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A Water-insoluble Polyanionic Derivative of Trypsin. II. Effect of the Polyelectrolyte Carrier on the Kinetic Behavior of the Bound Trypsin*

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The mode of action of the water-insoluble derivatives of trypsin (IMET), obtained by the covalent binding of trypsin to a copolymer of maleic acid and ethylene (1:1), has been investigated at 25°. The *p*H-activity profile of IMET at low ionic strength ($\Gamma/2 = 5.8 \times 10^{-3}$), using benzoyl-L-arginine ethyl ester as substrate, was found to be displaced by approximately 2.5 *p*H units toward more alkaline *p*H values, when compared with trypsin under similar conditions. At higher ionic strength, the *p*H-activity curve of IMET shifted toward more acid *p*H values, approaching the *p*H-activity curve of IMET–trypsin at $\Gamma/2 = 1.0$. The Michaelis constant ($K_m = 0.2 \pm 0.05 \times 10^{-3}$ M) measured for the benzoyl-L-arginine amide system at low ionic strength ($\Gamma/2 = 0.04$) and optimal *p*H (*p*H = 9.5) was found to be approximately thirty times lower than that of the trypsin–benzoyl-L-arginine amide system, at its optimal *p*H (*p*H 7.5) at $\Gamma/2 = 0.04$. The Michaelis constant at high ionic strength ($\Gamma/2 = 0.5$), measured for the IMET–benzoyl-L-arginine amide system at the *p*H of optimal activity (*p*H 9.5), approached that for the trypsin–benzoyl-L-arginine amide system ($K_m = 6.8 \pm 1.0 \times 10^{-3}$ M) when measured at its optimal *p*H (*p*H 7.5) and the same ionic strength. The effect of the polyanionic carrier on the *p*H-activity profiles and Michaelis constants of the bound enzymes investigated can be explained as resulting from the effect of the electrostatic potential of the polyelectrolyte carrier on the local concentration of hydrogen ions and positively charged substrate molecules in the microenvironment of the bound enzyme molecules. Theoretical analysis of the kinetic data allowed a quantitative correlation of the displacement in the *p*H-activity curves and the shifts in the Michaelis constants with the electrostatic potential prevailing in the domain of the polyelectrolyte carrier.

The preparation and properties of a new type of water-insoluble trypsin derivatives (IMET),[1] in which the enzyme is covalently bound via its ϵ-amino groups to the carboxyls of a copolymer of maleic acid and ethylene, was described in the preceding paper (Levin *et al.*, 1964). Each active trypsin molecule in this preparation is embedded in a highly negatively charged three-dimensional network formed by the many ionized carboxyl groups of the synthetic carrier. The enzyme is thus exposed to a strong electrostatic field which might markedly affect its mode of action. It seemed of interest, therefore, to investigate the effect of the polyelectrolyte environment prevailing in the IMET preparations on the kinetics of enzymatic hydrolysis of the classical low-molecular-weight trypsin substrates benzoyl-L-arginine ethyl ester (BAEE) and benzoyl-L-arginine amide (BAA).

The *p*H-activity curves of IMET at low ionic strength, when compared with those of trypsin, were found to be displaced by 2–3 *p*H units toward the alkaline region. The Michaelis constant of IMET, when determined under similar conditions, was markedly lower than that of trypsin. The differences recorded between the modified and intact enzyme could be explained by the effect of the negative electrostatic field on the local concentration of both the hydrogen ions and the positively charged substrate molecules in the domain of the insoluble IMET particles. A

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[1] The abbreviations used are: IMET, water-insoluble copoly-(maleic acid–ethylene)-trypsin; BAEE, benzoyl--arginine ethyl ester; BAA, benzoyl-L-arginine amide.

theoretical analysis of the experimental findings allowed the calculation of the electrostatic potential prevailing in the microenvironment surrounding the covalently bound trypsin molecules.

The effect of microenvironment on the mode of action of a given enzyme has been illustrated in the present case by the marked influence of the electrostatic field on the kinetic behavior of trypsin. It is pertinent to note, however, that other properties of the microenvironment, such as chemical composition and dielectric constant, might also be expected to affect the mode of action of various enzymes. A thorough analysis of these effects could shed new light on the action of enzymes in their native milieu. The techniques of covalent binding of enzymes to insoluble carriers, used by various authors (Grubhofer and Schleith, 1954; Bar-Eli and Katchalski, 1960, 1963; Cebra *et al.*, 1961; Mitz and Summaria, 1961; Manecke, 1962; Katchalski, 1962; Riesel and Katchalski, 1964), can be employed for the attachment of enzymes to polymers with various hydrophilic and hydrophobic groups. Different microenvironments can thus be formed artificially, from which the enzyme cannot be disentangled.

Finally it is worth mentioning, in accordance with the ideas expressed by McLaren and Babcock (1959), that a comparison of the properties of an enzyme in different artificial environments with those of the enzyme in its native microenvironment, e.g., while attached to subcellular particles or membranes, may serve as a means for characterizing the native microenvironment itself, the enzyme serving as a probe, or "molecular electrode."

Materials and Methods

Materials.—Trypsin (crystalline, salt-free, and lyophilized), was obtained from Worthington Biochemical Corp., Freehold, N. J. Acetyltrypsin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Benzoyl-L-arginine ethyl ester (BAEE), and benzoyl-L-arginine amide·HCl (BAA) were purchased from Yeda Research and Development Co., Rehovoth, Israel. The carrier, a copolymer of maleic anhydride and ethylene (1:1), DX-840, lot 949 possessing an average molecular weight of 40,000, was a gift from Monsanto Chemical Co., St. Louis, Mo. All the buffer salts used were of analytical grade.

