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Microenvironmental Effects on Enzyme Catalysis. A Kinetic Study of Polyanionic and Polycationic Derivatives of Chymotrypsin†

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ABSTRACT: A series of water-soluble polyanionic and polycationic derivatives of chymotrypsin were prepared by growing poly(glutamyl) or poly(ornithyl) side chains on the enzyme, by coupling chymotrypsin to an ethylene–maleic acid copolymer (EMA) and by partial succinylation or acetylation. The pH–activity profiles of the polyanionic derivatives of chymotrypsin were displaced toward more alkaline pH values as compared to the native enzyme; conversely the pH–activity profiles of the polycationic derivatives were displaced toward more acidic pH values. The k_{cat} values of the charged chymotrypsin derivatives acting on ester, amide, and anilide substrates were displaced symmetrically, relative to the native enzyme—to higher values in the case of the polyanionic derivatives (poly(glutamyl)chymotrypsin, EMA–chymotrypsin, succinylchymotrypsin, and acetylchymotrypsin), and to lower values in the case of the polycationic (poly(ornithyl)chymotrypsin) derivatives. The electrostatic effects on k_{cat} were much more pronounced when the substrate was amide or anilide than when it was an ester. Increasing the ionic strength caused an increase in the values of k_{cat} of both native chymotrypsin and the positively charged derivatives of the enzyme. The k_{cat} values of the negatively charged derivatives were not affected by the ionic strength. With ester substrates the values of K_m(app) of the polycationic derivatives were higher by an order of magnitude in comparison to the native enzyme; the K_m(app) values of the polyanionic derivatives were only slightly perturbed. The values of K_m(app) of all chymotrypsin derivatives acting on amide and anilide substrates were unperturbed and essentially identical with the value of the Michaelis constant of the native enzyme. These findings are discussed in the light of some recent ideas regarding the mechanism of action of chymotrypsin.

Studies on water-insoluble derivatives of several enzymes, in which the biologically active protein is covalently bound to a high molecular weight support material, have shown that the chemical nature, and in particular the charge of the carrier polymer, have a profound effect on both the stability and the overall kinetic behavior of the enzyme derivative (Goldstein

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and Katchalski, 1968; Goldstein, 1970; Goldman *et al.*, 1971a). Water-insoluble, polyanionic derivatives of trypsin, chymotrypsin, and papain exhibited enhanced stability in the alkaline pH range. The pH–activity profiles of the polyanionic enzyme derivatives were displaced toward more alkaline pH values as compared to tne native enzymes. Conversely, the pH–activity profiles of polycationic derivatives of chymotrypsin and papain were displaced toward more acidic pH values. These anomalies were canceled at high ionic strength (Goldstein *et al.*, 1964; Goldstein, 1970; Valenzuela and Bender, 1971; Pecht and Levin, 1972). The behavior of the polyelectrolyte enzyme derivatives could be explained by an essentially Donnan-type electrostatic model. This model assumed the redistribution of low molecular weight ionic species, such as hydrogen and hydroxyl ions, between the "polyelectrolyte phase"—the water-insoluble polyanionic or polycationic enzyme derivative and the outer solution; this effect leads to a modified microenvironment, *i.e.*, a lower or higher local pH, respectively, in the domain of the charged enzyme particle, as compared to the native enzyme in solution (Goldstein *et al.*, 1964). It should be pointed out that the model tacitly assumed that the intrinsic catalytic properties of the polyelectrolyte enzyme derivatives were independent of the charge characteristics of the microenvironment and identical with those of the native enzyme (see also Wharton *et al.*, 1968; Sundaram *et al.*, 1970).

The purpose of this study was to test the validity of these assumptions. A series of water-soluble polyanionic and polycationic derivatives of chymotrypsin were prepared by growing poly(glutamyl) or poly(ornithyl) side chains on the enzyme, by coupling chymotrypsin to an ethylene–maleic acid copolymer, and by partial succinylation or acetylation of the enzyme. The amount of active enzyme in these derivatives could be determined by the conventional spectrophotometric "active-site" titration methods. The intrinsic kinetic parameters of the various polyelectrolyte derivatives of chymotrypsin could therefore be compared with those of the native enzyme on an absolute basis. The kinetic data indicated that the overall rate constant (k_{cat}) of the polyanionic derivatives of chymotrypsin were considerably higher than the k_{cat} value of the native enzyme; conversely the k_{cat} values of the polycationic derivatives were lower. These findings give rise to some questions concerning the mechanism of chymotrypsin action.

Materials

Chymotrypsin, three-times crystallized, salt free and lyophilized, was purchased from Worthington Biochemical Corp., Ac-L-PheOMe,[1] Ac-L-PhepNA, Ac-L-TyrOEt, and Ac-L-TyrNH_2 were purchased from Miles Laboratories, Inc., Elkhart, Ind.

Ethylene–maleic anhydride (1:1) copolymer, EMA (molecular weight about 20,000) was obtained from Monsanto Company, St. Louis, Mo. About 70% of the carboxy groups of the EMA sample used were in the anhydride form. *N-trans*-Cinnamoylimidazole (Eastman Kodak) was recrystallized from dry (CaH_2-distilled) *n*-hexane, mp 133–134°. Spectrograde acetonitrile (Eastman Kodak) was used. All other reagents and buffers were of the best grade available commercially.

N-Carboxy-N^{δ}-trifluoroacetyl-L-ornithine anhydride was prepared by the method of Ariely *et al.* (1966); *N*-carboxy-γ-phthalimidomethyl-L-glutamic acid anhydride was prepared by the method of Wilchek *et al.* (1966). The anhydrides were stored at −20° in a desiccator. The freshly prepared *N*-carboxyanhydrides were completely soluble in dioxane. Upon prolonged storage they became less readily soluble in dioxane at room temperature, presumably due to partial spontaneous polymerization. In such cases the dioxane was slightly warmed and the *N*-carboxyanhydride solution filtered to remove insoluble material.

Poly(ornithyl)chymotrypsin (Pecht and Levin, 1972) was routinely prepared by double polyornithylation of chymotrypsin as follows. Chymotrypsin (200 mg) was dissolved in cold 0.05 M phosphate buffer (pH 7.8) (40 ml). The magnetically stirred chymotrypsin solution was cooled over ice and a dioxane solution of *N*-carboxy-N^{δ}-trifluoroacetyl-L-ornithine anhydride (450 mg in 14 ml of absolute dioxane) was added dropwise. The reaction was allowed to proceed in the cold room for about 20 hr. The milky suspension was dialyzed exhaustively against water in the cold. The protecting trifluoroacetyl groups were removed at pH 10.8, at room temperature. The pH was monitored by an automatic titrator using 4 M KOH as titrant. The cloudiness disappeared in the initial stages of the unblocking reaction. The reaction was continued until no significant uptake of alkali could be observed (about 2 days). The pH was then brought down to 2.5–3.0 with HCl, and the solution was dialyzed against 0.001 N HCl, using Visking dialysis tubing 8/32 (Union Carbide), and lyophilized. For the second ornithylation the lyophilized poly(ornithyl)chymotrypsin sample was dissolved in 0.05 M phosphate buffer (pH 7.8) (20 ml), and the polyornithylation reaction was initiated by the dropwise addition of a second portion of *N*-carboxy-N^{δ}-trifluoroacetyl-L-ornithine anhydride (450 mg dissolved in 5–6 ml of dioxane). The reaction was continued overnight at 4°, with stirring. The solution was dialyzed against water, and the trifluoroacetyl blocking groups were removed at pH 10.8, as described above. The poly(ornithyl)chymotrypsin solution was purified by dialysis against 10^{-3} M HCl and lyophilized. Using this procedure poly(ornithyl)chymotrypsin samples enriched by 200–300 ornithyl residues per molecule were obtained. When lower degrees of ornithylation (30–50 ornithyl residues per molecule) were desired, the polyornithylation reaction was carried out only once. For very high degrees of ornithylation a third polyornithylation was performed. Poly(ornithyl)chymotrypsin samples containing 500–800 ornithyl residues per molecule could be obtained upon triple ornithylation.

It is of interest to note that each ornithylation step was accompanied by a considerable loss of 280-nm-absorbing material (probably due to autodigestion). The recovery of protein of twice-ornithylated poly(ornithyl)chymotrypsin samples was in the range of 15–20%. The recovery of enzymic activity was 5–10%. The properties of the various polyornithyl)chymotrypsin samples are summarized in Table IA.

Poly(glutamyl)chymotrypsin. Chymotrypsin (200 mg) was dissolved in cold 0.05 M phosphate buffer (pH 7.8) (80 ml). *N*-Carboxy-γ-phthalimidomethyl-L-glutamic acid anhydride (600 mg) was dissolved in absolute dioxane (25 ml) and added dropwise to the magnetically stirred, ice-cooled enzyme solution. The reaction was continued at 4° for about 20 hr. The milky suspension was exhaustively dialyzed against water in the cold. The protecting γ-phthalimidomethyl groups were

[1] Abbreviations used are: Ac-L-PheOMe, acetyl-L-phenylalanine methyl ester; Ac-L-PhepNA, acetyl-L-phenylalanine-*p*-nitroanilide; Ac-L-TyrOEt, acetyl-L-tyrosine ethyl ester; Ac-L-TyrNH_2, acetyl-L-tyrosinamide; EMA-CHYM, ethylene–maleic acid copolymer–chymotrypsin conjugate; PGCH, poly(glutamyl)chymotrypsin; POCH, poly(ornithyl)chymotrypsin; DMF, dimethylformamide.

