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Enzyme and Nonenzyme Hydrolyses of Pendent Ester Units in the Copolymer of p-Nitrophenyl Methacrylate and Acrylamide

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ABSTRACT

The enzyme and nonenzyme hydrolyses of the pendent ester groups in the copolymer of p-nitrophenyl methacrylate and acrylamide [poly(p-NPMA/AAm)] were kinetically investigated. The pendent ester groups in poly(p-NPMA/AAm) were found to be cleaved by esterase and α-chymotripsin but were not cleaved by lipase. The catalytic ability of esterase to hydrolyze the ester groups in the copolymer was very smaller than that in p-nitrophenylacetate (p-NPA), which is a monomeric model. The maximal velocity for the esterase-catalyzed hydrolysis of ester groups in poly(p-NPMA/AAm) increased with an increase in the mole fraction of AAm units in poly-(p-NPMA/AAm), while the Michaelis constant was almost independent of the composition of the copolymer. The pendent ester groups in poly(p-NPMA/AAm) are known to be strongly hydrolyzed by alkaline solution but only slightly hydrolyzed by acidic solution. The alkaline hydrolysis velocity of poly(p-NPMA/AAm) was larger than that of monomeric p-NPA, and it was enhanced by increasing the mole fraction of AAm in poly(p-NPMA/AAm).

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INTRODUCTION

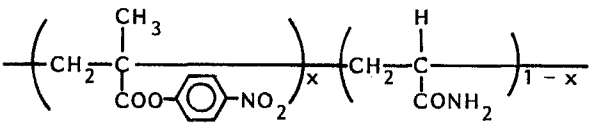
In order to provide polymeric drugs expected to give long-lasting pharmacological activity, we previously synthesized acryloyl-type polymers containing 5-fluorouracil (5-FU) residue through D-glucofuranose [1]. We are now interested in the release of 5-FU residues from such an acryloyl-type polymer.

As a model of hydrolysis of ester groups in the water-soluble acryloyl-type polymer, the present paper is concerned with the enzyme and nonenzyme hydrolyses of the ester groups contained in the copolymer of p-nitrophenyl methacrylate (p-NPMA) and acrylamide (AAm) [poly(p-NPMA/AAm)]. Here, the ability to hydrolyze poly(p-NPMA/AAm) is compared with that of monomeric p-nitrophenylacetate (p-NPA). Moreover, the effects of the composition of substrate poly(p-NPMA/AAm) on the velocity of hydrolysis of ester groups are studied.

EXPERIMENTAL

Materials

p-NPMA was prepared from p-nitrophenol and methacryl chloride according to the method of Morawetz et al. [2]. The copolymerization of p-NPMA with AAm was carried out in dioxane at 60°C using α,α-azobisisobutyronitrile as the initiator. After reaction, the contents were poured into a large amount of methanol to precipitate poly(p-NPMA/AAm). The copolymer obtained was purified three times by reprecipitation from dioxane solution with methanol. The copolymer compositions were determined on the basis of the optical density at 270 nm ($\epsilon_{270} = 1.01 \times 10^4$) for p-NPA. The following five kinds of poly(p-NPMA/AAm) were used as the substrates.



poly(p-NPMA/AAm)

where X = 20.0, 17.8, 14.5, 8.5, and 6.2.

p-NPA was of reagent commercial grade and used without further purification.

Esterase, α-chymotripsin, and lipase, used as enzymes for the hydrolysis of the ester bond, were the commercial dry type; Sigma

Chemical Co. type I was from porcine liver, Sigma Chemical Co. type II was from bovine pancreas, and Sigma Chemical Co. type VII was from Candia cylindracea and used without purification.

Buffer solutions were made up with special grade reagents and ion-exchanged distilled water. The pH of the reaction mixture was obtained by measurements with a Hitachi Horiba Model M-5 pH meter.