Preparation of IMET.—The water-insoluble trypsin derivative IMET-3, used in the present study was prepared according to the procedure of Levin *et al.* (1964) as follows: An aqueous 1% solution of hexamethylenediamine (1 ml) was added, as a cross-linking agent, to a homogenized, magnetically stirred suspension of maleic anhydride–ethylene copolymer (100 mg) in 0.2 M potassium phosphate buffer, pH 7.8 (10 ml) at 4°. Trypsin (50 mg, in 5 ml of the above buffer) was added after 2–3 minutes, and the magnetically stirred reaction mixture was left overnight at 4°. The gellike particles of IMET formed were centrifuged and washed about twenty times alternately with water and phosphate buffer (0.1 M, *p*H 7.8) until the supernatant, after filtration through a Millipore filter (type HP, 0.45 μ mean pore size), showed no residual enzymatic activity toward BAEE. The IMET-3 preparation obtained contained 16.8 mg protein/100 mg dry wt (see Levin *et al.*, 1964). The bound protein possessed an esteratic activity toward BAEE, at its optimal *p*H (*p*H 9.5 at $\Gamma/2 = 5.8 \times 10^{-3}$, see Fig. 1) corresponding to approximately 77% of the esteratic activity of an equal amount of native trypsin assayed at its optimal *p*H (*p*H 7.6, $\Gamma/2 = 0.035$, see Fig. 1). No decrease in the esteratic activity of IMET-3 could be detected on storage under 0.01 M NaCl, *p*H 7.0, at 4°, for 2–3 months.

Determination of the Esterase Activities of Trypsin and of IMET.—The rates of hydrolysis of BAEE by trypsin or by IMET were determined by the *p*H-stat method (Schwert *et al.*, 1948; Laskowski, 1955) with an automatic titrator (SBR-2 titrigraph and TTT-1a titrator, Radiometer, Copenhagen). NaOH (0.1 N) was used as titrant and the assays were in all cases performed at 25°. In the *p*H range investigated (*p*H 4.5–10.5), the amount of alkali added to maintain constant *p*H is equivalent to the amount of BAEE hydrolyzed.

Each of the reaction mixtures used for the evaluation of the *p*H-activity profiles of trypsin and IMET contained 5 ml of 5.8×10^{-3} M BAEE in water, and 0.1 mg trypsin or an amount of IMET possessing at its *p*H optimum (*p*H 9.5, see Fig 1) the esteratic activity of 0.1 mg intact trypsin at *p*H 7.5. Constant *p*H was maintained by the automatic titrator, no buffer being added. Ionic strengths were adjusted by the addition of sodium chloride.

Determination of the Amidase Activities of Trypsin and IMET.—BAA was used as substrate, and the extent of hydrolysis derived from the amount of ammonia liberated, as assayed by the Conway microdiffusion method (Conway, 1939; Schwert *et al.*, 1948; Laskowski, 1955), or from the amount of free carboxyl groups formed, was assayed by the *p*H-stat method of Bernhard (1955).

The Conway method.—One ml of BAA solution (3×10^{-3} to 5×10^{-2} M), 0.01 M in Tris buffer of *p*H 7.6 for trypsin, or *p*H 9.5 for IMET, was introduced into the outside compartment of the Conway vessel containing a micromagnetic stirrer. Boric acid (1%, 0.25 ml) and the Tashiro indicator were introduced into the central compartment. Trypsin (1.2 mg) or the enzymatically equivalent amount of IMET, in 0.2 ml of the corresponding Tris buffers, was added to the substrate in the outside compartment and the enzymatic reaction was allowed to proceed at 25° for 5 minutes with careful magnetic stirring. The reaction was stopped by the addition of 0.8 ml of saturated K_2CO_3 solution, and the ammonia was distilled for 3 hours at room temperature and assayed by titration with 0.1 N HCl, using an "Agla" syringe microburet.

The *p*H-stat method.—Bernhard's (1955) assay is based on the finding that the carboxyl groups liberated during the hydrolysis of BAA can be titrated in the presence of formaldehyde above *p*H 7.0. Trypsin (2–3 mg), or the enzymatically equivalent amount of IMET, in 0.01 M Tris buffer of *p*H 7.6 or *p*H 9.5, respectively, were added to 5 ml BAA solution (1×10^{-3} to 5×10^{-2} M) containing 0.4 ml formaldehyde (40%). The enzymatic reaction was followed *p*H-statically at 25° under nitrogen using 0.1 or 0.05 N NaOH as titrant.

Results

*p*H-*Activity Curve of IMET, Using BAEE as Substrate.*—The *p*H and ionic strength, $\Gamma/2$, dependence of the inital rates of hydrolysis of BAEE by trypsin and IMET-3, at 25°, is given in Figure 1. The enzymatic activities for each set of experiments carried out at constant ionic strength are expressed as the percentage of the maximum activity attained at the appropriate optimal *p*H (*p*H 8.0 for trypsin, and *p*H 8.5–9.5 for IMET). The *p*H-activity profile of IMET at low ionic strength ($\Gamma/2 = 5.8 \times 10^{-3}$), when compared with that of trypsin under similar conditions, was found to be displaced by approximately 2.5 *p*H units