TABLE I

Sample	Number of Poly-ornithylations	Mean Degree of Ornithylation[a]	Effective Normality (moles/mg)[b]	pK_1[c]	pK_2[c]	k_{cat}[d] (sec^{-1})	K_m(app)[d] (mM)
A. Polycationic Chymotrypsin Derivatives							
POCH-1	1	34	2.27×10^{-3}	6.3 ± 0.05	10.2 ± 0.1	172 ± 10	3.0 ± 0.2
POCH-2	1	76	0.358	5.55 ± 0.05	9.7 ± 0.1	165 ± 8	4.1 ± 0.3
POCH-7	2	136	0.331	5.5 ± 0.05	9.5 ± 0.1	141 ± 6	5.1 ± 0.3
POCH-3	2	223	0.427	5.4 ± 0.05	9.4 ± 0.1	135 ± 10	13.2 ± 1.5
POCH-4	2	250	0.418	5.4 ± 0.05	9.43 ± 0.1	119 ± 8	7.1 ± 1.0
POCH-8	2	270	0.272	5.4 ± 0.05	9.5 ± 0.1	123 ± 6	7.0 ± 1.0
POCH-5	3	330	0.381	5.4 ± 0.05	9.8 ± 0.1	84 ± 7	10.2 ± 1.3
POCH-10	3	450	0.179	5.4 ± 0.05	9.9 ± 0.1	63 ± 6	17.7 ± 2.0
POCH-6	3	820	0.093	5.4 ± 0.05	9.8 ± 0.1	63 ± 6	10.3 ± 2.0
B. Polyanionic Chymotrypsin Derivatives							
	Characterization						
PGCH-3	110 Glutamyl residues[a] per molecule		1.16×10^{-3}	7.80 ± 0.05	10.2 ± 0.1	246 ± 10	1.20 ± 0.10
PGCH-4	170 Glutamyl residues[a] per molecule		0.121	7.90 ± 0.05	10.25 ± 0.1	244 ± 10	0.84 ± 0.09
EMA–CHYM	54% protein by weight[a,e] (290 carboxyl groups per molecule)		0.895	7.90 ± 0.05	10.25 ± 0.1	296 ± 10	2.57 ± 0.25
Succinyl-chymotrypsin	11 Lysyl residues succinylated[f] ($\bar{Z} \approx -18$)[g]		2.36	7.9 ± 0.1	10.3 ± 0.1	245 ± 12	1.45 ± 0.20
Acetyl-chymotrypsin	10.5 Lysyl residues acetylated ($\bar{Z} \approx -6$)[f,g]		1.413	7.4 ± 0.1	9.9 ± 0.1	238 ± 12	1.90 ± 0.22
Crystalline chymotrypsin	x3 cryst, salt free, lyophilized (Worthington)		3.24	6.8 ± 0.05	9.95 ± 0.05	184 ± 5	0.74 ± 0.08

[a] Estimated from amino acid analysis of acid hydrolysates. [b] Determined by active-site titration with *N-trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961). [c] Calculated from the pH–activity profiles, determined at [Ac-L-TyrOEt] = 1.8×10^{-3} M and $\mu = 0.01$ using the method of Alberty and Massey (1954). [d] Kinetic runs carried out at the pH optimum for each derivative (see text), using Ac-L-TyrOEt as substrate ($\mu = 0.01$). K_m(app) and k_{cat} calculated from Lineweaver–Burk plots, using a least-squares procedure. [e] Estimated from total nitrogen content, assuming 16.5% nitrogen for chymotrypsin (Wilcox *et al.*, 1954). [f] Estimated from amine nitrogen determination by the Van Slyke method. [g] Calculated from amino acid composition of α-chymotrypsin (Dayhoff, 1969).

removed at pH 10.5, at room temperature. The pH was monitored by an automatic titrator, using 4 M KOH as titrant. The unblocking reaction was continued until no significant uptake of alkali could be observed (3–4 hr). The pH was then brought down to about 6, the solution dialyzed exhaustively against distilled water and lyophilized. Poly(glutamyl)chymotrypsin samples containing 100–170 glutamyl residues could be obtained by this procedure. The properties of the PGCH samples are summarized in Table IB.

Water-Soluble Ethylene–Maleic Acid Copolymer–Chymotrypsin Conjugate (EMA–Chymotrypsin). Water-soluble EMA–chymotrypsin was isolated from the supernatant of the coupling reaction mixture in the course of preparing water-insoluble EMA–chymotrypsin (Levin *et al.*, 1964; Goldstein, 1970). Chymotrypsin (600 mg) was dissolved in cold 0.1 M phosphate buffer (pH 7.8) (80 ml). Ethylene–maleic anhydride copolymer (600 mg) was homogenized in the same buffer (20 ml). The homogenized EMA suspension was added to the magnetically stirred, ice-cooled chymotrypsin solution, and the reaction continued overnight at 4°. The gelatinous precipitate of insoluble EMA–chymotrypsin was separated by centrifugation (Sorval, 12,000 rpm, 30 min). The supernatant was exhaustively dialyzed against water, clarified again by centrifugation, and lyophilized. The lyophilized powder was dissolved in a minimal amount of water and passed through a Sephadex G-25 column using distilled water as eluant. The fraction eluting with the void volume (containing most of the 280-nm absorption and essentially all the enzymic activity) was collected and lyophilized. The total weight of the lyophilized powder was 250 mg. The protein content of the soluble EMA–chymotrypsin sample as estimated from the nitrogen content, assuming 16.5% nitrogen for chymotrypsin (Wilcox *et al.*, 1954), total amino acid analysis, and the 280-nm absorption, using $\epsilon_{280} = 5 \times 10^4\ M^{-1}\ cm^{-1}$ (Oppenheimer *et al.*, 1966), was about 54%, corresponding to about 290 free carrier–polymer carboxyl groups per molecule of enzyme. Active-site titration indicated that about 40% of the bound protein was enzymically active (see Table IB).

Succinylchymotrypsin. Chymotrypsin (500 mg) was dissolved in cold distilled water (20 ml). Succinic anhydride (500 mg) was added in small portions in the course of 3 hr, the pH being maintained at 7.6 with an automatic titrator, using 2 M NaOH as titrant. The temperature was kept low by ice cooling. The reaction mixture was exhaustively dialyzed against distilled water at 4° and lyophilized (net weight of lyophilized powder 470 mg). Determination of the free amine nitrogen by the Van Slyke method indicated that 11 out of the 14 lysyl residues of chymotrypsin (Hartley, 1964; Dayhoff, 1969) had been succinylated (80% succinylation; see Table IB).

Acetylchymotrypsin. Chymotrypsin (500 mg) was dissolved in half-saturated sodium acetate (40 ml). Acetic anhydride (2 ml) was added dropwise in the course of 1 hr, to the ice-cooled, magnetically stirred, enzyme solution. The pH was maintained at 7.8 by means of an automatic titrator using 2 M NaOH as titrant. The reaction mixture was exhaustively dialyzed against distilled water at 4° and lyophilized. Determination of free amine nitrogen by the Van Slyke method indicated that about 10.5 out of the 14 lysyl residues of chymotrypsin had been acetylated (about 75% acetylation; see Table IB).

The average net charge ($\bar{Z}$) of the acylchymotrypsin derivatives at neutral pH could be estimated from the number of free amino groups and the known amino acid composition of chymotrypsin (Dayhoff, 1969). $\bar{Z}$ values of -18 and -6 units of electronic charge per molecule were calculated for the succinylchymotrypsin and acetylchymotrypsin preparations, respectively (Table IB).

Electrophoresis on cellulose acetate strips indicated that no peptide or unmodified enzyme contaminations were present in the purified samples of polyelectrolyte chymotrypsin derivatives.

Methods

Amino acid analysis of acid hydrolysates of chymotrypsin and its polyelectrolyte derivatives were carried out employing an automatic amino acid analyzer (Spackman, 1967). Total nitrogen was determined by the Dumas combustion method (Steyermark, 1961). Amine nitrogen was determined by the Van Slyke method (Van Slyke, 1929; Peters and Van Slyke, 1932).

Protein concentrations were determined spectrophotometrically at 280 nm using a molar extinction coefficient of 5×10^4 M^{-1} cm^{-1} for chymotrypsin (Dixon and Neurath, 1957; Oppenheimer *et al.*, 1966) and assuming a molecular weight of 25,000 (Wilcox *et al.*, 1957; Dixon *et al.*, 1958). Concentration of active enzyme was determined by the *N-trans*-cinnamoylimidazole method (Schonbaum *et al.*, 1961).

