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Inhibition of Bound Enzymes. II. Characterization of Product Inhibition and Accumulation†

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ABSTRACT: Diffusion resistances cause not only substrate depletion but also product accumulation in the microenvironment of bound enzymes. As a result product inhibition is more pronounced in the presence than in the absence of diffusional limitations at a given product concentration in the macroenvironment. The antienergistic interaction between chemical and diffusional inhibition, however, makes the activity of bound enzymes less sensitive to changing product concentration in the macroenvironment. The interplay between various types of product inhibition and diffusion resistances is illustrated by plots of the reaction rate against the ratio of the rate and the macroenvironmental substrate concentration. Combined diffusional and product inhibition can significantly affect the kinetic behavior of immobilized enzyme systems and the magnitude of feedback inhibition in cellular metabolism.

The inhibition of bound enzymes by a substance which is neither a substrate nor a product of the reaction has been treated in the preceding paper (Engasser and Horvath, 1974a). In this study the effect of product inhibition is investigated.

Many enzymes are inhibited by the product of the reaction and this feedback regulation is assumed to play a fundamental role in the control of biological systems. Therefore, product inhibition of enzymes in free solution has been extensively treated in the literature (Webb, 1963). In heterogeneous enzyme systems, however, diffusional resistances can give rise to both substrate depletion and product accumulation in the immediate vicinity of the enzyme. Since the degree of inhibition depends on the concentration of both the product and the substrate in the microenvironment, the product inhibition of naturally or artificially bound enzymes is more complex than that of enzymes in free solution (Goldman *et al.*, 1968a).

The following analysis encompasses the quantitative treatment of the combined effect of substrate depletion and product accumulation in the microenvironment on the kinetic behavior of bound enzymes. The results are presented in graphical form in order to facilitate the recognition of such phenomena from experimental data.

Theoretical Analysis

Product Accumulation and Inhibition. In the simplest case a heterogeneous enzyme reaction takes place at a surface covered with bound enzyme molecules. Both the substrate and the product diffuse to and from the surface through a nonreactive membrane or the surrounding liquid. The overall reaction consists of the following three steps. First the substrate is transported to the catalytic surface, *i.e.*, the microenvironment. Then the substrate is converted into product and finally the product is transported to the macroenvironment of the enzyme, which is often the bulk solution in practice.

If the reaction follows the Michaelis–Menten scheme and the product is a competitive inhibitor that only affects the K_M of the reaction, the rate of the enzymic reaction, v, is expressed by

$$v = \frac{V_{max}[S_0]}{K_M[1 + ([P_0]/K_p)] + [S_0]} \quad (1)$$

where K_p is the product inhibition constant, $[S_0]$ and $[P_0]$ are the concentrations of the substrate and product at the surface, respectively.

The rate of external transport of each species can be expressed as the product of the corresponding transport coefficient and the driving force. The latter is given by the concentration difference between the macro- and the microenvironment of the enzyme. At steady-state substrate transport, enzymatic reaction and product transport must proceed at the same rate so that

$$h_s([S] - [S_0]) = \frac{V_{max}[S_0]}{K_M[1 + ([P_0]/K_p)] + [S_0]} = h_p([P_0] - [P]) \quad (2)$$

where [S] and [P] are the concentrations in the macroenvironment, and h_s and h_p are the transport coefficients of the substrate and the product, respectively. Introducing a dimensionless substrate concentration

$$\beta = [S]/K_M \quad (3)$$

the substrate modulus without inhibition, μ, which expresses the diffusion resistance for the substrate

$$\mu = V_{max}/h_s K_M \quad (4)$$

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‡ Recipient of Yale University Fellowship.

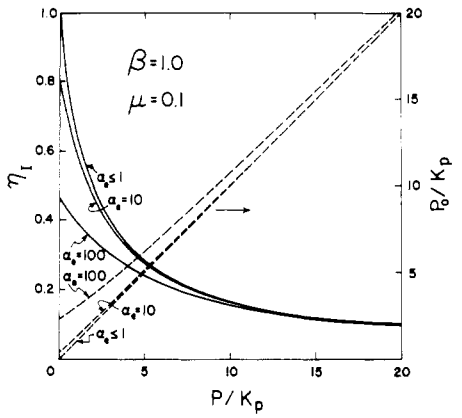

FIGURE 1: Graph illustrating the decrease of enzymic activity as expressed by the decrease of the efficiency factor, η_I, which represents the combined effect of diffusional and product inhibition, with increasing values of the dimensionless product concentration in the macroenvironment, $[P]/K_p$. In addition the broken lines show the dimensionless product concentration in the microenvironment, $[P_0]/K_p$, as a function of $[P]/K_p$. The external accumulation factor, α_e, is used as the parameter. The data have been calculated for competitive product inhibition with external diffusion resistances when the dimensionless substrate concentration, β, and the substrate modulus, μ, are 1 and 0.1, respectively.