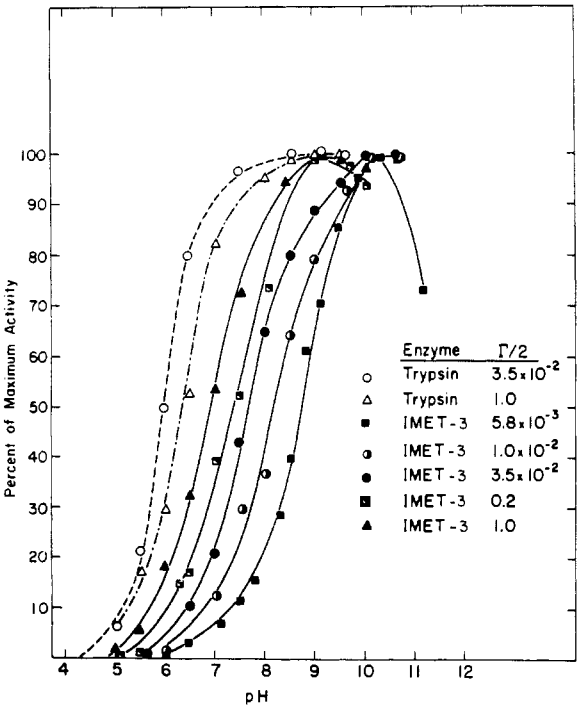

FIG. 1.—pH-Activity curves for trypsin and IMET-3 at different ionic strengths, using BAEE as substrate. Reaction mixture: 5.8×10^{-3} M BAEE (5 ml) and trypsin (0.1 mg) or IMET suspension of similar activity at the corresponding pH values of maximum activity. Substrate hydrolysis was determined potentiometrically at 25° using 0.1 N NaOH as titrant. Ionic strengths were adjusted with NaCl. The initial rates recorded at the pH values of maximum activity were 3×10^{-6} mole/min for trypsin and 3.5×10^{-6} mole/min for IMET-3.

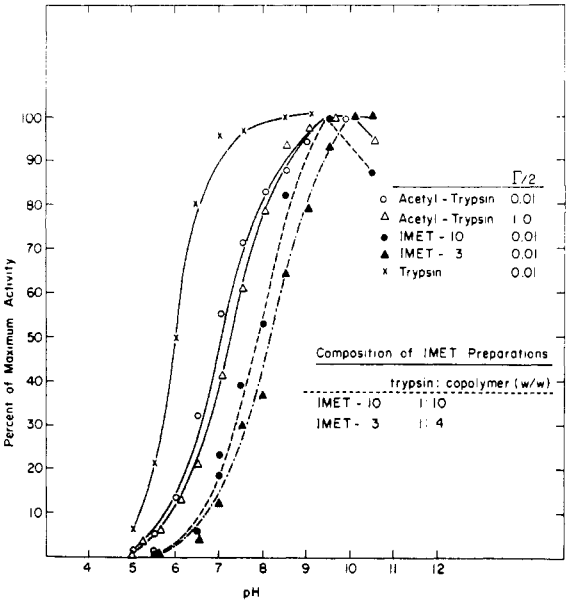

FIG. 2.—pH-Activity curves for acetyltrypsin and two different IMET preparations. Experimental conditions as in Fig. 1. The initial rates recorded at the pH values of maximum activity were 2.7×10^{-6} mole/min for acetyltrypsin (0.1 mg), 1.4×10^{-6} moles/min for IMET-10, and 4.5×10^{-6} mole/min for IMET-3.

toward more alkaline pH values. As the ionic strength is increased, the pH-activity curve of IMET shifts toward more acid pH values, approaching the pH-activity curve of trypsin at $\Gamma/2 = 1.0$.

A behavior similar to that recorded for IMET-3, in which the protein-to-carrier ratio is 1:4, was found also for IMET-10, in which the protein-to-carrier ratio is 1:10 (see Fig. 2).

In order to investigate the effect of acylation of the amino groups of trypsin on the pH-activity curve of the modified enzyme, and its dependence on ionic strength, the pH-activity profile of acetyltrypsin was determined at $\Gamma/2 = 0.01$ and $\Gamma/2 = 1.0$. The results recorded in Fig. 2 demonstrate that a 100-fold change in the ionic strength does not alter to a significant extent the pH-activity curve of this trypsin derivative.

Apparent Michaelis Constant for the IMET-BAA System.—In the study of the effect of substrate concentration on the initial rates of trypsin- and IMET-catalyzed hydrolysis of low-molecular-weight compounds, BAA was chosen as substrate. The Michaelis constant K_m for the BAA-trypsin system ($K_m \simeq 2$–4×10^{-3} M; Schwert and Eisenberg, 1949; Harmon and Niemann, 1949; Neurath and Schwert, 1950; Bernhard, 1955) is larger by about three orders of magnitude than that for the BAEE-trypsin system ($K_m \simeq 10^{-6}$ M; Schwert *et al.*, 1948; Schwert and Eisenberg, 1949; Neurath and Schwert, 1950). The measurement of the initial rates of the trypsin-catalyzed hydrolysis of BAEE in the concentration range required for the estimation of K_m, i.e., the concentrations at which the initial rates follow first-order kinetics (10^{-5} to 10^{-7} M), by the conventional analytical techniques is thus made difficult. The variation of the initial rates of hydrolysis, v, with substrate concentration, $[S]$, at low ionic strength ($\Gamma/2 = 0.04$) for the trypsin-BAA system (pH 7.5), and for the IMET-BAA system (pH 9.5), is given in Figure 3. The rates are expressed in the normalized Michaelis-Menten plots by v/V_{max}, where V_{max} denotes the corresponding limiting rates of hydrolysis attained at very high substrate concentrations. Under the experimental conditions a V_{max} of 0.53×10^{-6} mole/min was found for trypsin, and a V_{max} of 0.22×10^{-6} mole/min for IMET. The data given in the figure show clearly that the Michaelis constant for the IMET-BAA system at $\Gamma/2 = 0.04$, estimated from the value of $[S]$ at $v/V_{max} = 0.5$, is considerably smaller than that for the trypsin-BAA system under similar conditions.