Kinetic Measurements

The alkaline hydrolysis of the ester groups in substrate by 0.05 M tris-HCl buffer solution was carried out in a quartz cell at 37°C, keeping the concentration of KCl constant at 0.15 M. The extent of hydrolysis was determined by continuously measuring the following optical density at 400 nm for p-nitrophenol produced by the ester group cleavage:

pH	7.05	7.50	7.70	8.00	8.50	8.95
ϵ_{400}	1.08×10^4	1.55×10^4	1.70×10^4	1.83×10^4	1.93×10^4	1.98×10^4

The enzyme hydrolysis of the ester groups of substrates was also carried out in a quartz cell at 37°C, keeping the pH of aqueous solution constant at 6.50 by 0.05 M $NaHPO_4$-Na_2HPO_4 buffer solution. The enzyme was charged in the sample cell but not in the reference cell. The extent of initial hydrolysis was determined by continuously measuring the optical density at 318 nm ($\epsilon_{318} = 9.85 \times 10^3$ for p-nitrophenol) by means of a differential spectrophotometer.

The electronic absorption spectra were measured with a Simadzu double beam spectrophotometer UV-240 type.

RESULTS AND DISCUSSION

Nonenzyme Hydrolysis of Ester Groups in the Substrates

The alkaline hydrolyses of the ester groups in poly(p-NPMA 17.8/AAm 82.2) and p-NPA were done in 0.05 M tris-HCl buffer solution of pH 7.7. The results obtained are shown in Fig. 1. The hydrolysis rate of ester groups in poly(p-NPMA 17.8/AAm 82.2) was found to be larger than that in p-NPA. In order to know in detail the alkaline hydrolyses, the pseudo-first-order rate constants, k_1, for the alkaline hydrolyses of the ester groups in four kinds of poly(p-NPMA/AAm) and monomeric p-NPA were obtained in buffer solutions ranging in

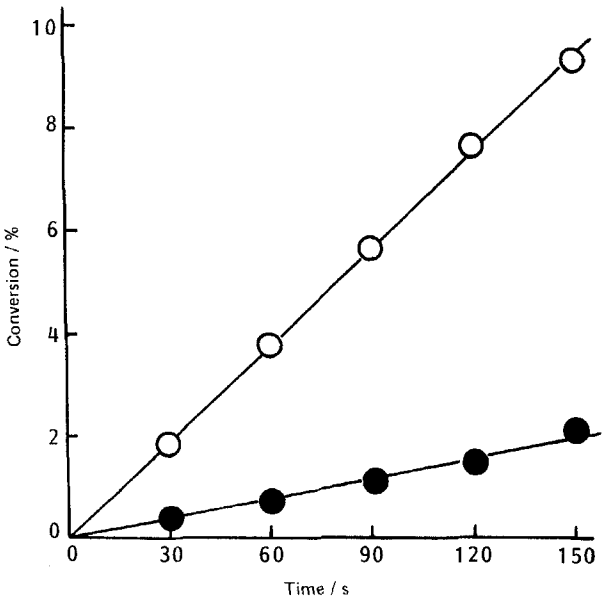


FIG. 1. Alkaline hydrolyses of poly(p-NPMA 17.8/AAm 82.2) and p-NPA in buffer solution at 37°C. Buffer solution: 0.05 M tris-HCl, pH 7.70. (○) Poly(p-NPMA 17.8/AAm 82.2) = 5.00×10^{-5} M of ester unit. (●) p-NPA = 5.00×10^{-5} M.

hydroxy anion, $[OH^-]$, concentration from 1.1×10^{-7} to 1.0×10^{-6} M. Plots of k_1 versus $[OH^-]$ are shown in Fig. 2, and they indicate, by application of Eq. (1) [3], that a good linear relationship exists in all cases.

$$k_1 = k' + k_{OH^-}[OH^-] \quad (1)$$

The second-order rate constants, k_{OH^-}, for the OH^--promoted hydrolysis were calculated from the slopes of the straight lines and are listed in Table 1. The k_{OH^-} values obtained for the OH^- ion indicated hydrolysis of the ester groups in the series poly(p-NPMA/AAm) was very much larger than the k_{OH^-} value for p-NPA. Moreover, the k_{OH^-} value tended to increase with an increase in mole fraction of AAm units in poly(PNPMA/AAm). In the other experiment, the alkaline hydrolysis of p-NPA was shown not to be affected by the addition of poly(AAm).