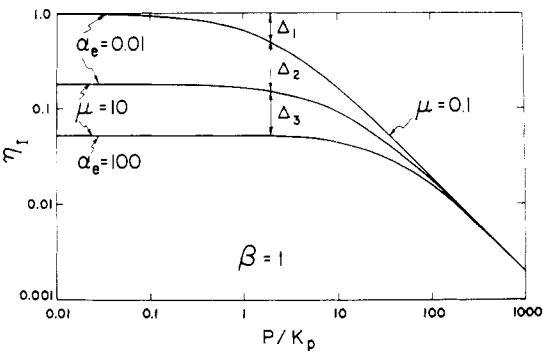

FIGURE 2: Double logarithmic plot of the efficiency factor, η_I, against the dimensionless product concentration in the macroenvironment, $[P]/K_p$, for different values of the external accumulation factor, α_e, and the substrate modulus, μ. The dimensionless substrate concentration, β, is unity. The graph shows that when competitive product inhibition and external diffusional inhibition occur simultaneously at α_e = 100 and μ = 10, the decrease in enzyme activity as shown by the decrease in the efficiency factor is, at a given value of $[P]/K_p$, the result of three effects, each of which decreases η_I by an incremental value: Δ_1 is the contribution of pure chemical inhibition without diffusion resistances, Δ_2 is due to diffusional inhibition caused by substrate depletion, and Δ_3 is the contribution of chemical inhibition due to product accumulation.

and the external accumulation factor

$$\alpha_e = h_s K_M / h_p K_p \quad (5)$$

we obtain from the two equalities in eq 2 the following dimensionless second-order equation for the substrate concentration at the surface. In order to analyze the effect of product inhibition, β_0, $[P_0]$, and the overall activity, v, are calculated from eq 6, 2, and 1 for given values of β, $[P]/K_p$, μ, and α_e.

$$(1 - \alpha_e)\beta_0^2 + [1 + (2\alpha_e - 1)\beta + \mu + ([P]/K_p)]\beta_0 - \beta - ([P]/K_p)\beta - \alpha_e\beta^2 = 0 \quad (6)$$

When the product is a noncompetitive inhibitor that only affects V_{max}, the enzyme activity is given by

$$v = \frac{V_{max}[S_0]}{[1 + ([P_0]/K_p)](K_M + [S_0])} \quad (7)$$

In this case β_0 is obtained by solving the following third-order equation

$$\alpha_e\beta_0^3 - [1 + ([P]/K_p) + 2\alpha_e\beta - \alpha_e]\beta_0^2 + \{\alpha_e\beta^2 + [1 + ([P]/K_p) - 2\alpha_e]\beta - 1 - ([P]/K_p) - \mu\}\beta_0 + \beta + ([P]/K_p)\beta + \alpha_e\beta^2 = 0 \quad (8)$$

According to eq 6 and 8 the interplay between product and diffusional inhibition is completely described, at fixed concentrations of the substrate and the product in the macroenvironment, by the two dimensionless parameters, μ and α_e. The modulus, μ, precisely expresses the degree of substrate depletion when inhibition is negligible. Nevertheless its magnitude reflects the importance of diffusion resistances also in the presence of product inhibition. The external accumulation factor, α_e, which combines all transport and affinity parameters according to eq 5, is a measure of the competition between the substrate and the product at the enzymic surface.

The combined effect of diffusional and product inhibition on the overall rate of reaction is quantitatively expressed by the efficiency factor, η_I, which is defined by

$$v = \eta_I[V_{max}[S]/(K_M + [S])] \quad (9)$$

The effect of the dimensionless product concentration, $[P]/K_p$, on the value of η_I, at β = 1 and μ = 0.1, is illustrated in Figure 1. The curves correspond to different values of the accumulation factor, α_e. Due to the negligible diffusion resistance for the substrate at μ = 0.1, there is no substrate depletion under the conditions illustrated in Figure 1 and the value of η_I is solely determined by product inhibition. Since the substrate concentration is fixed, the variation of η_I also represents the dependence of the enzymic activity on the product concentration in the macroenvironment.