The data of Figure 3 are replotted in a normalized Lineweaver-Burk form, as V_{max}/v versus $1/[S]$, in Figure 4. Straight lines intercepting the ordinate at $V_{max}/v = 1$ were obtained for trypsin and IMET, in accordance with the equation $V_{max}/v = K_m(1/[S]) + 1$. The pertinent V_{max} values, used in the evaluation of V_{max}/v, were calculated from regular Lineweaver-Burk plots (Lineweaver and Burk, 1934; Laidler, 1958). The Michaelis constants calculated from the slopes of the corresponding straight lines given for $\Gamma/2 = 0.04$ in Figure 4 were $K_m = 6.8 \pm 1.0 \times 10^{-3}$ M for trypsin-BAA, and $K_m = 0.2 \pm 0.05 \times 10^{-3}$ M for IMET-BAA. The K_m for the IMET-BAA system, at the low ionic strength investigated, is therefore approximately thirty times smaller than that of trypsin. On increasing the ionic strength to $\Gamma/2 = 0.5$, no marked change in the K_m of the trypsin-BAA system could be detected; however, the K_m of the IMET-BAA system increased to a value of $K_m = 5.2 \pm 0.5 \times 10^{-3}$ M, similar to that of native trypsin.

In the calculation of the K_m values for the interaction of trypsin and IMET with BAA, the inhibition of the enzyme by the product of the reaction, benzoyl-L-arginine (Schwert and Eisenberg, 1949; Harmon and Niemann, 1949; Bernhard, 1955), was ignored. This seems justified since only the initial rates of hydrolysis were taken into consideration, and the rates measured

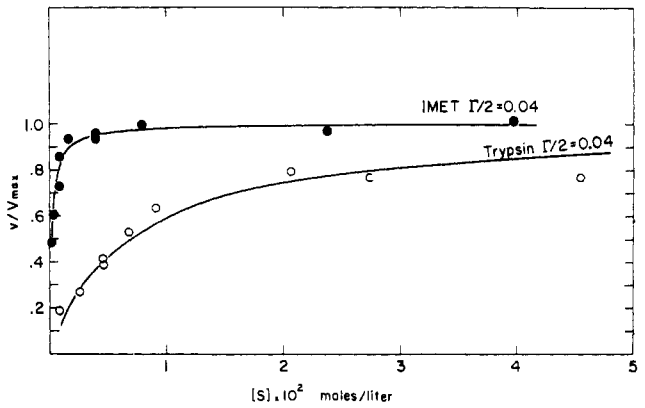

FIG. 3.—Normalized Michaelis-Menten plots for trypsin and IMET acting on BAA. The amidase activity of trypsin was determined at 25° by the Conway microdiffusion method, and potentiometrically (in the presence of formaldehyde) by the Bernhard method (see Experimental) at *p*H 7.6. The amidase activity of IMET was determined by the potentiometric method of Bernhard at *p*H 9.5. Solid lines calculated by equation (9), using the values $K_m = 6.85 \times 10^{-3}$ M for trypsin and $K_m = 0.2 \times 10^{-3}$ M for IMET.

The limiting rates (V_{max}) calculated from the appropriate Lineweaver-Burk plots were: $V_{max} = 0.53 \times 10^{-6}$ mole/min for trypsin and $V_{max} = 0.22 \times 10^{-6}$ moles/min for IMET-3.

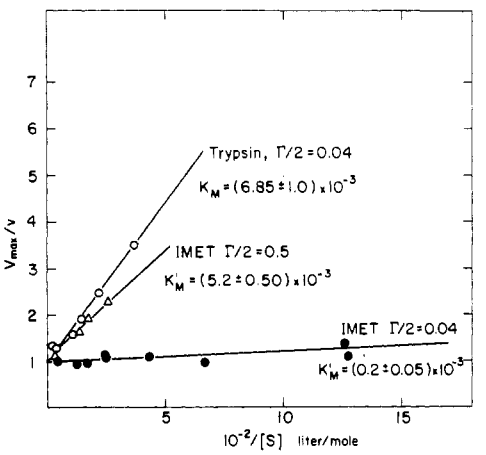

FIG. 4. Normalized Lineweaver-Burk plots for trypsin and IMET acting on BAA. Experimental data taken from Fig. 3.

were rather low. In most cases no more than 1–2% of the ester was hydrolyzed during an experimental run. A small but systematic error was probably introduced into our calculations by the omission of the product-inhibition correction, since the K_m calculated for trypsin acting on BAA from our data ($K_m = 6.8 \times 10^{-3}$ M) is somewhat higher than K_m values recorded in the literature (see, for example, the value given by Bernhard, 1955; $K_m = 3.3 \times 10^{-3}$ M).

Discussion

General Considerations.—In the preceding sections it has been shown that the covalently bound, negatively charged maleic acid–ethylene copolymer exhibits a pronounced effect on the *p*H-activity profile and the Michaelis constant of trypsin. Since covalent binding of enzymes to uncharged carriers via enzymatically nonessential functional groups does not affect their behavior toward low-molecular-weight substrates (Katchalski, 1962; Bar-Eli and Katchalski, 1963; Riesel and Katchalski, 1964), it is plausible to assume that the marked differences between the insoluble trypsin derivatives described in this paper, IMET, and the corresponding intact enzyme, are mainly owing to the strong electrostatic field of the charged carrier (Katchalsky and Michaeli, 1955; Michaeli and Katchalsky, 1957). This is supported by our findings that the effects recorded can be practically eliminated at sufficiently high ionic strength (Katchalsky, 1964).