Kinetic Measurements. The initial rates of the hydrolysis of Ac-L-TyrOEt or Ac-L-PheOMe catalyzed by chymotrypsin, or the various polyanionic and polycationic derivatives of chymotrypsin, were followed by measuring the rate of liberation of protons at a constant pH (Jacobsen *et al.*, 1957). A Radiometer pH-Stat consisting of the SBR2c/SBU-1/TTA3 titration assembly and the PHM 26c pH meter was used. The titrant was 0.05 N NaOH. In a typical titration, 5 or 10 ml of substrate solution, adjusted to the desired ionic strength with KCl, was placed in the titration vessel, thermostated at 25°. The solution was mechanically stirred, and nitrogen passed over the surface of the liquid. The solution was allowed to equilibrate, and 10–50 μl of an aqueous solution of chymotrypsin or its derivative was added. After the preset pH value had been reached, the rate of addition of titrant was a measure of the rate of the reaction. At low pH values the measured rates were corrected for the incomplete ionization of the product carboxyl groups, using a $pK_a = 3.64$ for acetyl-L-tyrosine and $pK_a = 3.70$ for acetyl-L-phenylalanine. For the determination of specific activities 0.018 M Ac-L-TyrOEt was used routinely. One unit of esterase activity was defined as that amount of enzyme which catalyzed the hydrolysis of 1 μmole of substrate per minute, at the optimal pH. The kinetic parameters (k_{cat} and K_m(app)) were determined at the appropriate pH optimum of each chymotrypsin derivative (pH 8.3 for chymotrypsin; pH 7.5 for poly(ornithyl)chymotrypsin; pH 9.5 for EMA–chymotrypsin and poly(glutamyl)chymotrypsin). Six to ten substrate concentrations in the range 1×10^{-3} M to 2×10^{-2} M for Ac-L-TyrOEt and 5×10^{-3} M to 4×10^{-2} M for Ac-L-PheOMe were used. The test solutions were (2–6) $\times 10^{-8}$ M in enzyme.

The initial rates of hydrolysis of Ac-L-Phe-*p*-nitroanilide catalyzed by chymotrypsin and its polyelectrolyte derivatives at 25° were followed spectrophotometrically at 410 nm using a Cary 14 recording spectrophotometer with a thermostated cell compartment. Buffer (2 ml) and a dimethylformamide solution of the substrate (250 μl) were mixed and brought to 25°. The reaction was initiated by the addition of 500 μl of enzyme solution. The initial rate of product formation was calculated using a value of $\epsilon_{410} = 10{,}400$ M^{-1} cm^{-1} for *p*-nitroaniline in 9% DMF. Six to ten substrate concentrations in the range $(0.5–5.0) \times 10^{-2}$ M were used. The test solutions were $(1–3.5) \times 10^{-6}$ M in enzyme. The following 0.01 M buffers adjusted to ionic strength 0.05 with KCl were used: pH 5.9–7.65, Tris-maleate; pH 8.0–8.5, Tris; pH 9.0, Veronal; pH 9.0–9.5, glycine–NaOH or carbonate. The pH of the test solutions was measured before and after each kinetic run.

The initial rates of hydrolysis of Ac-L-TyrNH_2, catalyzed by chymotrypsin and its polyelectrolyte derivatives at 25°, were followed "manually," by removing aliquots at fixed times and determining the ammonia liberated by the ninhydrin method (Moore and Stein, 1948). The kinetic runs were carried out at the appropriate optimal pH values (pH 8.5 for chymotrypsin, pH 7.5 for POCH-4, and pH 9.5 for EMA–CHYM). Six to ten substrate concentrations in the range $(0.4–2.0) \times 10^{-2}$ M were used. The test solutions were $(1–2) \times 10^{-6}$ M in enzyme.

The kinetic constants k_{cat} and K_m(app) were obtained from the Lineweaver–Burk plots (Dixon and Webb, 1964) using a least-squares procedure.

Results

Preparations and General Characterization. Several types of water-soluble polyanionic and polycationic derivatives of chymotrypsin were synthesized. Positively charged poly(ornithyl)chymotrypsin derviatives (POCH) of a varying degree of ornithylation were prepared by growing poly(ornithyl) side chains on the lysyl ϵ-amino groups of the enzyme. Negatively charged derivatives of chymotrypsin were prepared by several methods. Poly(glutamyl)chymotrypsin derivatives (PGCH) were prepared by growing poly(glutamyl) side chains on the enzyme. Another soluble negatively charged derivative of chymotrypsin, ethylene–maleic acid copolymer–chymotrypsin conjugate (EMA–CHYM) was prepared by the covalent binding of chymotrypsin to a 1:1 copolymer of ethylene and maleic anhydride (EMA). Chymotrypsin derivatives of lower overall negative charge were obtained by partial succinylation or acetylation of the free amino groups on the protein.

The properties of the polyelectrolyte derivatives of chymotrypsin employed in this study are summarized in Table I.

The activity retained by chymotrypsin, POCH-4, PGCH-4, and EMA–CHYM after incubation for 30 min at 37° at various pH values is shown in Figure 1. Both chymotrypsin and its derivatives are stable in the pH range 5–7. The stability of native chymotrypsin decreases steeply between pH 8.5 and 9; at acidic pH values the stability of chymotrypsin decreases gradually below pH 4. The polyanionic derivatives, PGCH-4 and particularly EMA–CHYM, show no significant loss of activity in the alkaline pH range, while in the acidic

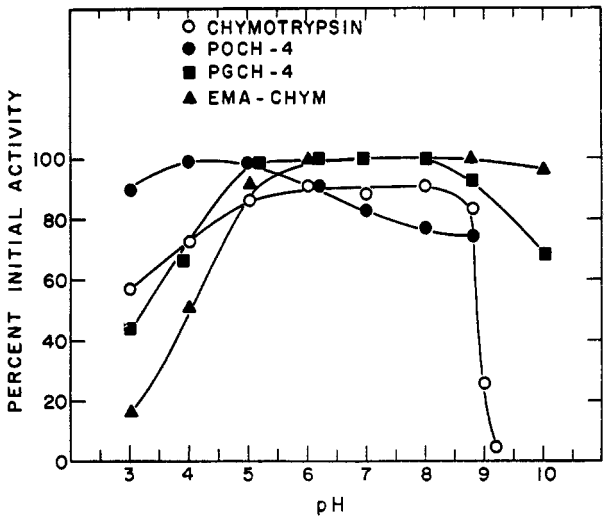

FIGURE 1: Effect of pH on the stability of chymotrypsin and several polyelectrolyte derivatives of chymotrypsin. The test solutions (0.5 ml) in the appropriate buffer, containing chymotrypsin or chymotrypsin derivative (about 15 esterase units per ml) were incubated at 37° for 30 min; 0.1-ml aliquots were withdrawn and the residual esterase activity was determined by the standard procedure (see Methods). The following buffer solutions were used to cover the pH range investigated: pH 3.0, 0.05 M citrate; pH 4–5, 0.1 M acetate; pH 6–9, 0.05 M phosphate; pH 10–10.7, 0.05 M carbonate.

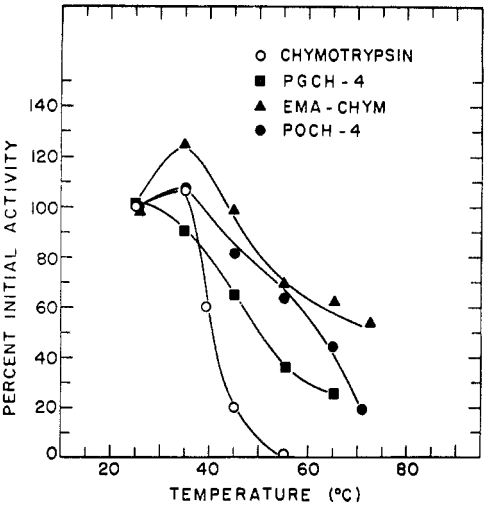

FIGURE 2: Stability of chymotrypsin and several polyelectrolyte derivatives of chymotrypsin at different temperatures. The test samples (0.5 ml) containing chymotrypsin or chymotrypsin derivative (about 15 esterase units per ml) dissolved in a buffer of pH of optimal stability (see Figure 1) were incubated at the specified temperature for 15 min; 0.1-ml aliquots were withdrawn, and the residual activity was determined by the standard procedure at 25° (see Methods).

pH region (pH 3–5) the decrease of stability with pH is much more pronounced than that of the native enzyme. The polycationic derivative, POCH-4, shows the reverse behavior: increased stability in the acidic pH region; above pH 6, gradual decrease in stability with increasing pH.

The temperature stabilities of chymotrypsin and of representative samples of polyanionic and polycationic derivatives of the enzyme are shown in Figure 2. In each case the stability experiments were carried out at the appropriate pH of maximal stability (see Figure 1). Figure 2 indicates that all polyelectrolyte derivatives of chymotrypsin exhibit better temperature stability than the native enzyme.