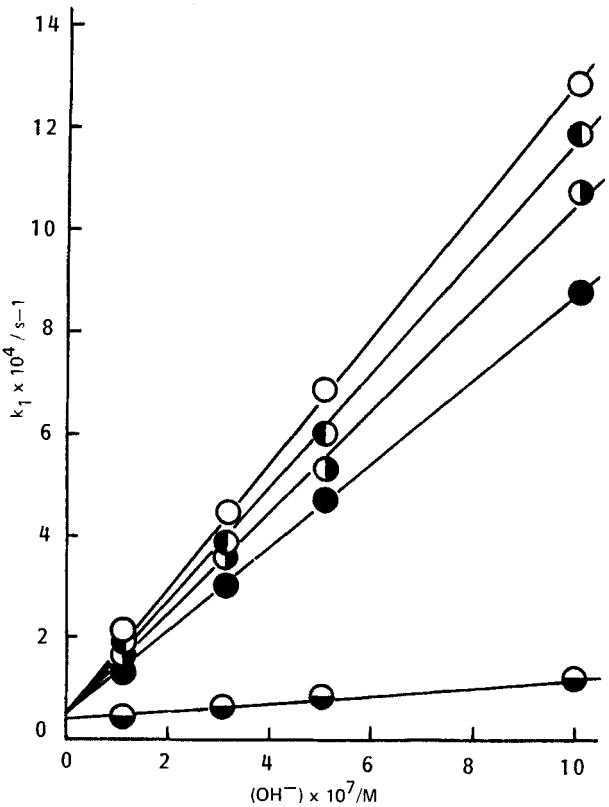


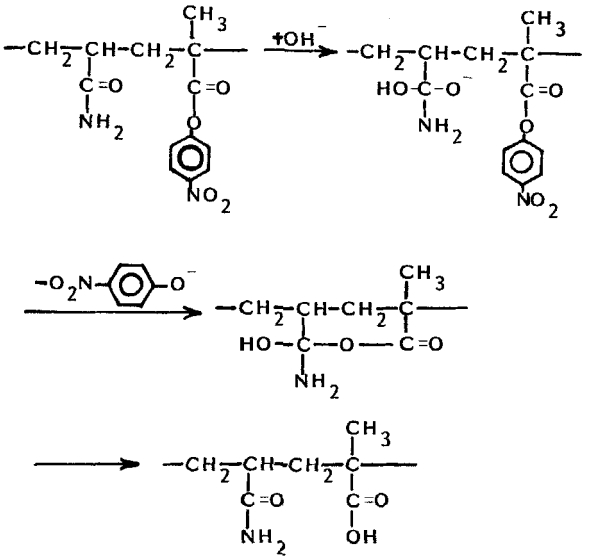
FIG. 2. Plots of k_1 versus $[OH^-]$. Buffer solution: 0.05 M tris-HCl in 0.15 M KCl, 37°C. Poly(p-NPMA 6.2/AAm 93.8) = 4.32 (○), poly(p-NPMA 8.5/AAm 91.5) = 4.57 (◐), poly(p-NPMA 14.5/AAm 85.5) = 2.86 (◑), poly(p-NPMA 20.0/AAm 80.0) = 2.92 (●), p-NPA = 4.43 (◒) × 10^{-5} M of ester unit.