The dependence of the dimensionless product concentration at the surface, $[P_0]/K_p$, on $[P]/K_p$ is also illustrated in Figure 1. As long as $\alpha_e \leq 1$, the concentration of the product is essentially the same in the micro- and macroenvironment of the bound enzyme, which is then inhibited as if it were in free solution. When α_e is larger than unity, however, product accumulates at the enzymic surface as illustrated by the dotted lines for α_e equal to 10 and 100. Consequently, the value of the efficiency factor is lower and the magnitude of product inhibition is greater than that obtained at α_e = 1 at the same value of $[P]/K_p$.

The concurrent effect of substrate depletion as well as product accumulation and inhibition is shown by the double logarithmic plot of η_I against $[P]/K_p$ at μ = 10 and α_e = 100 in Figure 2. Under such conditions the relatively low values of η_I are attributed to three different effects; each reduces the value of η_I by an increment Δ. These are as follows: Δ_1 represents the effect of pure chemical inhibition in the absence of any significant diffusion resistance, that is, when μ = 0.1 and α_e = 0.01; Δ_2 is due to diffusional inhibition which arises from substrate depletion when μ increases from 0.1 to 10 at α_e = 0.01; Δ_3 is the result of product accumulation when α_e increases from 0.01 to 100 at μ = 10 and manifests itself in an enhanced chemical inhibition.

As shown in Figure 2, the effects of both substrate depletion, Δ_2, and product accumulation, Δ_3, are greatest at low product concentrations, that is, when the activity of the enzyme is the highest for a given system. Since the enzyme activity and as a result the relative magnitude of diffusion resistances are reduced with increasing product concentration, the effects of both substrate depletion and product accumulation vanish at a

sufficiently high value of $[P]/K_p$. This antienergistic interaction (Engasser and Horvath, 1974a) between diffusional and chemical inhibition accounts for the relative insensitivity of the bound enzyme activity to changes in the macroenvironmental concentration of the product in the presence of diffusion limitations, as shown in Figures 1 and 2. The results are much the same also when the product is a noncompetitive inhibitor.

Product inhibition of enzymes embedded in a porous medium, when the relative slowness of the internal diffusion of the substrate and the product in the membrane affects the rate of reaction, is analogous to that described above for the surface reaction. The pertinent mathematical analysis presented in Appendix I shows that again two dimensionless parameters, the corresponding substrate modulus, ϕ, and the internal accumulation factor, α_i, suffice to quantify the interplay of chemical reaction, chemical inhibition, and internal diffusion at a given concentration of the substrate and the product in the macroenvironment in the absence of external diffusion resistances.

Effect of Substrate Concentration. Experimental and theoretical investigations have demonstrated that, with bound enzymes in the absence of an inhibitor, the dependence of the reaction rate on the substrate concentration in the macroenvironment, *i.e.*, in the bulk solution, no longer follows the Michaelis–Menten kinetic law when diffusional inhibition occurs. It has been shown that the departure from Michaelis–Menten kinetics is magnified, therefore, easily detected on Eadie–Hofstee type plots, which also facilitates the discrimination between internal and external diffusional inhibitions (Engasser and Horvath, 1973; Horvath and Solomon, 1972).

In this theoretical study plots of the reaction rate against the reaction rate divided by the substrate concentration in the bulk solution are used as graphical diagnostic tools for product accumulation and inhibition with both types of diffusional limitations. The different cases of combined diffusional and product inhibition are illustrated by such graphs in the following sections.

External Diffusion. When the substrate concentration is varied the combined effect of diffusional and product inhibition on the activity of heterogeneous enzyme systems is different for noncompetitive and competitive inhibitors at high substrate concentrations.

In the case of competitive inhibition only the K_M of the bound enzyme is affected, therefore, the saturation rate, V_{max}, is always reached at sufficiently high substrate concentrations where diffusional inhibition due to substrate depletion vanishes (Horvath and Engasser, 1974.). The two dimensionless graphs in Figure 3 illustrate the effect of competitive product inhibition with external diffusion resistance for μ equal to 0.1 and 10, in order to demonstrate the interplay of product inhibition with relatively mild and strong diffusional inhibition, respectively. In both cases the dimensionless reaction rate, v/V_{max}, is plotted against $v/V_{max}\,\beta$ with α_e as the parameter. The value of $[P]/K_p$ has been chosen 0.01 so that the inhibitory effect of the product present in the macroenvironment is negligible and both substrate depletion and product inhibition due to accumulation are greatest, as seen in Figure 2.

