Papain–Collodion Membranes. II. Analysis of the Kinetic Behavior of Enzymes Immobilized in Artificial Membranes*

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ABSTRACT: A theoretical treatment of the kinetic behavior of membranes with enzymic activity is presented. It is based on the assumption that a stationary state is attained within the membrane, and that the local concentrations of substrate and product are determined by the rate of the catalytic reaction and by the rates of diffusion of substrate and product. Two types of membrane are discussed. The first is a one-layer enzyme–membrane in which the enzyme is distributed homogeneously, and the second type is a two-layer enzyme–membrane in which one of the layers is devoid of enzyme. The mode of action of a one-layer enzyme–membrane separating two compartments of infinite volume (compartments 1 and 2) is analyzed for the following boundary conditions. Case Ia: The concentrations of substrate and product in compartment 1 exceed the corresponding concentrations in compartment 2. Case Ib: Substrate is present only in compartment 1, and both compartments are devoid of product. Case Ic: Both compartments contain the same concentrations of substrate and product. The mode of action of a two-layer enzyme–membrane separating two compartments of infinite volume is analyzed for a case (II) for which it is assumed that substrate is present only in compartment 1, and that both compartments are devoid of product. For all of the above cases expressions are derived for the sum of flows of substrate and product and the sum of substrate and product concentrations at any point in the membrane. With the assumption that local reaction rates obey first-order kinetics, explicit expressions are derived for the separate flows and concentration profiles of substrate and product in the membrane. The over-all rate of the membrane enzymic reaction is also evaluated. In case Ic an estimate is made of the over-all rate of enzymic reaction for a more general case in which the total enzymic reaction obeys Michaelis–Menten kinetics. A theoretical analysis is also made for an enzyme–membrane catalyzing a reaction in which acid is formed. The validity of some of the conclusions drawn was ascertained experimentally for papain–collodion membranes of different thickness acting on benzoyl-L-argininamide, benzoyl-L-arginine ethyl ester, and acetyl-L-glutamic acid diamide.

The understanding of the mode of action of enzymes embedded in native membranes requires the establishment of the correlation between enzyme activity and the flow of substrate, determination of the concentrations of substrate, product, and pH within the domain of the membrane, estimation of the rate of flow of product out of the membrane into the surrounding medium, and investigation of the effect of the structure of the membrane on its mode of action. Evaluation of the contributions of each of these parameters to enzyme activity in the organized cell structure is beyond the scope of available experimental techniques. Simple model systems in which enzymes are embedded in well-characterized synthetic membranes may be of use in the study of some of the above parameters individually, and thus may give a better insight into the factors governing the activity of enzymes in biological membranes. As a first attempt in this direction several papain–collodion membranes were prepared and their action on synthetic low molecular weight substrates was investigated (Goldman *et al.*, 1968). The pH–activity profiles of the papain–membranes with various synthetic substrates differed markedly from each other and from the corresponding normal pH–activity curves for the native enzyme. These differences could be accounted for qualitatively by the assumption that the enzyme in the membrane acts in a microenvironment different from that prevailing in the external solution. The microenvironment within the membrane is the result of a steady state which is rapidly established in the membrane phase and is characterized by a balance between the flows of substrate and the enzymic reaction. A characteristic pH gradient within the enzyme–membrane was shown to form on enzymic hydrolysis of substrates, such as benzoyl-L-arginine ethyl ester, which liberate hydrogen ions on hydrolysis.

In the present communication a theoretical analysis of the kinetic behavior of artificial membranes with enzymic activity is given. The kinetic characteristics of a one-layer enzyme–membrane, in which the embedded enzyme is homogeneously distributed, and of a two-layer enzyme–membrane, consisting of a homogeneous enzyme layer and an adjacent inert layer, was worked out in detail. The membranes were assumed to be exposed to different boundary conditions, *i.e.*, to different concentrations of substrate and product at their outer surfaces. A theoretical study enabled deduction of the

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magnitude and direction of the flows of substrate and product within the membrane. Furthermore, it was possible to derive the concentration profiles of substrate and product within the membrane for the various cases analyzed. Assuming first-order enzyme kinetics, we were able to correlate the over-all rate of enzyme–membrane reaction with the kinetic parameters of the enzyme–substrate system, the thickness of the enzyme–membrane, and the diffusion coefficient of the substrate within the membrane. An explicit expression was derived for the concentration gradient of hydrogen ions within a one-layer papain–membrane acting on benzoyl-L-arginine ethyl ester. Some of the theoretical results were confirmed experimentally for various papain–collodion membranes.

Kinetic Behavior of Membranes with Enzymic Activity. A membrane with enzymic activity immersed in a solution containing substrate represents a well-defined phase in a system consisting of the external solution and the membrane. After equilibration the concentration of substrate in the membrane equals that of the external solution, when the membrane has no enzymic activity and when no other factors, such as electrostatic field and specific adsorption, affect the substrate distribution between the two phases. The substrate concentration in a membrane possessing enzymic activity, on the other hand, differs from that of the external solution. The catalytic reaction taking place in the membrane prevents the establishment of equilibrium between internal and external solutions and leads to the appearance of local gradients in concentration of substrate and product. It is these concentration gradients which determine the rate and direction of flows of substrate and product into and out of the membrane. Symmetry requires that the flows of matter passing the membrane (J_i) be perpendicular to its surface, and that each of the J_i's has the same value at all points of the surface in a homogenous membrane (*i.e.*, a membrane possessing the same physical structure, and the same enzymic activity at each volume element).