TABLE 1. Rate Constants for the OH^- Promoted Hydrolyses of Poly(p-NPMA/AAm) and p-NPA in Buffer Solution[a]

Substrate	$10^5 \cdot [S]_0$ (M of ester unit)	k_{OH^-}[b] ($M^{-1}\ s^{-1}$)
Poly(p-NPMA 6.2/AAm 93.8)	4.32	1.30×10^3
Poly(p-NPMA 8.5/AAm 91.5)	4.57	1.14×10^3
Poly(p-NPMA 14.5/AAm 85.5)	2.86	1.03×10^3
Poly(p-NPMA 20.0/AAm 80.0)	2.92	8.54×10^2
p-NPA	4.43	7.46×10^1

[a] 0.05 M tris-HCl in 0.15 M KCl; pH = 7.05-8.95; 37°C.
[b] $k_1 = \overline{k'} + k_{OH^-}[OH^-]$.

Therefore, the large alkaline hydrolysis reactivity of poly(p-NPMA/AAm) can be explained by the neighboring-group assistance of the AAm unit, which is written in the following mechanism [4]:



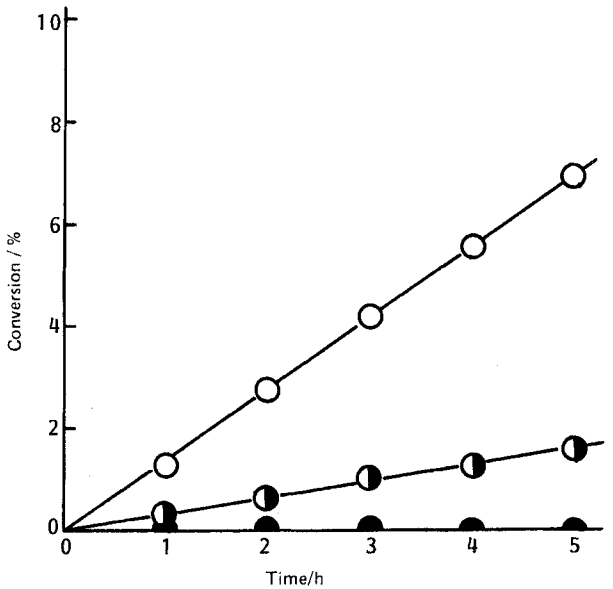


FIG. 3. Enzyme hydrolysis of poly(p-NPMA 17.8/AAm 82.2) in buffer solution at 37°C. Buffer solution: 0.05 M NaH_2PO_4-Na_2HPO_4, pH 6.5. Poly(p-NPMA 17.8/AAm 82.2) = 5.00×10^{-5} M of ester unit. Lipase = 3.13×10^5 (●), α-chymotrypsin = 4.96×10^3 (◑), esterase = 3.49×10^3 (○) unit/L.

Enzyme Hydrolysis of Ester Groups in the Substrates

First, the enzyme hydrolyses of poly(p-NPMA 17.8/AAm 82.2) were investigated using esterase, α-chymotripsin, and lipase as enzymes. The results obtained are shown in Fig. 3. It was clear that the ester groups of poly(p-NPMA/AAm) were very slowly hydrolyzed by esterase and α-chymotripsin but not by lipase; the specificity of the enzyme was recognized. Thus, the effective esterase was used as an enzyme in subsequent reactions.

Figure 4 shows the Lineweaver-Burk plots for the esterase-catalyzed hydrolyses of four kinds of poly(p-NPMA/AAm). The results in Fig. 4 suggest that these hydrolyses proceeded through the following Michaelis-Menten type of mechanism [5]:

$$E + S \underset{k_1^{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

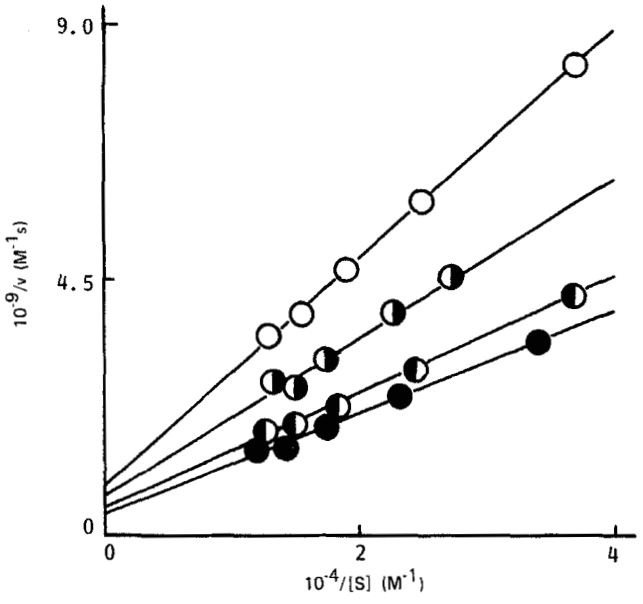


FIG. 4. Lineweaver-Burk plots for the esterase-catalyzed hydrolysis of poly(p-NPMA/AAm). The reactions were carried out in buffer solution at 37°C. Buffer solution: 0.05 M NaH_2PO_4-Na_2HPO_4, pH 6.5. $[E]_0 = 3.49 \times 10^3$ unit/L. (○) Poly(p-NPMA 17.8/AAm 82.2), (◑) poly(p-NPMA 14.5/AAm 85.5), (◐) poly(p-NPMA 8.5/AAm 91.5), (●) poly(p-NPMA 6.2/AAm 93.8).

where E, S, ES, and P are the enzyme, the substrate, the enzyme-substrate complex, and the product of the enzyme reaction, respectively.

Moreover, K_m and V_{max} are expressed by the following equations:

$$K_m = (k_{-1} + k_2)/k_1$$

$$V_{max} = k_2[E]_0$$

From the intercepts on the ordinate at zero and from the slopes of the straight lines, the maximal velocities, V_{max}, and the Michaelis constants, K_m, for poly(p-NPMA/AAm) were calculated. They are summarized together with those for p-NPA in Table 2. From this table it is found that the V_{max} and K_m values for poly(p-NPMA/AAm)

TABLE 2. Esterase-Catalyzed Hydrolyses of Poly(p-NPMA/AAm) and p-NPA[a]

Substrate	$10^5 \cdot [S]$ (M of ester unit)	$10^4 \cdot K_m$ (M)	$10^9 \cdot V_{max}$ (M s^{-1})
Poly(p-NPMA 6.2/AAm 93.8)	7.33-2.60	2.36	2.26
Poly(p-NPMA 8.5/AAm 91.5)	7.53-3.65	2.20	2.21
Poly(p-NPMA 14.5/AAm 85.5)	7.90-2.72	2.11	1.52
Poly(p-NPMA 17.8/AAm 82.2)	7.60-2.70	2.32	1.18
p-NPA	5.09-2.52	0.961	3.13

[a] $[E]_0 = 3.49 \times 10^3$ unit/L in 0.05 M NaH_2PO_4-Na_2HOP_4 buffer solution (pH = 6.5); 37°C.

are about 1/1000 times and twice as much as those for p-NPA, respectively. These results suggest that the stability of the complex consisting of esterase and poly(p-NPMA/AAm) was lower owing to the steric hindrance of polymer chain than that consisting of esterase and monomeric p-NPA, and the decomposition of the complex to the product for the copolymer was remarkably depressed compared to that for the monomeric compound. In addition, it is known from Table 2 that the K_m values for poly(p-NPMA/AAm) are almost constant at $2.11\text{-}2.36 \times 10^4$ M regardless of their composition, and the V_{max} values for poly(p-NPMA/AAm) increased with an increasing mole fraction of AAm units in the copolymer substrate. The decomposition rate of the complex to the product was also suggested to be accelerated by the introduction of hydrophilic AAm units into the polymer substrate.