In both graphs the convex curves for $\alpha_e = 0.01$ represent the sole effect of substrate depletion since at such a low value of the accumulation factor no product accumulation occurs. As seen the high diffusion resistance at $\mu = 10$ yields a stronger curvature than that obtained at $\mu = 0.1$. All curves for $\alpha_e < 1$ are convex suggesting the preponderant effect of substrate depletion. On the other hand, high product accumulation, at $\alpha_e > 1$, results in concave curves whose curvature also increases with diffusional limitations. Figure 3 shows that when the product is

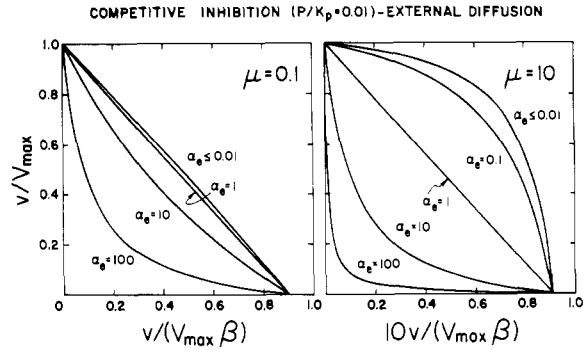

FIGURE 3: Illustration of the effect of competitive product inhibition with external diffusion resistances by plots of the dimensionless rate of reaction, v/V_{max}, against $v/V_{max}\,\beta$, where β is the dimensionless substrate concentration in the macroenvironment, for different values of the external accumulation factor, α_e, and at two values of the substrate modulus, μ. The dimensionless product concentration in the macroenvironment, $[P]/K_p$, is 0.01.

a competitive inhibitor all curves representing various values of α_e converge at limiting low and high β's because the effect of product accumulation disappears at sufficiently low and high substrate concentrations.

Interestingly both graphs show a straight line for $\alpha_e = 1$, indicating that the dependence of the reaction rate on the bulk concentration of the substrate also follows a Michaelis–Menten scheme when the accumulation factor is unity. This finding is easily verified analytically since eq 6 reduces to a first-order equation in β_0 when $\alpha_e = 1$; consequently the reaction rate, v, is expressed as a function of β by the Michaelis–Menten equation with a K_M^* value given by

$$K_M^* = K_M[1 + \mu + ([P]/K_p)] \qquad (10)$$

Generally, the rate of an enzymic reaction does not show the Michaelis–Menten dependence on the substrate concentration in the macroenvironment when diffusional inhibition occurs. Therefore this singular case of combined diffusional and competitive product inhibition with $\alpha_e = 1$ has to be considered a unique situation which, however, may occur in practice.

When the product is a noncompetitive inhibitor, which lowers the saturation rate of the enzymic reaction, V_{max}, product accumulation decreases the rate of reaction at high substrate concentrations, and this effect increases with the accumulation factor, α_e. As a result the corresponding curves shown in Figure 4 intercept the ordinate at different points depending on the values of μ and α_e. At large values of α_e noncompetitive inhibition results in plots having very small curvature in comparison to those obtained for competitive product inhibition.

Internal Diffusion. The interaction between product inhibition and internal diffusion is quantitatively treated in Appendix I and the results are presented graphically in Figures 5 and 6 for competitive and noncompetitive product inhibition, respectively.

The graphs are analogous to those presented in Figures 3 and 4 but ϕ and α_i replace the previously used μ and α_e, respectively. At low internal diffusional resistances, when $\phi = 0.1$, the curves for both competitive and noncompetitive inhibition are similar to those obtained previously for $\mu = 0.1$, except that the effect of product accumulation is much weaker at a given α_i than at the same value of α_e. On the other hand at $\phi = 10$, the curves for sufficiently low α_i values are sigmoidal. With increasing values of α_i, however, the sigmoidal shape disappears and the resulting curves become similar to those shown in Figures 3 and 4 for $\mu = 10$.

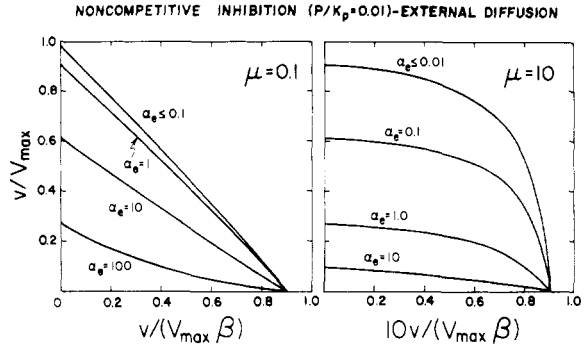

FIGURE 4: Illustration of the effect of noncompetitive product inhibition with external diffusion resistances by plots of the dimensionless rate of reaction, v/V_{max}, against $v/V_{max}\beta$, where β is the dimensionless substrate concentration in the macroenvironment, for different values of the external accumulation factor, α_e, and at two values of the substrate modulus, μ. The dimensionless product concentration in the macroenvironment, $[P]/K_p$, is 0.01.