pH Dependence of Activity. The pH dependence of the specific activity (V/E_0) of poly(ornithyl)chymotrypsin (POCH-4), poly(glutamyl)chymotrypsin (PGCH-4), EMA–chymotrypsin, and crystalline chymotrypsin with acetyl-L-tyrosine ethyl ester (0.018 M) as substrate is shown in Figure 3. The apparent dissociation constants of the ionizing groups involved in the enzyme catalysis (pK_a) calculated from the two limbs of the bell-shaped pH–activity profiles (pK_1 and pK_2) are given in columns 5 and 6 of Table I. The pK_a values are essentially the pH values at which V/E_0 has one-half its maximum value (Dixon and Webb, 1964; Alberty and Massey, 1954; Hammond and Gutfreund, 1955). The acid limb of the pH–activity profile of the positively charged poly(ornithyl)chymotrypsin is displaced at low ionic strength (μ = 0.01) toward lower pH values by 1.4 pH units as compared to native chymotrypsin (pK_1 = 6.8 for chymotrypsin, pK_1 = 5.4 for POCH-4). The acid limb of the pH–activity profiles of the polyanionic derivatives, poly(glutamyl)-chymotrypsin and EMA–chymotrypsin, are displaced toward higher pH values (pK_1 = 7.90) by about the same magnitude (Figure 3A and Table I). The basic limbs of the pH–activity profiles of both the cationic and the anionic derivatives are perturbed to a considerably lesser extent relative to the native enzyme (for POCH-4, chymotrypsin, EMA– and poly(glutamyl)chymotrypsin the values of pK_2 are 9.45, 9.95, and 10.3, respectively; Table I). The behavior of succinyl-chymotrypsin and acetylchymotrypsin was similar to that of PGCH and EMA–CHYM (pK_1 = 7.90 and 7.40, pK_2 = 10.3 and 9.9 for succinyl- and acetylchymotrypsin, respectively; Table I). On increasing the ionic strength (Figure 3B), the anomalies in the apparent pK's of the various polyelectrolyte chymotrypsin derivatives are practically abolished. These results are in good agreement with previous findings on water-insoluble polyanionic (ethylene–maleic acid copolymer) derivatives of trypsin, chymotrypsin, and papain (Goldstein *et al.*, 1964; Goldstein, 1970) and can be related to "local pH" effects, *i.e.*, to the unequal distribution of hydrogen and hydroxyl ions between the polyelectrolyte enzyme derivative and the bulk of the solution (Goldstein *et al.*, 1964). The data of Figure 3 also show that at low ionic strength (μ = 0.01) the specific activity of the positively charged POCH-4 in the optimal pH range is considerably lower than that of the native enzyme; the specific activity of the polyanionic derivatives PGCH-4 and EMA–CHYM, on the other hand, is higher than that of native chymotrypsin (Figure 3A). Increasing the ionic strength causes an increase in the activity of poly(ornithyl)chymotrypsin and of native chymotrypsin. The activity of the polyanionic derivatives PGCH-4 and EMA–CHYM is unaffected by increase in the ionic strength.

Kinetics of Hydrolysis of Ester, Amide, and Anilide Substrates. A better understanding of the way in which electrostatic parameters affect the kinetic behavior of chymotrypsin can be obtained from a study of the Michaelis–Menten kinetics of the various polyelectrolyte derivatives of the enzyme. Table I (last two columns) lists the values of k_{cat} and K_m(app) determined at the optimal pH and μ = 0.01 for native chymotrypsin and for all derivatives of the enzyme, using Ac-L-TyrOEt as substrate. Representative Lineweaver–Burk plots for chymotrypsin, poly(ornithyl)chymotrypsin (POCH)-4, poly(glutamyl)chymotrypsin (PGCH-4), and EMA–CHYM (at μ = 0.01) are given in Figure 4.

The data of Table I and Figure 4 show that the k_{cat} values of the polyanionic derivatives of chymotrypsin are con-

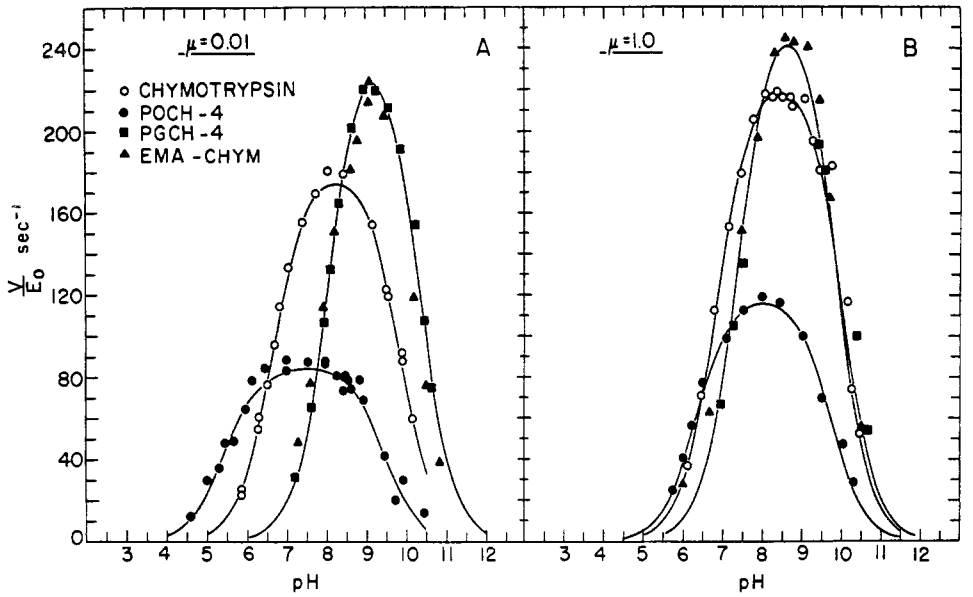

FIGURE 3: pH–activity curves for chymotrypsin and several polyelectrolyte derivatives of chymotrypsin acting on acetyl-L-tyrosine ethyl ester, at different ionic strengths. The assay solutions were 0.018 M in Ac-L-TyrOEt and 0.01 M (A) or 1 M (B) in KCl. The amount of active enzyme used for each assay was determined by *N-trans*-cinnamoylimidazole titration (Schonbaum *et al.*, 1961). Substrate hydrolysis was determined potentiometrically using 0.05 N NaOH as titrant. The solid lines were calculated using the appropriate values of k_{cat}, K_m(app), pK_1, and pK_2 given in Table I.

siderably higher than the k_{cat} value for the native enzyme ($k_{cat} \simeq$ 240–300 sec^{-1} for the various polyanionic chymotrypsin derivatives; k_{cat} = 184 sec^{-1} for chymotrypsin at μ = 0.01). The K_m(app) values of these derivatives are only slightly perturbed (K_m(app) $\simeq$ (1–2.5) $\times$ 10^{-3} M). The polycationic derivatives of chymotrypsin when compared with the crystalline enzyme exhibit lower values of k_{cat} and much higher values of K_m(app) ($k_{cat} \simeq$ 63–165 sec^{-1}, K_m(app) $\simeq$ (4–17) $\times$ 10^{-3} M for the various POCH derivatives; K_m(app) = 0.74 $\times$ 10^{-3} M for chymotrypsin; μ = 0.01).

The data of Table I show a rough proportionality between the number of charged groups on the modified enzyme molecule and the magnitude of the perturbation of k_{cat}, the effect levelling off at high degrees of overall charge. This phenomenon is illustrated in Figure 5, where the variation of k_{cat} with the degree of polyornithylation for the various POCH samples is presented. The data on the poly(ornithyl)chymotrypsin derivatives also indicate that the values of K_m(app) increase with increasing degree of ornithylation.

Table II gives the ionic strength dependence of k_{cat} and K_m(app) for chymotrypsin, POCH-4, PGCH-4, and EMA–CHYM acting on Ac-L-TyrOEt. The values of k_{cat} for chymotrypsin increase regularly with ionic strength (k_{cat} = 184 sec^{-1} at μ = 0.01; k_{cat} = 240 sec^{-1} at μ = 2.0). A similar increase was found for the various poly(ornithyl)chymotrypsin samples (*e.g.*, for POCH-4: k_{cat} = 120 sec^{-1} at μ = 0.01,

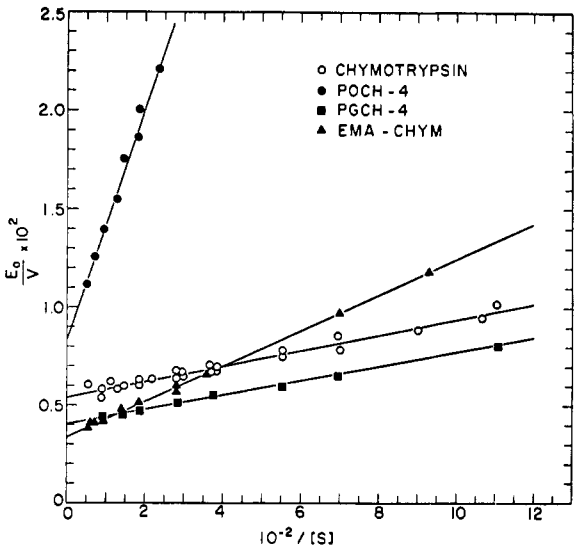

FIGURE 4: Lineweaver–Burk plots for chymotrypsin and several polyelectrolyte derivatives of chymotrypsin acting on acetyl-L-tyrosine ethyl ester at μ = 0.01. The enzymic activities were determined potentiometrically (see Methods) at the optimal pH of each enzyme derivative (chymotrypsin, pH 8.25; PGCH-4 and EMA–CHYM, pH 9.50; POCH-4, pH 7.5). The amount of active enzyme used for each assay was determined by *N-trans*-cinnamoylimidazole titration (Schonbaum *et al.*, 1961). The solid lines were calculated using the values of k_{cat} and K_m(app) given in Table I.

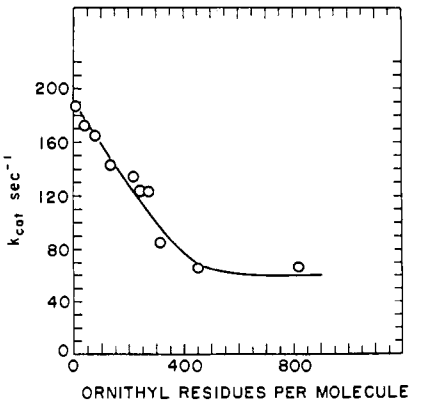

FIGURE 5: Dependence of k_{cat} of the poly(ornithyl)chymotrypsin derivatives on the degree of ornithylation. For experimental details, see text and Table I.

TABLE II: Kinetic Parameters of Chymotrypsin, Poly(glutamyl)chymotrypsin, EMA–Chymotrypsin, and Poly(ornithyl)chymotrypsin Acting on Acetyl-L-tyrosine Ethyl Ester at Different Ionic Strengths.