From polyelectrolyte theory it is well known that the relative concentration of hydrogen ions in the domain of a charged polyelectrolyte gel is different from that of the solution with which it is in equilibrium; an enzyme embedded in the gel phase will thus be exposed to a *p*H different from that measured by the standard physicochemical methods, for the bulk of the solution. The hydrogen-ion concentration within a negatively charged polyelectrolyte gel is higher than in the bulk of the solution, while in a positively charged polyelectrolyte gel it is lower than in the bulk of the solution. The *p*H-activity curve of an enzyme embedded in a charged gel should therefore be displaced toward more alkaline *p*H values when the gel is negatively charged, and toward more acid *p*H values when the gel is positively charged.

The electrostatic field prevailing in a charged carrier gel might be expected to affect also the distribution of charged low-molecular-weight substrates between the gel phase and the external solution. Substrates with a charge opposite in sign to that of the carrier should concentrate in the domain of the polyelectrolyte gel as a result of electrostatic attraction, while substrates with a charge of the same sign as the carrier, on the other hand, should have a lower concentration in the gel phase than in the bulk of the solution because of electrostatic repulsion. An enzyme bound to a charged polyelectrolyte, acting on a substrate of opposite charge, will reach the limiting rate, V_{max}, at lower bulk concentrations of substrate than those recorded for the corresponding unbound enzyme. The Michaelis constant of the bound enzyme will therefore be lower than that of the free enzyme. With a substrate equal in sign of charge to the carrier the bound enzyme will attain V_{max} at higher bulk concentrations of substrate, and the Michaelis constant will be correspondingly higher than that of the intact enzyme.

In the following the *p*H-activity curves and Michaelis constants of IMET are derived theoretically assuming an electrostatic potential ψ in the neighborhood of the bound enzyme molecules.

*Effect of the Electrostatic Potential of the Microenvironment on the p*H*-Activity Curve.*—Particles of an enzyme, covalently bound to a polyelectrolyte carrier suspended in an aqueous medium, can be represented as a two-phase system in equilibrium (see Fig. 5). Phase I consists of the gel particles of the insoluble enzyme, phase II of the external soluion. An average electrostatic potential ψ is assumed to prevail in the neighborhood of the enzyme molecules in phase I. In the elementary treatment to be given it was deemed unnecessary to calculate ψ from molecular parameters.

The equilibrium condition between the two phases requires that the electrochemical potential of the hydrogen ions in phase I, $\tilde{\mu}^{I}_{H^+}$, equal that of the hydrogen ions in phase II, $\tilde{\mu}^{II}_{H^+}$ (Guggenheim, 1929).

$$\tilde{\mu}^{I}_{H^+} = \tilde{\mu}^{II}_{H^+} \quad (1)$$

The electrochemical potentials of the hydrogen ions of the two phases are related to the corresponding chemical potentials $\mu^{I}_{H^+}$ and $\mu^{II}_{H^+}$ by equations (2) and (3), where ϵ designates the positive electronic charge.

$$\tilde{\mu}^{I}_{H^+} = \mu^{I}_{H^+} + \epsilon\psi \quad (2)$$

$$\tilde{\mu}^{II}_{H^+} = \mu^{II}_{H^+} \quad (3)$$

Correlating the chemical potentials with the corresponding hydrogen-ion activities in phase I, $a^{I}_{H^+}$, and phase II, $a^{II}_{H^+}$, one obtains equations (4) and (5),

$$\mu^{I}_{H^+} = \mu^{\circ}_{H^+} + kT \ln a^{I}_{H^+} \quad (4)$$

$$\mu^{II}_{H^+} = \mu^{\circ}_{H^+} + kT \ln a^{II}_{H^+} \quad (5)$$

in which $\mu^{\circ}_{H^+}$ is the standard chemical potential of the hydrogen ion, k is the Boltzmann constant, and T is the absolute temperature. Equations (2), (3), (4), (5), and (1) give:

$$\ln a^{II}_{H^+} - \ln a^{I}_{H^+} = \epsilon\psi/kT \quad (6)$$

Since $-\log a_{H^+} = p\mathrm{H}$, equation (6) can be rewritten in the form:

$$\Delta p\mathrm{H} = p\mathrm{H}^{I} - p\mathrm{H}^{II} = 0.43\epsilon\psi/kT \quad (7)$$

where $\Delta p\mathrm{H}$ is the difference between the $p\mathrm{H}$ of the gel phase ($p\mathrm{H}^{I}$) and that of the external solution ($p\mathrm{H}^{II}$).

The $p\mathrm{H}$ of the external solution, $p\mathrm{H}^{II}$, can be measured potentiometrically with a standard glass electrode. The $p\mathrm{H}$ of the gel phase, $p\mathrm{H}^{I}$, cannot be measured directly; it can, however, be derived from a comparison of the normalized $p\mathrm{H}$-activity profiles of the polyelectrolyte-bound enzyme and the corresponding free enzyme (e.g., Fig. 1). The $p\mathrm{H}^{I}$ is equal to the $p\mathrm{H}$ at which the native enzyme shows a catalytic activity identical with that of the bound enzyme under the conditions specified. The experimentally determined values of $p\mathrm{H}^{I}$ and $p\mathrm{H}^{II}$ allow the calculation of the electrostatic potential ψ prevailing in the gel with the aid of equation (7). The values of ψ calculated for IMET at different ionic strengths are given in Table I.