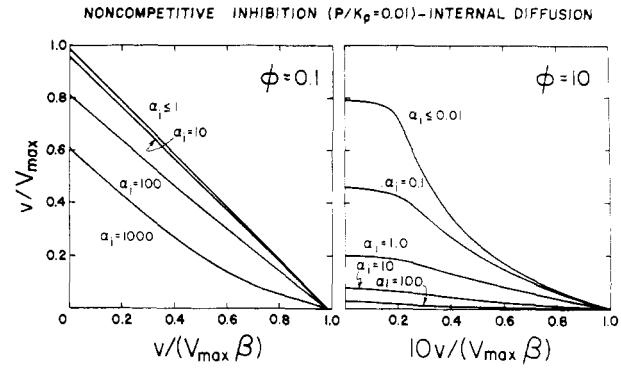

FIGURE 6: Illustration of the effect of noncompetitive product inhibition with internal diffusion resistances by plots of the dimensionless rate of reaction, v/V_{max}, against $v/V_{max}\beta$, where β is the dimensionless substrate concentration in the macroenvironment, for different values of the internal accumulation factor, α_i, and at two values of the substrate modulus, ϕ. The dimensionless product concentration in the macroenvironment, $[P]/K_p$, is 0.01.

Discussion

According to the present study diffusion resistances can greatly affect the kinetic behavior of heterogeneous enzyme systems which are inhibited by the product of the reaction. The relatively slow removal of the product from the bound enzyme by diffusion results in a higher product concentration in the microenvironment than in the macroenvironment, *i.e.*, at a certain distance from the bound enzyme. Therefore the extent of product inhibition is greater with than without diffusional limitations at the same macroenvironmental product concentration. Since the enzymic activity decreases with increasing product concentration, the magnitude of both substrate depletion and product accumulation in the microenvironment of the enzyme also decreases. As a result of this antienergism between diffusional and chemical inhibition, the enzyme activity is less sensitive to changes in the concentration of the product in the macroenvironment when diffusion resistances are present.

In recent years the study of enzymes immobilized on solid supports received great attention because of their promising practical applications. On the other hand, the properties of membrane-bound enzymes are increasingly investigated in order to elucidate their behavior in the cellular milieu. The experiments with such heterogeneous enzyme systems give deeper insight into enzyme action in nature than those with enzymes in free solution. Under such conditions it is very difficult to measure the microenvironmental concentrations experimentally and the kinetic studies are performed by measuring the concentrations of both the substrate and the product in the bulk solution, *i.e.*, in the macroenvironment. As shown in this study, when diffusional limitations impede the communication of the substrate and product between the micro- and macroenvironment the observed dependence of the enzyme activity on the concentration of these species may significantly differ from the intrinsic dependence.

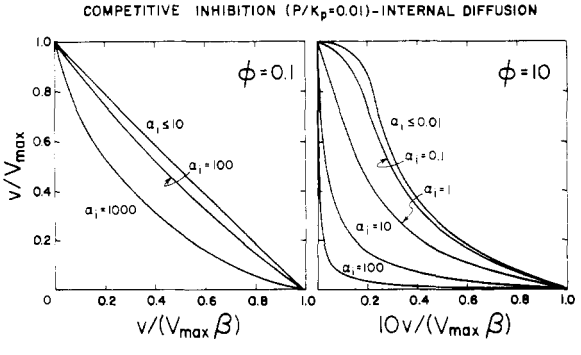

FIGURE 5: Illustration of the effect of competitive product inhibition with internal diffusion resistances by plots of the dimensionless rate of reaction, v/V_{max}, against $v/V_{max}\beta$, where β is the dimensionless substrate concentration in the macroenvironment, for different values of the internal accumulation factor, α_i, and at two values of the substrate modulus, ϕ. The dimensionless product concentration in the macroenvironment, $[P]/K_p$, is 0.01.

Plots of the effective reaction rate against the reaction rate divided by the bulk concentration of the substrate have been found most useful to characterize the type of the product inhibition or the nature of the diffusion resistances. The use of this plot to detect the accumulation and the competitive inhibitory effect of hydrogen ions in ester hydrolysis by immobilized trypsin is illustrated in Appendix II.

