characteristics were the same with the three BI–VP copolymers. The variation of the composition of these copolymers appears not to affect the nature of the catalytic site noticeably, although more subtle influence may be present as shown by the data of Table IV.

Overberger, *et al.*, observed an enhanced esterolytic activity of poly(vinylbenzimidazole) relative to benzimidazole and explained the results in terms of the cooperative action of the polymeric imidazole group in the overall second-order kinetics.[42–44] It is similarly expected that the intramolecular association of the imidazole group in the type III polymer might cause an enhancement of the catalytic efficiency due to the formation of a cooperative multifunctional catalytic site. However, the increase in the extent of association of the BI unit as inferred from the titration and viscosity data (BI–AA-1 *vs.* BI–AA-2) did not affect $k_{cat.}$ and unexpectedly decreased the binding capacity (K_m, 53 mM *vs.* 77 mM, Table III). Therefore, BI units may be associated in the catalytic site in a conformation as to be not suitable for the cooperative action. More specifically, a plane-to-plane conformation of the associated BI units would certainly not be suitable for creation of a cooperative multifunctional catalytic site.

In the previous study MVI–VP copolymers (low MVI contents) showed an approximately seven times greater capacity than an MVI–AA copolymer. This difference may be considered to be a measure of the contribution of the VP unit in a catalytic loop to substrate binding (type I polymer). As the binding capacity of the catalytic unit increases, the interaction of a bound substrate with the catalytic unit will increase and, therefore, the contribution of the neighboring VP units to binding relative to that of the catalytic unit will decrease. This is confirmed by the smaller ratio of K_m values for the BI–VP and BI–AA copolymers ($2 \sim 3$), compared to the ratio (~ 7) of MVI–VP and MVI–AA copolymers. Therefore, the catalytic loop of the type I polymer can be said to be tighter than that of the type II polymer. In the PI polymers this ratio was about 4, probably indicating a smaller contribution of the PI unit relative to the BI unit to the overall binding capacity.

In the absence of the cooperative character in the catalytic site of the BI–AA polymer, a greater $k_{cat.}$ value for this polymer relative to those for the BI–VP polymers indicates that the product formation on the surface of the BI unit is faster than that proceeding in the catalytic loop of the BI–VP polymer. The intracomplex process ($k_{cat.}$) was much slower than would be anticipated from the rate of the intramolecular catalysis by the imidazole group,[45] and this can be ascribed to destabilization of the highly polar transition state of the product formation in hydrophobic environments of the catalytic site. As will be described in the accompanying publication,[31] the intracomplex rate is inversely correlated with the binding capacity which is based on hydrophobic forces.

Intracomplex Pathway. It has been shown that, in the reaction of benzimidazole with *p*-acetoxybenzoic acid, the path through *N*-acetylbenzimidazole accounts for at least 90% of the conversion of ester to hydrolytic products.[46,47] Probably this applies to the bimolecular

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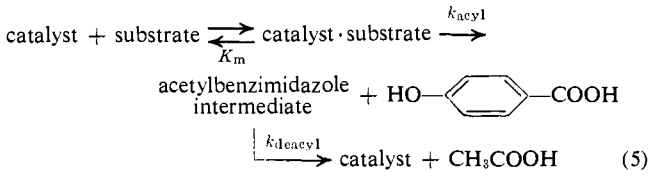
(47) T. C. Bruice and S. J. Benkovic, see ref 46, Chapter 1.

catalysis by acetamidobenzimidazole. However, since there is no assurance that the intracomplex reaction follows the same route as that of the bimolecular path, the general base catalysis of the BI unit remains as a mechanistic possibility.

In the case of general base catalysis, the BI unit acts as a true catalyst, and $k_{cat.}$ is identical with the turnover constant. On the other hand, in the case of nucleophilic catalysis, the intracomplex process involves acylation and deacylation steps



(5)

Then the kinetic parameters observed will be expressed by[48]

$$k_{cat.} = \frac{k_{acyl}k_{deacyl}}{k_{acyl} + k_{deacyl}} \quad (6)$$

$$K_m = K_s\frac{k_{deacyl}}{k_{acyl} + k_{deacyl}} \quad (7)$$

where K_s, substrate constant, is the equilibrium constant of dissociation of the catalyst–substrate complex into catalyst and substrate.