TABLE I
EFFECT OF IONIC STRENGTH ON THE pH-ACTIVITY PROFILE OF BOUND TRYPSIN

Ionic Strength ($\Gamma/2$)	$\Delta p\mathrm{H}$ [a]	ψ (volts $\times 10^3$) [b]
0.6×10^{-2}	2.4 ± 0.2	145 ± 15
1.0×10^{-2}	2.0	124 ± 13
3.5×10^{-2}	1.6	96 ± 12
0.2	1.3	81 ± 10
1.0	0.4	27 ± 8

[a] $\Delta p\mathrm{H} = p\mathrm{H}^{I} - p\mathrm{H}^{II}$, gives the difference in the $p\mathrm{H}$ values at which trypsin and IMET show 50% of maximum activity, at the ionic strength specified. [b] ψ is the electrostatic potential prevailing in the polyelectrolyte gel as calculated from equation (7).

The $\Delta p\mathrm{H}$ values used were obtained from the difference in the $p\mathrm{H}$ values at which IMET and intact trypsin show 50% of their maximal activities. The values of ψ for $\Gamma/2 = 6 \times 10^{-3}$ ($\psi \simeq 160$ mv) is of the order of magnitude to be expected for a practically completely ionized polyelectrolyte of the maleic acid–ethylene copolymer type (e.g., a value of $\psi = 180$ mv at $\Gamma/2 = 0.002$, $\psi = 150$ mv at $\Gamma/2 = 0.01$, $\psi = 90$ mv at $\Gamma/2 = 0.05$, and $\psi = 60$ mv at $\Gamma/2 = 0.10$ has been reported for 80%-ionized polymethacrylic acid [Katchalsky *et al.*, 1954]). Furthermore, the magnitude of ψ decreases with ionic strength in accord with polyelectrolyte theory.

It has been shown by Gutfreund (1955) that the $p\mathrm{H}$ dependence of the catalytic activity of trypsin can be represented by a curve corresponding to the ionization of a group with pK 6.25. The value of this pK, combined with other available information (Bernhard and Gutfreund, 1958), led to the assumption that an uncharged imidazole group of histidine is probably essential for the hydrolytic activity of trypsin. In the foregoing theoretical explanation of the $p\mathrm{H}$-activity curve of IMET it was tacitly assumed that the pK_{Im} of the imidazole of the active site does not vary and that the shift in the $p\mathrm{H}$-activity profile is owing solely to the redistribution of the hydrogen ions between the polyelectrolyte-gel phase and the external solution. It should be mentioned, however, that the "displaced" $p\mathrm{H}$-activity curve of IMET can be alternatively explained by assuming a corresponding shift in the ionization constant of the imidazole of the active site, caused by the electrostatic field of the microenvironment surrounding the bound enzyme. The apparent dissociation constant of the imidazole of the bound enzyme, pK'_{Im}, will thus be given by the expression, $pK'_{Im} = pK_{Im} + 0.43\epsilon\psi/kT$. It is obvious that the two explanations suggested for the $p\mathrm{H}$-activity profile of IMET lead to identical free-energy terms.

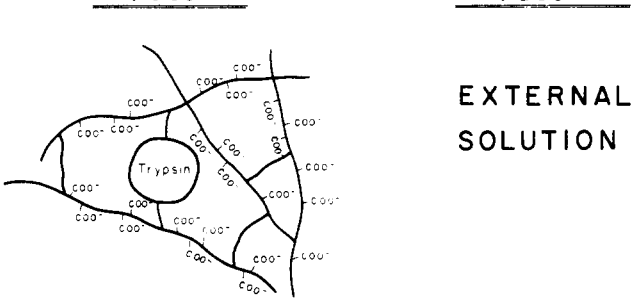

FIG. 5.—Schematic presentation of an IMET particle in suspension.

Trypsin has an isoelectric point of 10.8 (Terminiello *et al.*, 1955). It thus bears a positive net charge in the $p\mathrm{H}$ range ($p\mathrm{H}$ 5.0–7.0) of the ascending side of its $p\mathrm{H}$-activity curve (see Fig. 1). Acetyltrypsin, on the other hand, has an isoelectric point of 4.0 (Sri Ram *et al.*, 1954). The pK of the imidazole of the active site of this trypsin derivative should therefore be greater than the pK_{Im} of trypsin. The $p\mathrm{H}$-activity curve of acetyltrypsin might therefore be expected to be displaced to $p\mathrm{H}$ values more alkaline than those of intact trypsin (see Fig. 2). The insensitivity of the $p\mathrm{H}$-activity profile of acetyltrypsin to ionic strength is most likely owing to the small electrostatic potential, ψ, produced by the relatively low negative net charge of the acetyltrypsin molecules in the $p\mathrm{H}$ range of 4.0–9.0 (Sri Ram *et al.*, 1954, 1962).

Effect of the Electrostatic Potential of the Microenvironment on the Apparent Michaelis Constant.—In the following it is assumed that the covalently bound enzyme is embedded in a polyelectrolyte network exerting an electrostatic potential ψ, and that the low-molecular-weight substrate bears a charge $z\epsilon$ (z being a positive or a negative integer). The distribution of the charged substrate between the gel phase and the external solution, at equilibrium, is given by equation (8),

$$[S]^{i} = [S]^{o} e^{ze\psi/kT} \quad (8)$$

in which $[S]^{i}$ and $[S]^{o}$ denote the concentration of substrate inside the gel phase and in the external solution (outside), respectively. This equation can be obtained analogously to equation (7), substituting substrate concentrations for the corresponding hydrogen-ion activities.