Ionic Strength	k_{cat}[a] (sec^{-1})	K_m(app)[a] (mM)	pK_1[b]	pK_2[b]
		Chymotrypsin		
0.01	184 ± 5	0.74 ± 0.08	6.80 ± 0.05	9.95 ± 0.1
0.10	190 ± 5	0.81 ± 0.08	6.70 ± 0.05	9.90 ± 0.1
0.50	217 ± 6	0.86 ± 0.09	6.85 ± 0.05	10.00 ± 0.1
1.00	225 ± 6	0.55 ± 0.07	6.85 ± 0.05	10.00 ± 0.1
2.00	240 ± 8	0.60 ± 0.08		
		Poly(glutamyl)chymotrypsin (PGCH-4)[c]		
0.01	240 ± 10	0.88 ± 0.1	7.90 ± 0.05	10.25 ± 0.1
0.05	242 ± 10	0.89 ± 0.1	7.90 ± 0.05	10.3 ± 0.1
0.10	239 ± 10	0.97 ± 0.1	7.80 ± 0.05	10.3 ± 0.1
0.50	248 ± 10	1.30 ± 0.12	7.45 ± 0.05	10.3 ± 0.1
1.00	242 ± 10	1.40 ± 0.14	7.30 ± 0.05	10.3 ± 0.1
		EMA–Chymotrypsin[c]		
0.01	300 ± 15	2.50 ± 0.3	7.90 ± 0.05	10.25 ± 0.1
0.10	281 ± 14	2.93 ± 0.3	7.50 ± 0.05	10.14 ± 0.1
0.50	271 ± 14	1.95 ± 0.2	7.30 ± 0.05	10.0 ± 0.1
1.00	278 ± 14	1.93 ± 0.3	7.30 ± 0.05	9.9 ± 0.1
		Poly(ornithyl)chymotrypsin (POCH-4)[c]		
0.01	119 ± 10	7.10 ± 0.8	5.40 ± 0.05	9.4 ± 0.1
0.10	132 ± 12	5.70 ± 0.6	5.95 ± 0.05	9.75 ± 0.1
0.50	140 ± 12	5.80 ± 0.6	6.30 ± 0.05	9.8 ± 0.1
1.00	156 ± 14	5.90 ± 0.7	6.35 ± 0.05	9.7 ± 0.1
2.00	165 ± 14	5.82 ± 0.7		

[a] Kinetic experiments carried out at the optimal pH of each derivative (see text) using Ac-L-TyrOEt as substrate at the specified ionic strength; K_m(app) and k_{cat} calculated from Lineweaver–Burk plots using a least-squares procedure. [b] Calculated from pH–activity profiles, determined at [Ac-L-TyrOEt] = 1.8×10^{-3} M and specified ionic strength using the method of Alberty and Massey (1954) (see text and Figure 3). [c] For preparation and characterization, see text and Table I.

k_{cat} = 165 sec^{-1} at μ = 2.0; Table II). The values of k_{cat} of the polyanionic derivatives, PGCH-4 (k_{cat} = 240 sec^{-1}) and EMA–CHYM (k_{cat} = 300 sec^{-1}), are, on the other hand, essentially independent of ionic strength. Increasing the ionic strength lowers the values of K_m(app) of crystalline chymotrypsin and poly(ornithyl)chymotrypsin (Table II). K_m(app) of the polyanionic derivatives of chymotrypsin is not significantly affected by ionic strength.

The values of pK_1 calculated from the acid limbs of the pH–activity profiles are very sensitive to ionic strength (Table II); the values of pK_2, calculated from the basic limbs of the pH–activity profiles, are relatively insensitive to changes in ionic strength.

It should be noted that pK_1 has been ascribed to the ionization of the active-side histidine of chymotrypsin (His-57), controlling the pH dependence of k_{cat} (Hammond and Gutfreund, 1955; Schoellman and Shaw, 1962; Bender *et al.*, 1964; Ong *et al.*, 1964; Hess *et al.*, 1970). pK_2 has been ascribed to the ionization of the N-terminal isoleucine of chymotrypsin (Ile-16), controlling the pH dependence of K_m(app) through conformational changes involving an Ile-16 . . . Asp-194 ion pair (Hess *et al.*, 1970; Birktoft *et al.*, 1970; Sigler *et al.*, 1968; Himoe *et al.*, 1967; Himoe and Hess, 1966).

For chymotrypsin acting on specific ester substrates such as Ac-L-TyrOEt, used in the above experiments, the deacylation step has been shown to be rate limiting (Zerner *et al.*, 1964). To determine whether the acylation and deacylation steps in the chymotrypsin-catalyzed hydrolysis of specific substrates are affected to the same extent by changes in the electrostatic parameters, kinetic experiments using amide and anilide substrates were carried out. In the case of amides and anilides the acylation step has been shown to be rate limiting (Gutfreund and Sturtevant, 1956a,b; Bender *et al.*, 1964; Inagami and Sturtevant, 1964; Brandt and Hess, 1966; Bundy and Moore, 1966; Inagami *et al.*, 1965,1969; Parker and Wang, 1968). The pH dependence of the Michaelis–Menten parameters of chymotrypsin and its polyelectrolyte derivatives was also investigated. Acetyl-L-phenylalanine methyl ester and acetyl-L-phenylalanine-*p*-nitroanilide were used as substrates in these studies in order to eliminate possible complications arising from the ionization of the phenolic hydroxyl of tyrosine at alkaline pH values. Because of the low solubility of Ac-L-PhepNA in water, the assays with this substrate were carried out in a medium containing 9% (v/v) DMF. The values of k_{cat} and K_m(app) for chymotrypsin, EMA–CHYM, and POCH-4 acting on Ac-L-TyrNH_2 at low and high ionic strength are given in Table III. The values of the kinetic parameters with Ac-L-TyrOEt, taken from Table II, are also included for comparison.

The pH dependence of the Michaelis–Menten parameters for chymotrypsin and its derivatives acting on Ac-L-PheOMe

TABLE III: Kinetic Parameters of Chymotrypsin, Poly(ornithyl)chymotrypsin, and EMA–Chymotrypsin Acting on Acetyl-L-tyrosine Ethyl Ester and Acetyl-L-tyrosinamide.[a]

Enzyme	Ionic Strength	Ac-L-TyrOEt		Ac-L-TyrNH$_4$	
		k_{cat} (sec^{-1})	K_m(app) (mM)	$k_{cat} \times 10^2$ (sec^{-1})	K_m(app) (mM)
Chymotrypsin	0.05	184 ± 5	0.74 ± 0.07	8 ± 0.8	34 ± 4
	1.00	230 ± 6	0.55 ± 0.06	14.1 ± 1.0	21 ± 2
EMA–CHYM	0.05	300 ± 15	2.50 ± 0.25	33.6 ± 3.0	30 ± 4
	1.00	280 ± 14	1.93 ± 0.20	29 ± 3.0	35 ± 3.6
POCH-4	0.05	119 ± 10	7.10 ± 0.9	3.9 ± 0.4	38 ± 4
	1.00	165 ± 14	5.82 ± 0.6	5.9 ± 0.5	25 ± 3

[a] Kinetic runs carried out at the pH optimum of each derivative (for details, see text). K_m(app) and k_{cat} calculated from the Lineweaver–Burk plots using a least-squares procedure.

in an aqueous medium (μ = 0.05) is shown in Figure 6. The optimal values of k_{cat} and K_m(app) and the values of pK_1 obtained from the k_{cat} *vs.* pH data of Figure 6 are given in Table IV.

The pH dependence of k_{cat} and K_m(app) for chymotrypsin, POCH-4, and EMA-CHYM acting on Ac-L-PhepNA and Ac-L-PheOMe in a medium containing 9% DMF (μ = 0.05) is shown in Figures 7 and 8. The optimal values of the kinetic parameters at μ = 0.05 and μ = 1.0 are summarized in Table V.

The calculated curves of Figures 6–8 were drawn according to the equation $k_{cat} = k_{cat}(\text{lim})/(1 + (H/K_1))$ (Dixon and Webb, 1964). Here k_{cat}(lim) is the maximal value of k_{cat} attained in the plateau region where this parameter is independent of pH and K_1 is the dissociation constant for the enzyme obtained by best fit of the k_{cat} *vs.* pH data (Alberty and Massey, 1954; Hammond and Gutfreund, 1955; Dixon and Webb, 1964).

The kinetic data show that with both ester and amide or anilide substrates, k_{cat} is symmetrically perturbed toward higher and lower values for the polyanionic and polycationic derivatives of chymotrypsin, respectively. A comparison of the values of k_{cat} obtained with an ester or amide and anilide substrate (Tables III and V) shows, however, that the magnitude of the perturbation of k_{cat} effected by changes in the electrostatic parameters is considerably larger when the substrate is amide or anilide. Thus, increasing the ionic strength to μ = 1.0 results in roughly a twofold increase in the value of k_{cat} of native chymotrypsin acting on Ac-L-TyrNH$_2$ or Ac-L-PhepNA as compared to an increase of 20–30% when the substrate is the *N*-acetyltyrosine or phenylalanine ester. The values of k_{cat} of the negatively charged EMA–CHYM are roughly twofold and fourfold larger than those of the native enzyme when acting on Ac-L-PhepNA and Ac-L-TyrNH$_2$, respectively. With the corresponding esters as substrates the increase in the k_{cat} values of the polyanionic derivative is about 50% (Tables III and V). The k_{cat}

TABLE IV: Kinetic Parameters of Chymotrypsin, Poly(ornithyl)-chymotrypsin, and EMA–Chymotrypsin Acting on Acetyl-L-phenylalanine Methyl Ester at Low Ionic Strength (μ = 0.05).[a]

Enzyme	pK_1	k_{cat} (sec^{-1})	K_m(app) (mM)
Chymotrypsin	6.70 ± 0.02	108 ± 8	1.5 ± 0.2
EMA–CHYM	7.82 ± 0.05	160 ± 12	2.6 ± 0.4
POCH-4	6.10 ± 0.05	70 ± 7	8.0 ± 1

[a] Kinetic runs carried out at the pH optimum of each derivative (for details, see text and Figure 6). K_m(app) and k_{cat} calculated from the Lineweaver–Burk plots, using a least-squares procedure. The values of pK_1 obtained by best fit of the data of k_{cat} *vs.* pH of Figure 6.