Additive Effects of Poly(AAm), Poly(MAA), and Poly(MAA/AAm) on the Esterase-Catalyzed Hydrolysis of Substrates

In order to investigate the effects of the comonomer AAm unit in poly(p-NPMA/AAm) and the methacrylic acid (MAA) unit produced by hydrolysis of the esterase-catalyzed hydrolysis of the substrate, the hydrolyses of monomeric p-NPA were carried out in the presence or absence of poly(AAm), poly(MAA), and poly(MAA 6.2/AAm 93.8). The results obtained are shown in Fig. 5. The parameters of V_{max} and K_m, calculated from Lineweaver-Burk plots, are shown in Table 3. It was found from this table that the hydrolysis of p-NPA was depressed by poly(MAA) and poly(MAA/AAm) but not by poly(AAm). Moreover, it is suggested that the enzyme reaction proceeded through a complete noncompetitive inhibition mechanism, because the K_m value obtained was constant [6].

Additive effects on the esterase-catalyzed hydrolysis of poly(p-NPMA/AAm) were subsequently studied and the results obtained are shown in Figs. 6 and 7. These figures show that the hydrolysis of poly(p-NPMA/AAm) was completely inhibited by the addition of poly(MAA) or poly(MAA/AAm) but was not affected by poly(AAm).

From the results obtained above, it is concluded that the ester units pending in the water-soluble methacryloyl-type polymer were hydrolyzed by the alkaline solution but not by the acidic solution, and they were very gradually hydrolyzed by enzymes such as esterase and lipase. Furthermore, it is concluded that in order to design a water-soluble methacryl-type polymeric drug, AAm is appropriate for use as a comonomer.

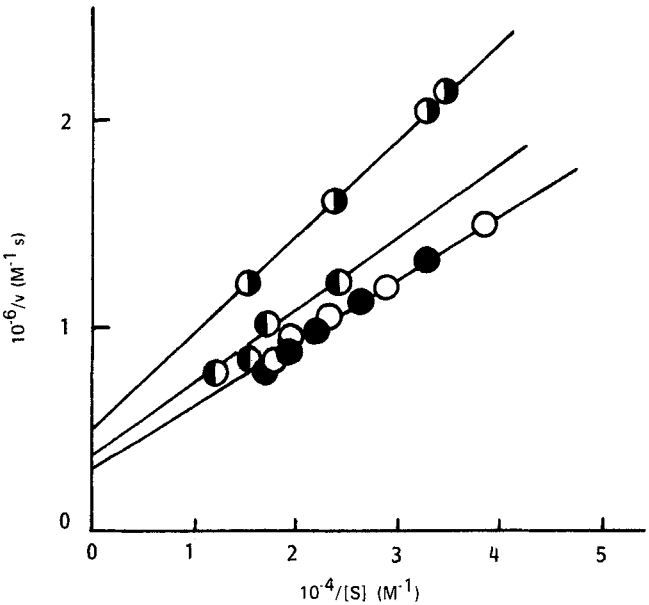


FIG. 5. Additive effects of poly(AAm), poly(MAA), and poly(MAA 6.2/AAm 93.8) on the esterase-catalyzed hydrolysis of p-NPA. $[E]_0 = 3.49 \times 10^3$ unit/L in 0.05 M NaH_2PO_4-Na_2HPO_4 buffer solution (pH = 6.5), 37°C. Additives: (●) poly(AAm) = 5.88×10^{-4}, (◑) poly(MAA) = 1.54×10^{-4}, (◐) poly(MAA 6.2/AAm 93.8) = 2.27×10^{-5} (MAA), 4.15×10^{-4} (AAm) M of unit; (○) none.

TABLE 3. Additive Effects of Poly(AAm), Poly(MAA), and Poly(MAA 6.2/AAm 93.8) on the Esterase-Catalyzed Hydrolysis of p-NPA[a]

Additive (M of unit)		$10^5 \cdot [S]_0$ (M of ester unit)	$10^5 \cdot K_m$ (M)	$10^6 \cdot V_{max}$ (M s^{-1})
None	-	5.09-2.52	9.61	3.13
Poly(AAm)	5.88×10^{-4}	6.07-3.06	"	"
Poly(MAA 6.2/AAm 93.8) MAA	2.27×10^{-5}	8.34-4.08	9.50	2.67
Poly(MAA 6.2/AAm 93.8) AAm	4.15×10^{-4}			
Poly(MAA)	1.54×10^{-4}	7.40-4.24	9.49	2.04

[a] $[E]_0 = 3.49 \times 10^3$ unit/L in 0.05 M NaH_2PO_4-Na_2HPO_4 buffer solution (pH = 6.5); 37°C.