Working with papain membranes Goldman *et al.* (1968b) found that at low pH values the activity of immobilized papain decreases with pH to a lesser extent than that of papain in free solution. This phenomenon is readily accounted for by the antienergism between diffusional and product inhibition and can be quantitatively interpreted in view of the results of the present study. The effect of proton accumulation in the microenvironment on the pH profile of bound enzymes was treated theoretically in a previous study (Engasser and Horvath, 1974b). It has been demonstrated quantitatively that buffers present in the system can facilitate proton transport and reduce the concentration difference of H^+ between the micro- and macroenvironment.

Although electrostatic phenomena are not taken into account in this investigation, it should be kept in mind that fixed charges in the microenvironment can also have a significant effect on the kinetic behavior of bound enzymes. The magnitude of product inhibition, for instance, may be increased or decreased by the Donnan distribution of charged species between the bulk solution and an enzymic membrane depending on the sign of the charges carried by the product and the membrane (Katchalski *et al.*, 1971; Engasser and Horvath, 1974c).

The results of the present study may also be relevant to the control of metabolic events in the heterogeneous cellular milieu where product inhibition plays a major role in feedback regulation. The present regulatory schemes for metabolic pathways have almost exclusively evolved from the knowledge obtained by the investigation of the kinetics of isolated enzymes in free

solution. Diffusion resistances, however, may limit the transport of metabolites across membranes in the cell and the actual regulatory effect of product inhibition may be different from that suggested by quantitative data obtained for product inhibition with enzymes in free solution at cellular product concentrations.

Appendix I

Theoretical Model for Product Inhibition with Internal Diffusion Resistance. A bound enzyme is uniformly distributed in a membrane which is immersed in a solution of the substrate. The reaction follows Michaelis–Menten kinetics with competitive inhibition by the product. At steady state and in the absence of electrical effects the concentration change of the substrate and product in the membrane is described by the following differential equations

$$D_s \frac{d^2[S_0]}{dx^2} = \frac{V_{max}'''[S_0]}{K_M[1 + ([P_0]/K_p)] + [S_0]} \quad \text{(AI-1)}$$

$$D_p \frac{d^2[P_0]}{dx^2} = \frac{-V_{max}'''[S_0]}{K_M[1 + ([P_0]/K_p)] + [S_0]} \quad \text{(AI-2)}$$

where V_{max}''' is the saturation rate per unit volume of the membrane and x is the distance from the surface into the membrane. $[S_0]$ and $[P_0]$ are the local concentrations, D_s and D_p are the diffusivities of the substrate and the product in the membrane, respectively. By adding eq AI-1 to AI-2 we obtain the following differential relation for the concentration of the substrate and product

$$D_s \frac{d^2[S_0]}{dx^2} + D_p \frac{d^2[P_0]}{dx^2} = 0 \quad \text{(AI-3)}$$

Integration of eq AI-3 with the following boundary conditions

$$[S_0] = [S] \text{ and } [P_0] = [P] \text{ at } x = 0 \quad \text{(AI-4)}$$

$$\frac{d[S_0]}{dx} = 0 \text{ and } \frac{d[P_0]}{dx} = 0 \text{ at } x = l \quad \text{(AI-5)}$$

yields an algebraic relation between $[S_0]$ and $[P_0]$ given by

$$[P_0] = [P] + (D_s/D_p)([S] - [S_0]) \quad \text{(AI-6)}$$

where [S] and [P] are the concentrations of the substrate and the product at the membrane surface, respectively.

Combination of eq AI-1 and AI-6 gives the following equation for the dimensionless substrate concentration in the membrane

$$\frac{d^2\beta_0}{dz^2} = \frac{\phi^2\beta_0}{1 + ([P]/K_p) + \alpha_i\beta + (1 - \alpha_i)\beta_0} \quad \text{(AI-7)}$$

where z is the dimensionless distance given by

$$z = x/l \quad \text{(AI-8)}$$

ϕ is the internal substrate modulus in the absence of an inhibitor defined by

$$\phi = l(V_{max}'''/K_M D_s)^{1/2} \quad \text{(AI-9)}$$

and α_i is the internal accumulation factor given by

$$\alpha_i = D_s K_M / D_p K_p \quad \text{(AI-10)}$$

When the product is a noncompetitive inhibitor the differential equation corresponding to eq AI-7 is given by

$$\frac{d^2\beta_0}{dz^2} = \frac{\phi^2\beta_0}{1 + ([P]/K_p) + \alpha_i\beta + [1 + ([P]/K_p) + \alpha_i\beta - \alpha_i]\beta_0 - \alpha_i\beta_0^2} \quad \text{(AI-11)}$$

Equations AI-7 and AI-11 are integrated numerically first to calculate β_0, then the overall enzymatic activity, v, as a function of ϕ, α_i, β, and $[P]/K_P$.