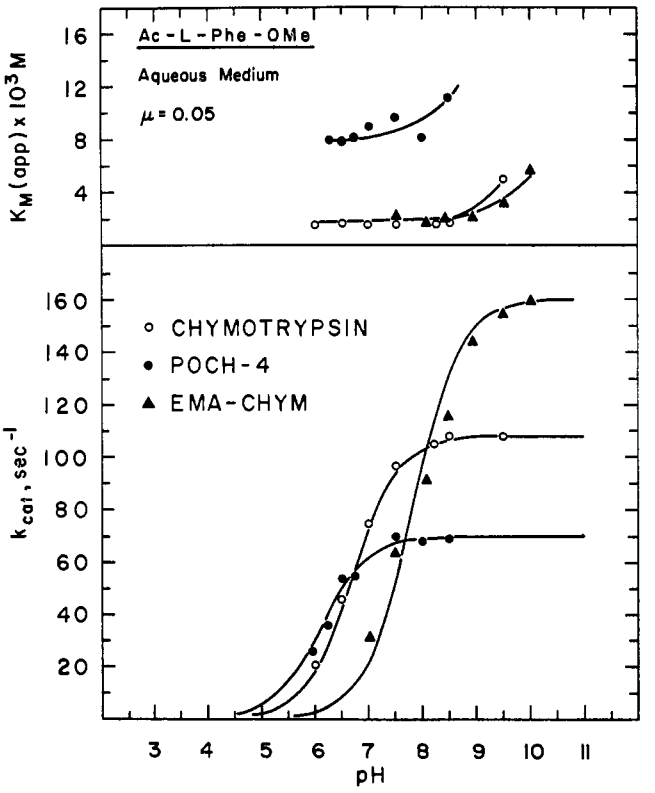

FIGURE 6: pH dependence of k_{cat} and K_m(app) of chymotrypsin, poly(ornithyl)chymotrypsin (POCH-4), and EMA–chymotrypsin acting on acetyl-L-phenylalanine methyl ester in an aqueous medium at μ = 0.05 (for experimental details, see text). The smooth curves were calculated using k_{cat}(lim) = 108 sec^{-1}, pK_1 = 6.70 for chymotrypsin; k_{cat}(lim) = 70 sec^{-1}, pK_1 = 6.10 for POCH-4; k_{cat}(lim) = 160 sec^{-1}, pK_1 = 7.80 for EMA-CHYM.

TABLE V: Kinetic Parameters of Chymotrypsin, Poly(ornithyl)chymotrypsin, and EMA–Chymotrypsin Acting on Acetyl-L-phenylalanine Methyl Ester and Acetyl-L-phenylalanine-*p*-nitroanilide in 9% Dimethylformamide–Water Mixture.[a]

Enzyme	Ionic Strength	Ac-L-PheOMe		Ac-L-Phe-*p*-nitroanilide	
		k_{cat} (sec^{-1})	K_m(app) (mM)	$k_{cat} \times 10^2$ (sec^{-1})	K_m(app) (mM)
Chymotrypsin	0.05	95 ± 7	6 ± 1	2.0 ± 0.1	0.92 ± 0.1
	1.00	120 ± 10	2.4 ± 0.4	3.55 ± 0.2	0.54 ± 0.07
EMA–CHYM	0.05	162 ± 15	6.0 ± 1	3.4 ± 0.2	0.94 ± 0.1
	1.00	165 ± 15	4.7 ± 0.6	4.6 ± 0.3	0.60 ± 0.07
POCH-4	0.05	73 ± 8	17 ± 2	0.83 ± 0.08	0.91 ± 0.1
	1.00	90 ± 8	9 ± 1	1.85 ± 0.2	1.27 ± 0.15

[a] Kinetic runs carried out at the pH optimum of each derivative (for details, see text and Figures 7 and 8). K_m(app) and k_{cat} calculated from Lineweaver–Burk plots using a least-squares procedure.

values of EMA–CHYM are essentially ionic strength independent with all substrates. The k_{cat} values of the positively charged POCH-4 are lower than the k_{cat} of native chymotrypsin by a factor of about two when the substrate is amide or anilide as compared with a lowering of about 30% when the substrate is an ester of either phenylalanine or tyrosine. Increasing the ionic strength causes a much larger increase in the k_{cat} values of POCH-4 when Ac-L-PhepNA or Ac-L-TyrNH_2 are used as substrates.

In contrast to the large electrostatic effects on the values of k_{cat} for amide or anilide substrates the data of Tables III and V show that the values of K_m(app) with Ac-L-TyrNH_2 and Ac-L-PhepNA of all chymotrypsin derivatives are unperturbed and essentially identical with the value of the Michaelis constant of the native enzyme (K_m(app) = 30×10^{-3} M with Ac-L-TyrNH_2; K_m(app) $\simeq 0.9 \times 10^{-3}$ M with Ac-L-PhepNA). This finding is of particular interest in view of the fact that the values of K_m(app) of POCH-4 with the ester substrates Ac-L-TyrOEt and Ac-L-PheOMe are highly perturbed (Tables III and V). The data of Figures 7 and 8 show another unusual feature of the polycationic POCH-4, *i.e.*, the steep pH-dependent increase in k_{cat} observed above pH 8. At the highest pH tested (pH 9.5) the values of k_{cat} of POCH-4 reach the values recorded for the native enzyme with both ester and anilide substrate. This phenomenon could be related to the deprotonation of the polyornithyl side chains of the polycationic derivatives of chymotrypsin, and thus to the loss of positive charge occurring at alkaline pH values.

The effects of the organic solvent (DMF) used in the kinetic

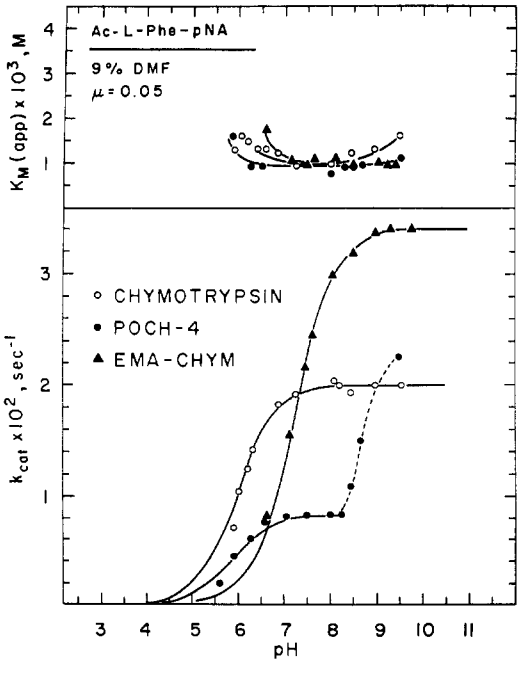

FIGURE 7: pH dependence of k_{cat} and K_m(app) of chymotrypsin, poly(ornithyl)chymotrypsin (POCH-4), and EMA–chymotrypsin acting on acetyl-L-phenylalanine-*p*-nitroanilide in a 9% (v/v) DMF–water mixture, at μ = 0.05 (for experimental details, see text). The smooth curves were calculated using k_{cat}(lim) = 20×10^{-2} sec^{-1}, pK_1 = 5.95 for chymotrypsin; k_{cat}(lim) = 0.83×10^{-2} sec^{-1}, pK_1 = 6.00 for POCH-4; k_{cat}(lim) = 3.4×10^{-2} sec^{-1}, pK_1 = 7.20 for EMA–CHYM.