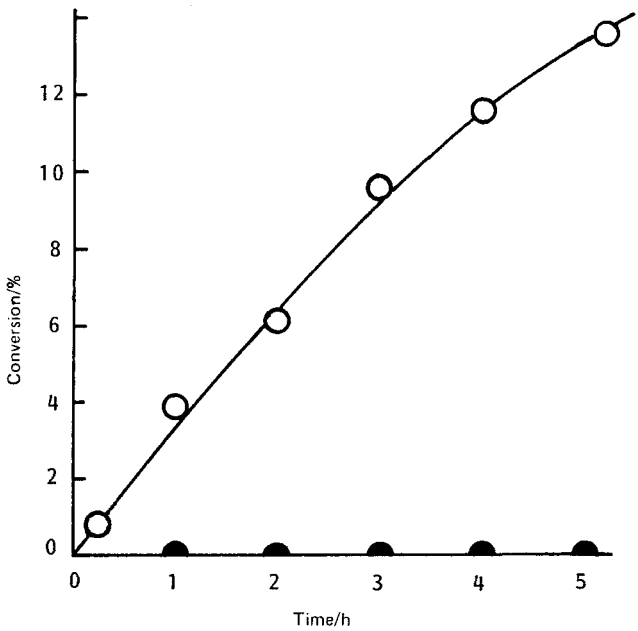


FIG. 6. Additive effects of poly(AAm), poly(MAA), and poly-(MAA 6.2/AAm 93.8) on the esterase-catalyzed hydrolysis of poly(p-NPMA 6.2/AAm 93.8). $[E]_0 = 3.49 \times 10^3$ unit/L in 0.05 M NaH_2PO_4-Na_2HPO_4 buffer solution (pH = 6.5), 37°C. Additives: (•) poly(MAA) = 2.25×10^{-6}, poly(MAA 6.2/AAm 93.8) = 2.27×10^{-5} (MAA), 4.15×10^{-4} (AAm) M of unit; (○) none.

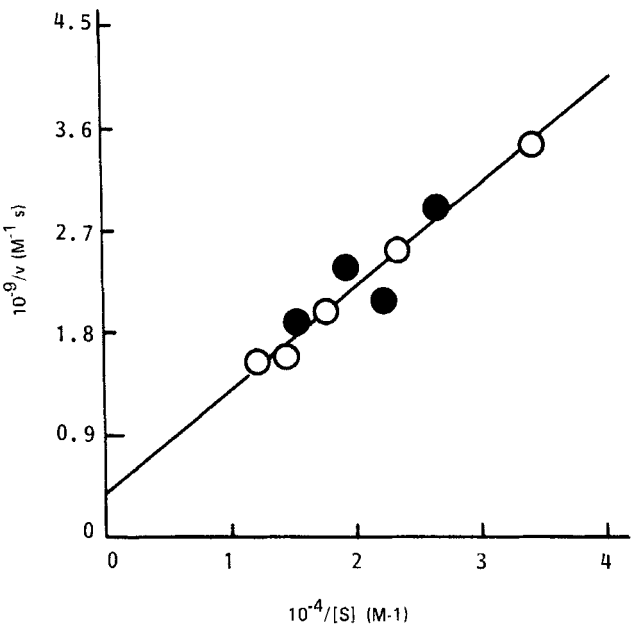


FIG. 7. Additive effects of poly(AAm) on the esterase-catalyzed hydrolysis of poly(p-NPMA 6.2/AAm 93.8). $[E]_0 = 3.49 \times 10^3$ unit/L in 0.05 M Na_2PO_4-Na_2HPO_4 buffer solution (pH = 6.5), 37°C. Additives: (•) poly(AAm) = 5.88×10^{-4} M of unit; (○) none.

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