Appendix II

Experimental Study of Product Inhibition with Supported Spherical Membranes of Immobilized Trypsin. Hydrogen ions are the product of esterolytic reactions and under circumstances act as inhibitors for the enzyme. The effect of this type of product inhibition on trypsin immobilized in a polycarboxylic gel has been investigated in a kinetic study of the hydrolysis of *N*-benzoyl-L-arginine ethyl ester.

Materials and Methods. Trypsin and *N*-benzoyl-L-arginine ethyl ester (BzArgOEt)[1] were purchased from Worthington Biochemical Co. Urea, NaCl, EDTA, and acetone were reagent grade and supplied by Fisher Scientific Co. Gantrez AN, copolymer of maleic anhydride, and vinyl methyl ether were a gift of GAF Co. Glass beads having a mean particle diameter of 60 μm were used. The pellicular trypsin resin has been prepared by the technique described previously (Horvath, 1974); 10 g of beads were coated with a mixture of 60 mg of Gantrez AN and 60 mg of hydrolyzed and lyophilized Gantrez AN in dimethylformamide solution containing acetone and the solvents were evaporated. The polycarboxylic trypsin conjugate was formed by treating 5 g of polymer coated beads with 100 mg of trypsin dissolved in 5 ml of 0.1 M phosphate buffer (pH 7.0) at 4° overnight. The product was washed with phosphate buffer until the washing showed no esterolytic activity, dried *in vacuo*, and stored at 5°.

The pH-Stat method with the instrument of the Radiometer Co. was used to measure the esterolytic cavity of both soluble and immobilized enzymes. The tryptic activity was assayed with 0.1 or 0.01 M NaOH as titrant at pH 8.0 and 25° under nitrogen and vigorous stirring. The reaction mixture (14 ml) contained 4 ml of 4 M urea, 2.5 ml of 1.7 M NaCl, 3 ml of acetone, 0.5 ml of 0.1 M EDTA, and 4 ml of BzArgOEt solution in water. Only the concentration of the BzArgOEt solution was varied in the different experiments. 50 mg of pellicular trypsin was used in each measurement. In experiments with trypsin in free solution the same conditions were employed and the amount of soluble trypsin was chosen to obtain reaction rates comparable to those measured with immobilized trypsin.

Results and Discussion. The rate of BzArgOEt hydrolysis by soluble and immobilized trypsin was measured in the substrate concentration range from 10^{-6} to 10^{-2} M and the results have been plotted according to the Eadie–Hofstee scheme as shown in Figure 7. The data obtained with trypsin in free solution give a straight line which indicates that the reaction obeys Michaelis–Menten kinetics with a K_M value equal to 7×10^{-6} M.

On the other hand, the plot of data measured with the immobilized enzyme yields a concave curve. As seen in Figure 7 the rate of reaction sharply increases at high substrate concentrations and the saturation rate is not reached even at 10^{-2} M BzArgOEt concentration. In free solution the activity of trypsin at pH 8.0 is not affected by the presence of the maleic acid–vinyl methyl ether copolymer even at ten times higher concentrations (w/v) than that of the enzyme. Therefore the possibility that a chemical effect by the conjugant is responsible for the obtained concave plot has been ruled out.

The experimental results strongly resemble the theoretically obtained concave curves shown in Figures 3 and 5 which illus-

[1] Abbreviation used is: BzArgOEt, *N*-benzoyl-L-arginine ethyl ester.

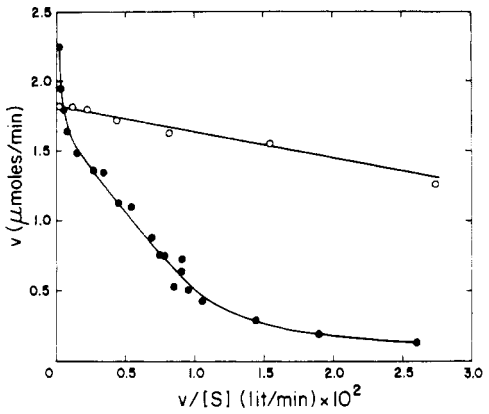

FIGURE 7: Eadie–Hofstee plots for the hydrolysis of *N*-benzoyl-L-arginine ethyl ester by trypsin immobilized in a polyanionic gel (●) and by trypsin in free solution (○) at pH 8.0. The reaction rate was measured with the pH-Stat and the mixture contained 1.2 M urea, 3.5×10^{-3} M EDTA, 0.3 M NaCl, and 22% (v/v) acetone.