In the present study, the kinetic constants were determined from the initial rate, $v_{cat.}$. If deacylation is very slow with respect to acylation in the case of nucleophilic catalysis, $v_{cat.}$ may represent the rate of acylation. That this is not the case is readily seen from the amount of acid released. For instance, in column no. 4 (BI–VP-3) of Table III, the amount of the BI unit in the reaction system (25 ml) was 2.75×10^{-5} mol ($= 1.10 \times 10^{-3}\ M \times 25$ ml/1000 ml). Assuming the quantitative acylation of the BI unit, the amount of alkali required to neutralize *p*-hydroxybenzoic acid released is 1.65×10^{-6} mol ($= 2.75 \times 10^{-5}\ M \times 0.06$, the degree of dissociation of the phenol group is 6% at pH 8.0, and the carboxyl group has been neutralized prior to addition of catalyst). The hydrolysis was usually followed up to $5 \sim 15 \times 10^{-5}$ mol of alkali consumption, and the alkali consumption increased with time linearly or with slight downward or upward curvature. Therefore, the amount of alkali added is much greater than that expected from acylation alone, and it is concluded that $v_{cat.}$ determined from the slope of alkali consumption curves represents the catalytic (turnover) rate. The same conclusion was obtained for the catalysis of the model compound, AcBI.

Although the rates of acylation and deacylation cannot be evaluated separately at present, the linearity of the alkali consumption curves and greater efficiencies of the polymer catalysts relative to that of the model compound suggest that k_{deacyl} is greater than k_{acyl}. The observation of second-order kinetics for the catalytic hydrolysis with acetamidobenzimidazole (rate determined from alkali consumption) preclude that deacylation is rate determining for this system. The same appears to be true for the polymer catalyst, though further studies are needed for confirmation.

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Rate Enhancement Due to Bound Phenols. In the catalytic hydrolysis with the BI–VP polymers, rate accelerations were induced by accumulation of phenolic compounds in the reaction system. In contrast, several other additives decreased the catalytic rate. The binding constants of some of these additives with poly(vinylpyrrolidone) have been determined by equilibrium dialysis (30°, 0.1 *M* NaCl):[34] phenol, 14.5 M^{-1}; sodium *p*-hydroxybenzoate, 69 M^{-1}; sodium benzoate, 33 M^{-1}; nitrobenzene, 88 M^{-1}. These values are in the same range as the binding constant of substrate with the BI–VP polymer (K_m^{-1} = 41–43 M^{-1}, 30°, 1.0 *M* KCl). Interestingly, the order of the influence of additives on $v_{cat.}$ is correlated with their binding tendencies with aqueous poly(vinylpyrrolidone) given above, irrespective of whether the influence is positive (rate acceleration) or negative (rate depression): *p*-hydroxybenzoate is more effective than phenol in rate enhancement and the rate-depressing influence of nitrobenzene is stronger than that of benzoic acid. These correlations and the closeness of the binding constant of substrate and additives strongly indicate that both acceleration and depression of rates were caused by substances bound on the polymer chain. Inhibition of the catalytic hydrolysis with several neutral and charged molecules was previously observed in a related system,[8] and, therefore, the rate-depressing additives can be assumed to inhibit the catalytic hydrolysis in a competitive fashion.