The Michaelis-Menten equation (Michaelis and Menten, 1913; Laidler, 1958)

TABLE II

THE MICHAELIS CONSTANTS (K_m) OF TRYPSIN AND IMET ACTING ON BAA AT DIFFERENT IONIC STRENGTHS[a]

Ionic Strength ($\Gamma/2$)	$K_m \times 10^3$ (M)	$K_m' \times 10^3$ (M)	K_m/K_m'	ΔpK_m[b]	ψ (volts $\times 10^3$)[c]
0.04	6.85 ± 1.0	0.2 ± 0.05	34 ± 10	1.53 ± 0.2	92 ± 12
0.50	6.85 ± 1.0	5.2 ± 0.5	1.32 ± 0.3	0.12 ± 0.05	7.0 ± 3.0

[a] The Michaelis constants of trypsin (K_m), and IMET (K_m') were calculated from the data given in Fig. 4. [b] $\Delta pK_m = pK_m' - pK_m = \log K_m/K_m'$. [c] ψ calculated by equation (12).

$$v = \frac{V_{max}[S]}{K_m + [S]} \tag{9}$$

relates the initial rate of reaction, v, with the substrate concentrations, $[S]$, for a soluble enzyme. In the case of an enzyme bound to an insoluble polyelectrolyte carrier, acting on a charged substrate, the concentration $[S]$ in equation (9) should be substituted by the concentration of substrate in the gel phase, $[S]^i$. Since, however, only the external concentration of substrate, $[S]^o$, is readily measurable, $[S]^i$ is expressed in terms of $[S]^o$ using equation (8). The Michaelis-Menten equation for an insoluble enzyme can thus be rewritten in the form:

$$v' = \frac{V_{max}[S]^o e^{ze\psi/kT}}{K_m + [S]^o e^{ze\psi/kT}} \tag{10}$$

Primed symbols indicate quantities pertaining to water-insoluble enzymes.

Equation (10) shows that $v' = V_{max}/2$ when $[S]^o = K_m e^{-ze\psi/kT}$. The determination of the outside substrate concentration, $[S]^o$, at which half-maximal velocity is attained, leads, therefore, to an apparent Michaelis constant for the insoluble enzyme, K_m', related to the true Michaelis constant, K_m, by the expression:

$$K_m' = K_m e^{-ze\psi/kT} \tag{11}$$

Hence

$$\Delta pK_m = pK_m' - pK_m = \log (K_m/K_m') = 0.43ze\psi/kT \tag{12}$$

Equation (11) shows that when z and ψ are of the same sign, i.e., when the substrate is positively charged and the polyelectrolyte carrier is negatively charged or vice versa, $K_m' < K_m$. On the other hand, when z and ψ are of opposite signs $K_m' > K_m$. Furthermore, an experimental determination of K_m' combined with a knowledge of K_m allows the calculation of the electrostatic potential ψ.

Calculation of ψ for the IMET-BAA system at two different ionic strengths ($\Gamma/2 = 0.04$ and 0.5) by the procedure outlined above yielded the values given in Table II. The potential calculated from the data at low ionic strength, $\psi = 92 \pm 12$ mv, is in good agreement with the value of $\psi = 96 \pm 12$ mv, calculated from the *p*H-activity curve for the IMET-BAEE system at $\Gamma/2 = 0.035$ (see Table I).

Derivation of the Integral Michaelis-Menten Equation for an Enzyme Bound to a Polyelectrolyte Water-insoluble Carrier.—In the following an integral expression is derived for the variation of total concentration of substrate with time in a closed system containing the water-insoluble enzyme. The total volume V of the system is given by:

$$V = v^i + v^o \tag{13}$$

where v^i and v^o denote the volume of the polyelectrolyte-gel phase and the external solution, respectively. The total amount of product formed per unit time in the whole system, dP/dt, is given by equation (14):

$$\frac{dP}{dt} = -\frac{d\{[S]^i v^i + [S]^o v^o\}}{dt} = \frac{k_2[E]^i[S]^i v^i}{K_m + [S]^i} \tag{14}$$

in which K_m, $[S]^o$, and $[S]^i$ have their previous meaning, k_2 is the rate constant (Laidler, 1958), and $[E]^i$ is the concentration of enzyme in the gel phase. Expressing $[S]^o$ in terms of $[S]^i$ by equation (8), denoting the term $e^{-ze\psi/kT}$ by γ, equation (14) yields on integration, equation (15) (see Appendix):

$$\{[S]_0 - [S]_t\} + \left(\frac{v^i + \gamma v^o}{V}\right) K_m \ln \frac{[S]_0}{[S]_t} = k_2[E]t \tag{15}$$

In this equation $[S]_0$ and $[S]_t$ denote the total average concentrations of substrate in the whole system at times $t = 0$ and t, respectively, and $[E]$ is the total average concentation of enzyme in the whole system.