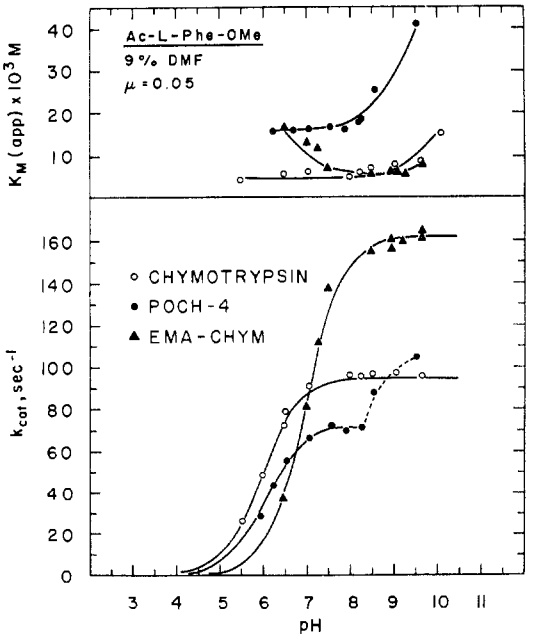

FIGURE 8: pH dependence of k_{cat} and K_m(app) of chymotrypsin, poly(ornithyl)chymotrypsin (POCH-4), and EMA–chymotrypsin acting on acetyl-L-phenylalanine methyl ester in a 9% (v/v) DMF–water mixture at μ = 0.05 (for experimental details, see text). The smooth curves were calculated using k_{cat}(lim) = 95 sec^{-1}, pK_1 = 5.95 for chymotrypsin; k_{cat}(lim) = 73 sec^{-1}, pK_1 = 6.05 for POCH-4; k_{cat}(lim) = 162 sec^{-1}, pK_1 = 6.95 for EMA–CHYM.

investigations with phenylalanine derivatives could be estimated from a comparison of the experiments with Ac-L-PheOMe carried out in an aqueous medium (Figure 6 and Table IV) and in the presence of 9% DMF (Figure 8 and Table V). The values of k_{cat} of chymotrypsin, EMA–CHYM, and POCH do not seem to be significantly perturbed in the presence of organic solvent. The values of K_m(app) with Ac-L-PheOMe, on the other hand, are higher in the mixed solvent for all three enzymes and may be due to specific interactions with the organic solvent. The values of pK_1 obtained from k_{cat} *vs.* pH plots show a lowering of the value of pK_1 for both chymotrypsin and EMA–CHYM in the presence of 9% DMF by about 0.8–1.0 pK unit. (See legends to Figures 7 and 8.) The value of pK_1 for poly(ornithyl)chymotrypsin appears to be practically the same ($pK \simeq 6.10$) in both the aqueous and mixed solvent media. Potentiometric determination of the pK_a values of imidazole and of acetic acid in water and in a 9% DMF–water mixture (at $\mu = 0.01$) showed that the pK values of acetic acid were increased by 0.2 pK unit in the mixed solvent ($pK = 4.7$ in water and 4.9 in 9% DMF). The pK_a values of imidazole were lowered by about the same amount in the mixed solvent ($pK = 7.20$ in water; $pK = 7.0$ in 9% DMF). A similar effect has been reported by Sager and Parks (1964) and by Parker and Wang (1968) for dimethyl sulfoxide–water mixtures. According to these authors the pK_a values of acids of unit positive charge were depressed by about 0.4 pK unit in 30% ME_2SO–water.

The lowered values of pK_1 of chymotrypsin and EMA–CHYM in 9% DMF–water could thus be attributed to effects of the organic component of the solvent on the ionization of the active-site imidazole (His-57) of the enzyme. The absence of this effect in the case of poly(ornithyl)chymotrypsin is probably due to differences in the distribution of the two components of the solvent in the domain of the enzyme derivative, and may reflect interactions of a more specific nature between the ornithyl side chains of the enzyme and the organic solvent component.

Discussion

The preparation of polyanionic and polycationic derivatives of chymotrypsin of widely varied mean net charge (Table I) has made possible the systematic study of microenvironmental effect on the kinetic behavior of chymotrypsin—the most extensively investigated of the serine proteases.

The gross features of the currently accepted three-step mechanism of action of chymotrypsin (Bender and Kézdy, 1965) are given in eq 1, where ES′ is the acyl enzyme inter-

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+P_1}{ES'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

mediate, P_1 and P_2 are the leaving group (alcohol or amine) and the acyl moiety of the substrate, respectively, and k_2 and k_3 the acylation and deacylation rate constants. It can be shown (Gutfreund and Sturtevant, 1956a; Zerner and Bender, 1964) that the steady-state rate parameters of eq 1 are related to the Michaelis–Menten overall kinetic parameters k_{cat} and K_m(app) by

$$k_{cat} = k_2k_3/(k_2 + k_3) \qquad (2)$$

$$K_m(app) = (k_3/(k_2 + k_3))K_s \qquad (3)$$

where $K_s = k_{-1}/k_1$ is the true equilibrium constant for the dissociation of the enzyme–substrate complex.

When the deacylation step is rate limiting, *i.e.*, when $k_3 \ll k_2$, as in the case of ester substrates, eq 2 and 3 reduce to

$$k_{cat} = k_3 \qquad (4)$$

and

$$K_m(app) = (k_3/k_2)K_s \qquad (5)$$

When the acylation step is rate limiting, $k_2 \ll k_3$ as in the case of amide and anilide substrates

$$k_{cat} = k_2 \qquad (6)$$

$$K_m(app) = K_s \qquad (7)$$

From the kinetic data it can be seen that the k_{cat} values of the various polyelectrolyte derivatives of chymotrypsin could not be related in a straightforward manner to pK_1(app) (attributed to His-57) by a linear free energy relationship of the Brönsted type (Brönsted, 1928a; Jencks, 1969; Hammett, 1970). That is to say that although qualitatively all negatively charged derivatives had higher pK_1 and higher k_{cat} values relative to native chymotrypsin, while the positively charged derivatives exhibited lower pK_1 and k_{cat} values, a linear log k_{cat} *vs.* pK_1 relationship was not obeyed. This is best seen from Table IA; the values of k_{cat} of the poly(ornithyl)chymotrypsin derivatives decrease with increasing degree of ornithylation, while pK_1 is perturbed to essentially the same value ($pK_1 \simeq 5.50$) for practically all POCH samples. Moreover, increasing the ionic strength (Table II) canceled the perturbation of pK_1 while the k_{cat} values of each charge type of chymotrypsin derivative exhibited different dependencies on the ionic strength. Comparison of the kinetic parameters in aqueous and DMF-containing media (Figures 6 and 8) also showed that the perturbation in the values of pK_1 of the various enzyme derivatives in the mixed solvent was not reflected in a parallel perturbation of the k_{cat} values. These findings did not allow the use of a simple general base catalysis model (Brönsted, 1928a; Bell, 1941; Goldstein and Katchalski, 1968; Jencks, 1969; Bender, 1971), involving the active-site imidazole (His-57), for a unified interpretation of the kinetic data. The kinetic data also show that the perturbation in the values of k_{cat} of the polyanionic and polycationic derivatives of chymotrypsin cannot be attributed to conformational changes induced by long-range electrostatic interactions (*i.e.*, repulsion) between the charged side chains on the enzyme molecule; such a model would be expected to lead to analogous kinetic effects for both negatively and positively charged derivatives of chymotrypsin, contrary to our data. Moreover, the k_{cat} values of the uncharged poly(γ-phthalimidomethylglutamyl)chymotrypsins and poly(δ-trifluoroacetylornithyl)chymotrypsins were similar to those of native chymotrypsin. Perturbation of k_{cat} is observed only after the removal of the blocking groups (phthalimidomethyl, or trifluoroacetyl), and the formation of the appropriate polyelectrolyte derivative of chymotrypsin.

In view of the foregoing, the electrostatic effects were separated into two classes: (a) microenvironmental effects stemming mainly from the redistribution of low molecular weight ionic species such as hydrogen and hydroxyl ions, as a result of the electrostatic field generated by the charged side chains, *e.g.*, displaced pK_a's and modified patterns of enzyme stability as a function of pH. These effects have been analyzed else-

where (Goldstein *et al.*, 1964; Goldstein, 1970) and will not be discussed further. (b) Local charge effects which perturb the course of the chemical reactions occurring at the active site of the enzyme during the catalytic process. The main features of these effects can be summarized as follows.

The k_{cat} values of the charged derivatives of chymotrypsin were symmetrically displaced—relative to the native enzyme—to higher values in the case of the polyanionic derivatives (poly(glutamyl)chymotrypsin, EMA–chymotrypsin, succinylchymotrypsin, and acetylchymotrypsin), and to lower values in the case of the polycationic derivatives (the various poly(ornithyl)chymotrypsins). The perturbation of k_{cat} of both the polyanionic and the polycationic derivatives was much larger with amide or anilide substrates, indicating that the acylation step was much more strongly affected by the electrostatic parameters (Tables III and V). The magnitude of the perturbation of k_{cat} appears to be related to the number of charged groups on the enzyme molecule as indicated by the data of Table I and Figure 5. Moreover, in the case of the poly(ornithyl)chymotrypsin derivatives, the perturbation of k_{cat} is cancelled at high pH values where the poly(ornithyl) side chains lose their positive charge (Figures 7 and 8).

Increasing the ionic strength caused an increase in the values of k_{cat} of both native chymotrypsin and the positively charged poly(ornithyl)chymotrypsin derivatives. Here again the effects were much larger when the substrate was amide or anilide. The k_{cat} values of the negatively charged derivatives, on the other hand, were not significantly affected by the ionic strength (Tables II, III, and V).

The values of K_m(app) of the polycationic derivatives (POCH) acting on ester substrates were higher, by as much as an order of magnitude, than those of the native enzyme, depending on the degree of ornithylation. The K_m(app) values of the polyanionic derivatives (EMA–CHYM and PGCH) were only slightly higher. The values of K_m(app) of all chymotrypsin derivatives acting on amide or anilide substrates were unperturbed and almost identical with the Michaelis constant of the native enzyme (Tables III and V).