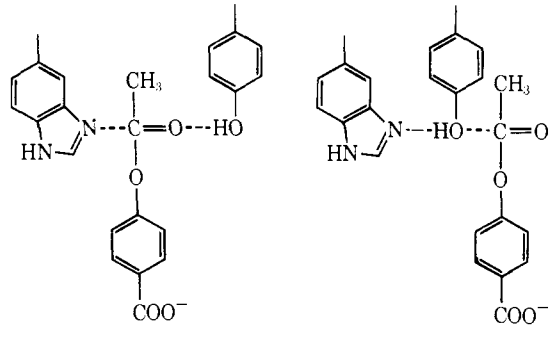
In a catalytic pathway of the Michaelis–Menten type, the rate enhancement due to additives may be caused by the increase in the amount of the Michaelis complex and/or by the increase in $k_{cat.}$. Since both enhancement and depression were observed in the presence of additives, the rate enhancement cannot be attributed to the increase, if any, in the amount of the catalyst–substrate complex, and the rate of the intracomplex product formation must have been affected by phenolic compounds bound on the polymer chain. The rate acceleration was not observed in the catalytic hydrolysis with the BI–AA polymers, where *p*-hydroxybenzoic acid simply showed an inhibitory action (Figure 6). Therefore, phenolic compounds directly bound with the BI unit cannot be involved in the rate enhancement and the noncatalytic binding site (VP sequence) must play an essential role.

We now consider that the acceleration was caused by participation in the product-forming step of the phenolic sustances bound *near* the catalytic site. With the BI–VP polymer, the substrate molecule is bound in the catalytic loop and surrounded by the BI unit and the neighboring VP units simultaneously. The catalytic loop of the BI–VP copolymer can be considered to be loose (see above), and an additive molecule may be able to bind at a site located close to the catalytic site. If the additive molecule contains the phenolic function, imidazole and phenol functions may cooperatively act on the bound substrate. This means that substrate and additive molecules coexist in an expanded catalytic loop. This situation is represented schematically in Figure 8. The phenolic additives can also be bound directly at the BI unit and, in this case, only the inhibitory effect will be observed. The influence of phenolic additives on $v_{cat.}$ (Figures 4 and 5) can be explained by a combination of these two effects. At low

additive concentrations, rate-enhancing contribution of the additive bound near the catalytic site prevails. The catalytic site becomes occupied with the additive molecule increasingly with increasing concentrations of the additive, and rate depression ensues. From the binding constant given above, the total amount of the bound additive at the rate maximum was estimated to be 20–25 mol % of the BI unit present.

As the tightness of a catalytic loop (contribution of the VP unit to substrate binding) increases, formation of an expanded catalytic loop becomes difficult. This is probably the reason why the rate acceleration was not observed with the MVI–VP and PI–VP polymers.[31]

The benzimidazole group may catalyze the hydrolysis of the substrate as a general base or, more preferably, nucleophilic catalyst as mentioned above. Therefore, the properly located phenol group may be involved in the catalytic process as a general acid catalyst (**1**) or as an imidazole-assisted nucleophilic catalyst (**2**). This



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cooperative catalytic effect is mechanistically somewhat different from the cooperative action of benzimidazole and phenolate anion on phenyl ester assumed in the overall second-order process by Overberger, *et al.*[11]

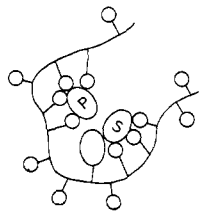

Figure 8. Schematic representation of an expanded catalytic loop accommodating substrate and phenol: S, substrate; P, bound phenol. For explanation, see text.

Absence of the Cooperative Action in the Terpolymer. From the above discussion, it is expected that suitably located imidazole and phenol functions in the polymer chain show a cooperative esterolytic effect. However, when the esterolytic action of the BI–AP–AA terpolymer was examined, the result was disappointing.

The AP unit in the terpolymer increased the binding capacity and at the same time decreased $k_{cat.}$ (Table III). These changes are explicable by increased hydrophobicity of the catalytic site as shown in the correlation of the binding tendency and the intracomplex rate.[31] It is interesting that bound phenols are effective for the cooperative action whereas the pendent phenol group is not. The conformation of the catalyst–substrate complex, the spatial arrangement of the substrate molecule and the catalytic groups, may influence the occurrence of cooperativity. At present we do not know how to construct intentionally cooperative catalytic sites.

Acknowledgment. We are very grateful to Professor Chuji Aso, head of our research group, for his unfailing encouragement and advice.