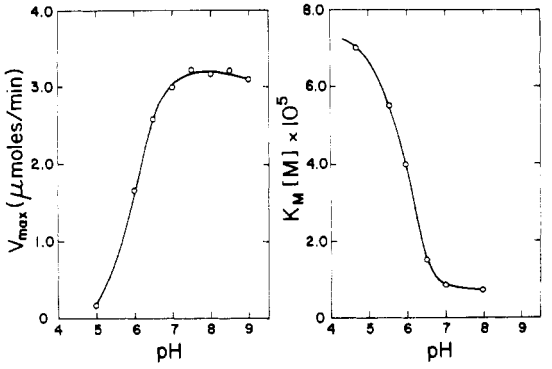

FIGURE 8: Plots of V_{max} and K_M against the pH for the tryptic hydrolysis of *N*-benzoyl-L-arginine ethyl ester in free solution. Each point on the K_M *vs.* pH curve has been determined graphically from a corresponding Eadie–Hofstee plot. The reaction rate was measured with the pH-Stat in the same mixture as stated in Figure 7.

trate the combined effect of diffusional and competitive product inhibition when the accumulation factor, α_e or α_i, is greater than unity. The effect of pH on the enzymic activity has been extensively treated in the literature. It is known that usually both kinetic parameters, V_{max} and K_M, are affected by the hydrogen ion concentration. Our experiments with the immobilized enzyme were carried out at pH 8.0, the optimum pH of the activity of trypsin in free solution. In the case of product accumulation inside the tryptic gel the local hydrogen ion concentration is greater, that is, the pH is lower than in the bulk solution. Under such conditions the effect of the hydrogen ions on the enzymic activity can be treated as a particular case of product inhibition.

In order to characterize the inhibition of trypsin by H^+ in free solution having the same composition as that used with the immobilized enzyme the pH dependence of both V_{max} and K_M has been measured and the data are plotted in Figure 8. As seen from the pH profile of the saturation rate, the effect of H^+ is tantamount to that of a noncompetitive inhibitor which reduces the value of V_{max}. The corresponding inhibition constant is estimated from the pH of the half-maximum activity as 10^{-6} M. On the other hand, the K_M of the soluble trypsin increases with decreasing pH as shown in Figure 8. Therefore the effect of H^+ between pH 5 and 8 is equivalent to that of a competitive inhibitor. The corresponding inhibition constant has been estimated by curve fitting as 3×10^{-7} M. From these results we conclude that hydrogen ions which are generated in the tryptic hydrolysis of BzArgOEt act both as competitive and noncompetitive inhibitors at pH values lower than 8.

The experimental results shown in Figure 8 were obtained with immobilized trypsin at low buffer concentrations (3.5×10^{-3} M EDTA) but at relatively high ionic strength (0.25 M NaCl). Therefore electrical effects were negligible and accumulation of hydrogen ions in the membrane due to diffusional effects could explain the observed concave curves on the *v vs.* $v/[S]$ plot, if the internal, α_i, or external, α_e, accumulation factor for the competitive inhibition by H^+ were significantly greater than unity.

In order to estimate α_i, we assume that K_M and K_p for immobilized trypsin are the same as those for soluble trypsin in the bulk solution of the same composition. Electroneutrality requires that two oppositely charged species diffuse with the same velocity (Helfferich, 1962). Therefore the effective diffusivities of H^+ and the acid formed in the reaction are assumed to be equal. Because the diffusivity of the acid is likely to be very similar to that of the substrate we assume the effective diffusivities of H^+ and BzArgOEt also to be equal. Then the value of α_i, defined by eq AI-10, is determined by the ratio K_M/K_p and we obtain that for competitive product inhibition $\alpha_i \simeq 20$. The external transport is assumed to be mainly convective in the vigorously stirred solution, therefore, the mass transfer coefficients for BzArgOEt and H^+ (h_S and h_P) have approximately the same value. Then the estimated value of α_e, defined by eq 5, is the same as that of α_i. Unfortunately the values of the substrate moduli, ϕ and μ, cannot be inferred in such a simple fashion, because V_{max}''' and the absolute value of the diffusivities are unknown.

According to the theoretical results, the combined effect of diffusional and competitive product inhibition yields concave plots in a wide range of ϕ and μ when the accumulation factor is 20. Consequently the experimental results are easily explained by this phenomenon even if noncompetitive product inhibition concurrently occurs.

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