Comparison of equation (15) with the standard form of the integrated Michaelis-Menten equation (Laidler, 1958) reveals that the apparent Michaelis constant for a water-insoluble enzyme, K_m', defined in the preceding section, is correlated to the true Michaelis constant, K_m, by the expression:

$$K_m' = \left(\frac{v^i + \gamma v^o}{V}\right) K_m \tag{16}$$

An analysis of the relation between K_m' and K_m given in equation (16) for a few special cases seems desirable at this point. (1) When $\psi = 0$, i.e., when the carrier is devoid of charge, $\gamma = 1$, and $K_m' = K_m$. The Michaelis constant measured for an enzyme bound to an uncharged carrier is thus equal to that of the free enzyme. (2) When $ze\psi/kT >> 1$, i.e., when z and ψ are of the same sign and the absolute value of ψ is large, e.g., a highly negatively charged carrier and a positively charged substrate, $\gamma << 1$ and $K_m' = (v^i/V)K_m$. In this case practically all the substrate concentrates in the gel phase. Enzyme saturation is thus attained already at a relatively low total substrate concentration. (3) When $ze\psi/kT << 1$, i.e., when z and ψ are of opposite sign and the absolute value of ψ is large, e.g., a highly positively charged carrier and a positively charged substrate, $\gamma >> 1$ and $K_m' = \gamma v^o/VK_m$. (4) When $v^i \simeq 0$ ($v^o \simeq V$), i.e., when the volume of the gel is negligible in comparison with the total volume of the system V, $K_m' = ([v^i/V] + \gamma)K_m$. If, in addition, $\gamma > 1$, one obtains the relationship $K_m' = \gamma K_m = (e^{-z/e\psi kT})K_m$ (equation 11). In the derivation of this equation in the preceding section, the volume of the gel phase of the water-insoluble enzyme was ignored. It should be noted that equation (11) describes satisfactorily our experimental findings concerning the apparent Michaelis-Menten constant of IMET. (5) When $v^o \simeq 0$, and practically the whole volume of the system is occupied by the enzyme-gel phase, $K_m' = K_m$, as would be expected for a one-phase system.

Concluding Remarks.—The data presented in this article demonstrate that a polyelectrolyte carrier might markedly affect the "local *p*H" and the "local substrate concentration" of the microenvironment in which an enzyme is embedded. It is obvious that other parameters of the microenvironment, such as chemical composition, dielectric constant, and affinity for substrate, may also affect the mode of action of enzymes. Since many of the enzymes act in the cell while attached to

characteristic structural elements (e.g., membranes, reticular networks, microsomes, and mitochondria), or while limited to specific media, conclusions drawn as to the mode of action of enzymes *in vivo* based on the study of the properties of isolated and purified enzymes in dilute aqueous solutions may often be invalid. A thorough investigation of the effect of different environments on the specificity, kinetics, and mode of action of enzymes on low- and high-molecular-weight substrates is required before the elucidation of the behavior of enzymes in their natural environment will be possible.

In the case of IMET a strong electrostatic field was imposed on trypsin by its covalent binding to a suitable polyelectrolyte. By the use of similar techniques (Katchalski, 1962) it is possible to surround an enzyme by various hydrophilic or hydrophobic groups. Furthermore, since polymers possessing different affinities for the components of a mixed-solvent system can be prepared, the choice of the proper carrier will determine the solvent composition in the domain of the insoluble enzyme.

Some of the ideas expressed here as to the role of environment in determining enzyme kinetics have been applied to substrate-enzyme interactions at interfaces (McLaren and Babcock, 1959). A comparison of the action of chymotrypsin on lysozyme in solution and on lysozyme adsorbed on negatively charged kaolinite particles (McLaren and Estermann, 1957), revealed that the *p*H of half-maximal activity for the suspension is shifted by about two units toward higher *p*H values, showing that chymotrypsin behaves in accordance with a concentration of hydrogen ions at the surface which is greater than in the surrounding buffer medium.

Finally it should be reemphasized that the data now available indicate that under suitable conditions it might be possible to use enzymes as "molecular probes" for the determination of some of the characteristics of a given microenvironment. In the use of such probes the magnification of any environmental change as a result of the catalytic nature of the "enzymic probe" might be of considerable value.

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Appendix

Equation (15) can be derived from equation (14) as follows. Substitution of $[S]^o$ in equation (14) by $[S]^i e^{-ze\psi/kT} = [S]^i\gamma$ (see equation 8) gives:

$$-\frac{d[S]^i}{dt}(v^i + \gamma v^o) = \frac{k_2[E]^i[S]^i v^i}{K_m + [S]^i} \quad (17)$$

Integration of equation (17) in the time limits $t = 0$ to $t = t$, yields:

$$\{[S]_0{}^i - [S]_t{}^i\} + K_m \ln \frac{[S]_0{}^i}{[S]_t{}^i} = \frac{k_2[E]^i v^i}{v^i + \gamma v^o} t \quad (18)$$

The concentrations $[S]_0{}^i$ and $[S]_t{}^i$ in equation (18) can be expressed in terms of the corresponding total average substrate concentrations $[S]_0$ and $[S]_t$ by making use of the relations:

$$V[S]_t = v^i[S]_t{}^i + v^o[S]_t{}^o$$

and

$$[S]_t{}^o = [S]_t{}^i\gamma$$

from which one obtains:

$$[S]_t{}^i = \frac{V[S]_t}{v^i + \gamma v^o} \quad (19)$$

$$[S]_0{}^i = \frac{V[S]_0}{v^i + \gamma v^o} \quad (20)$$

Equation (15) is finally obtained from equations (17), (19), and (20), $[E]$ denoting the average concentration of enzyme in the whole system.

$$\{[S]_0 - [S]_t\} + \left(\frac{v^i + \gamma v^o}{V}\right) K_m \ln \frac{[S]_0}{[S]_t} = k_2[E]t \quad (15)$$

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