An overall interpretation of the kinetic data could be made assuming that the increase in k_{cat} of native chymotrypsin with ionic strength could be attributed to a kinetic salt effect (Bjerrum, 1924, 1925, Brönsted, 1928b; Castanēda-Agullo *et al.*, 1961; Hammett, 1970). Such an assumption would require that the hydrolytic reactions catalyzed by chymotrypsin involve at some stage along the reaction pathway the interaction of two charged groups. If the charges are of the same sign, increase in the ionic strength would facilitate the approach of the reacting species, and thus the formation of the transition-state complex, leading to an increase in the observed overall rate. If the charges are of opposite sign, increase of the ionic strength would lead to a decrease in the rate constants. This model does not consider the possibility of salt-dependent conformational changes which may affect the catalytic rate constants. The available data on the effects of ionic strength on chymotrypsin do not, however, provide any positive indication for the existence of salt-induced conformational changes at neutral and slightly alkaline pH values in the presence of a specific substrate (see, for example, Castanēda-Agullo *et al.*, 1961; Warren and Cheatum, 1966; Kahana and Shalitin, 1969; Garel and Labouesse, 1970; Rossi and Bernhard, 1971; Kurosky *et al.*, 1971; Royer *et al.*, 1971; Cuppett *et al.*, 1971; Gaudin and Viswanatha, 1972).

Thus, the simplest kinetic model which could explain the ionic strength dependence of k_{cat} of native chymotrypsin can be based on the assumption that a kinetically significant step in chymotrypsin catalysis involves two charged groups of the same sign.

The interpretation of the kinetics of the polyanionic and polycationic derivatives of chymotrypsin, given below, follows this hypothesis, with the additional assumption that no specific binding of ions occurs.

Examination of the kinetic data shows that the k_{cat} values of the polyanionic derivatives are similar to the values of k_{cat} attained by native chymotrypsin at very high ionic strengths (*viz.*, with Ac-L-TyrOEt as substrate $k_{cat} \simeq 240$ sec^{-1} for PGCH-3, PGCH-4, succinylchymotrypsin, and acetylchymotrypsin; $k_{cat} = 295$ sec^{-1} for the more heavily charged EMA–CHYM; for native chymotrypsin $k_{cat} = 184$ sec^{-1} at $\mu = 0.01$, and $k_{cat} \simeq 240$ sec^{-1} at $\mu = 2.0$; with Ac-L-PhepNA as substrate $k_{cat} = 2 \times 10^{-2}$ sec^{-1} at $\mu = 0.05$; $k_{cat} = 3.6 \times 10^{-2}$ sec^{-1} at $\mu = 1.0$ for chymotrypsin; $k_{cat} = 3.4 \times 10^{-2}$ sec^{-1} for EMA–CHYM at $\mu = 0.05$–1.0). It could therefore be postulated that the effect of a polyanionic microenvironment on the k_{cat} value of chymotrypsin is analogous to that of an increase in ionic strength; conversely, the decrease in k_{cat} in the case of the polycationic derivatives could be interpreted as a negative salt effect (*e.g.*, for POCH-4, $k_{cat} = 120$ sec^{-1} with Ac-L-TyrOEt; $k_{cat} = 0.83 \times 10^{-2}$ sec^{-1} with Ac-L-PhepNA). In terms of the hypothesis presented above such an approach would lead to the conclusion that an intermediate step in chymotrypsin catalysis involves two closely spaced *positive* charges. This view is reinforced by the finding that the values of k_{cat} of the polyanionic derivatives are independent of μ, while the k_{cat} values of the polycationic derivatives show strong ionic strength dependence and assymptote at very high μ's to the k_{cat} values of the native enzyme.

A further indication for the plausibility of the above assumption is the finding that the perturbation of k_{cat} of the poly(ornithyl)chymotrypsin derivatives is cancelled at high pH values, where the ornithyl side chains lose their positive charge.

The currently available structural and kinetic information (see for example, Bender and Kézdy, 1965; Hess, 1971) does not allow for the identification or the assignment of a specific role, in a "minimum mechanism" of chymotrypsin, to the two positive charges, the existence of which was assumed on the basis of our experiments. An intermediate step, involving two positive charges, located along the reaction pathway *before* the formation of acyl enzyme could, however, explain the larger electrostatic effects observed with amide or anilide substrates, as compared to esters. These conclusions should be complementary to some recent suggestions on the pretransition-state protonation of substrate along oriented hydrogen bonds (Wang and Parker, 1967; Parker and Wang, 1968; Wang, 1968; Wang, 1970), as incorporated in the "charge-relay system"—Asp-102....His-57....Ser-195—proposed by Blow and coworkers (1969) to explain the nucleophilicity of the Ser-195 hydroxyl group. This model involves however only one formal positive charge—on His-57. One could postulate the participation of a second positively charged group which, although not directly implicated in the catalytic process, does affect the reactivity of one of the catalytically significant groups, either as a general acid or as a strategically located cationic nearest neighbor.

It is of interest to note that a cationic group (tentatively identified as Arg-145) was invoked by Epstein *et al.* (1968) to explain the unusual nucleophilicity of the Ser-195 hydroxyl of chymotrypsin. The authors based their conjecture on the finding that certain nucleophiles, containing cationic groups, have higher reactivities in displacement reactions with neutral

substrates than would be expected from their aqueous basisities.

An increase in k_{cat} and k_3 of a negatively charged derivative of chymotrypsin (succinylated δ-chymotrypsin) accompanied by an increase in pK_1 has been recently observed by Valenzuela and Bender (1971). A positively charged derivative, ethylenediamine-amidated δ-chymotrypsin, exhibited the expected shift in pK_1 toward the acid region. No change in k_{cat} or k_3 of this derivative was, however, found (Valenzuela and Bender, 1971). The reason for this discrepancy with our results may be the low charge density of this cationic chymotrypsin derivative, as well as the rather low sensitivity of k_3 to charge effects.

The closely similar values of K_m(app) of chymotrypsin and its polyelectrolyte derivatives when amide or anilide substrate are used (Tables III and V), seem to indicate, in view of eq 7, that the equilibrium binding constant, K_s, is not significantly affected by electrostatic parameters.

The perturbed values of K_m(app) of the polyanionic and polycationic derivatives of chymotrypsin, when ester substrates are used, can be understood when taking into account at least two factors: (1) diffusional limitations on the effective substrate concentration; (2) kinetic effects on K_m(app) arising from changes in the magnitude of the ratio k_3/k_2 (eq 5).

Diffusional limitations on the effective substrate concentration arise most probably from the fact that the enzyme, located at the core of a highly branched molecule, is surrounded by a physically constrained layer of solvent, which the substrate has to cross in order to reach the site of the enzymic reaction; a concentration gradient of substrate is thus established across the domain of the enzyme phase, leading to an increase in K_m(app). Similar effects, reported for enzymes immobilized on solid supports, have been ascribed to the existence of substrate gradients across the unstirred layers of solvent at the solid–solution interphase (Goldman *et al.*, 1971b). Diffusional limitations would be expected to be more pronounced with ester substrates, where the rates of hydrolysis are higher by a factor of 10^3–10^4 as compared to amides or anilides and would depend mainly on the number and length of side chains grown on the enzyme molecule. Diffusional effects would thus be expected to lead to increases of comparable magnitude in the values of K_m(app) of all derivatives of chymotrypsin modified to a similar extent, regardless of the charge characteristics of the enzyme derivative, since the substrates Ac-L-TyrOEt and Ac-L-PheOMe are electrically neutral (see, for example, Goldman *et al.*, 1971a, and Sundaram *et al.*, 1970).

The kinetic effects on K_m(app) derive from the fact that the acylation and deacylation steps in chymotrypsin catalysis are not equally affected by the overall net charge of the enzyme. Since for ester substrates, where deacylation is rate limiting, the apparent Michaelis constant is given by $K_m(app) = (k_3/k_2)K_s$ (eq 5), the perturbation of K_m(app) can be attributed to changes in the ratio k_3/k_2. Thus in the case of the polycationic derivatives, a larger decrease in k_2, as compared to the decrease in k_3 would lead to an increase of k_3/k_2, and by eq 5, since K_s is unperturbed, to an increase in the value of K_m(app). By the same reasoning the K_m(app) values of the polyanionic derivatives would be expected to be lower than K_m(app) of native chymotrypsin (due to a larger increase in k_2).

The data show that with ester substrates the values of K_m(app) of both polyanionic and polycationic derivatives of chymotrypsin are higher than the Michaelis constant of the native enzyme. It is significant, however, that the K_m(app) of the negatively charged derivatives is higher by a factor of about 2 only, while the values of K_m(app) of the positively charged derivatives are higher by more than an order of magnitude in the extreme cases. The data thus seem to indicate that, when ester substrates are used, the perturbations of K_m(app) derive from a combination of both diffusional and kinetic effects. In the case of the polycationic derivatives of chymotrypsin kinetic and diffusional effects reinforce each other, leading to highly perturbed K_m(app) values. In contrast, in the case of polyanionic derivatives, the kinetic effects partially cancel the K_m(app) perturbation due to diffusion. The kinetic and diffusional effects, however, cannot be separated.

In a study of the hydrolysis of acetyl-L-tryptophan methyl ester by succinylated, and ethylenediamine-amidated δ-chymotrypsins, the values of K_m(app) of the negatively and positively charged derivatives of chymotrypsin were found to be respectively lower and higher, relative to the Michaelis constant of the native enzyme (Valenzuela and Bender, 1971). In the case of these derivatives, where the chemical modification is relatively mild, it is reasonable to assume that diffusional effects are negligible and only the ratio k_3/k_2 determines the value of K_m(app).

In conclusion, the approach used in this investigation, namely the creation of an artificial polyelectrolyte microenvironment around an enzyme molecule, may serve as a new tool for the study of the individual steps in enzyme catalysis. Furthermore, the study of the kinetic behavior of water-soluble polyelectrolyte enzyme derivatives may supply valuable information as regards the various factors which can affect the kinetic pattern in analogous immobilized enzyme systems, and in particular of enzymes embedded in biological membranes.

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