From the laws of nonequilibrium thermodynamics (Prigogine, 1961; De Groot and Mazur, 1963) it can be readily concluded that a reactive system containing a membrane with enzymic activity immersed in a substrate solution will attain a stationary state within a relatively short time determined by the boundary conditions of the system. The stationary state is characterized by the equations $(\partial S/\partial t)_{x,y,z} = 0$ and $(\partial P/\partial t)_{x,y,z} = 0$, where S and P denote the local concentrations of substrate and of product in the membrane. The local concentrations of substrate and product do not vary with time because of two simultaneous processes acting in opposite directions at each volume element of the membrane. The disappearance of substrate as a result of the enzymic reaction is compensated by the net flow of substrate into the volume element as a result of diffusion. On the other hand, accumulation of product is counterbalanced by the diffusion of product out of the volume element. Assuming Fick's law for the diffusion of substrate and product, the relationship between the enzymic reaction and diffusion process at the stationary state can be summarized by eq 1 and 2, where f(S) denotes the local rate of

$$D_s'\frac{d^2S}{dx^2} - f(S) = 0 \tag{1}$$

$$D_p'\frac{d^2P}{dx^2} + f(S) = 0 \tag{2}$$

enzymic reaction and D_s' and D_p' are the apparent diffusion coefficients of substrate and product in the membrane. Henceforth it will be assumed that both diffusion coefficients are independent of substrate and product concentrations, and that they remain constant throughout the membrane phase. Summation of eq 1 and 2 and integration with respect to x give

$$D_s'\frac{dS}{dx} + D_p'\frac{dP}{dx} = a \tag{3}$$

where a is an integration constant. $-D_s'(dS/dx) = J_s$ and $-D_p'(dP/dx) = J_p$ represent the local flows of substrate and product per centimeters squared of membrane, respectively. Equation 3 thus shows that at the stationary state, the sum of flows of substrate and product is independent of time, and is the same at any point of the membrane. The relation between the local concentrations of substrate and product is given by eq 4, derived by integration of eq 3. The integration constants a and b are determined, as usual, by the appropriate boundary conditions.

$$D_s'S + D_p'P = ax + b \tag{4}$$

The determination of the separate flows of substrate and product at each point of the membrane, as well as the evaluation of the substrate and product concentration profiles in the membrane phase, require the definition of the local rate of enzymic reaction, *i.e.*, the definition of the function f(S). In most of the cases to be treated below it will be assumed, for the sake of simplicity, that the local rates of enzymic reaction obey first-order kinetics and thus are represented by eq 5

$$f(S) = kS \tag{5}$$

where k is a first-order reaction rate constant. The relation given by eq 5 holds for all enzymic reactions which are fully described by the Michaelis–Menten equation when the Michaelis constant, K_m(app), markedly exceeds substrate concentration. Under these conditions

$$k = k_{cat}E_0/K_m\,(\text{app}) \tag{6}$$

where E_0 denotes concentration of enzyme, and k_{cat} is the turnover number.

In the following we will discuss in some detail the mode of action of two types of membrane with enzymic activity. The first consists of a one-layer enzyme–membrane in which the enzyme is distributed homogeneously throughout the membrane, and the second type consists of a two-layer enzyme–membrane in which one of the layers is devoid of enzyme. A system describing a one-

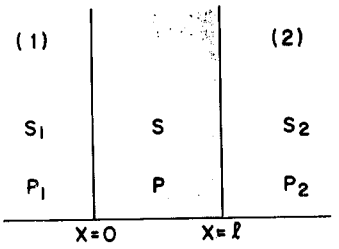

FIGURE 1: Scheme describing a one-layer enzyme–membrane of thickness l, separating the two compartments 1 and 2 each containing the corresponding concentrations of substrate (S) and product (P).

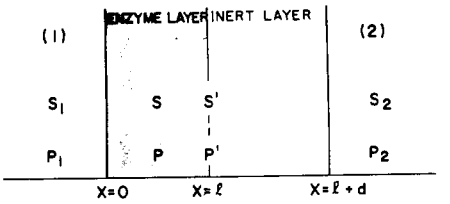

FIGURE 2: Scheme describing a two-layer enzyme membrane of thickness $l + d$, separating the two compartments 1 and 2, each containing the corresponding concentrations of substrate (S) and product (P). The two-layer enzyme membrane consists of an enzyme layer of thickness l, and an inert layer void of enzymic activity of thickness d. S' and P' denote the concentration of substrate and product, respectively, at the interface, $x = l$, between the two layers.

layer enzyme–membrane separating two compartments of infinite volume, containing well-stirred solutions of substrate and product, is given in Figure 1. A system describing a two-layer enzyme–membrane is given in Figure 2. The mode of action of a one-layer enzyme–membrane in a system of the type represented by Figure 1 was analyzed for three different boundary conditions. (Ia) The concentrations of substrate and product in compartment 1 exceed the corresponding concentrations in compartment 2, *i.e.*, $S_1 > S_2$ and $P_1 > P_2$. (Ib) Substrate is present only in compartment 1, and both compartments are devoid of product, *i.e.*, $S_1 \neq 0$ and $S_2 = P_1 = P_2 = 0$. (Ic) Both compartments contain the same concentrations of substrate and product, *i.e.*, $S_1 = S_2 = S_0$ and $P_1 = P_2 = P_0$. The mode of action of a two-layer enzyme–membrane in a system of the type represented by Figure 2 was analyzed for a case (II) for which it had been assumed that substrate is present only in compartment 1, and that both compartments are devoid of product. The boundary conditions for this case are thus similar to those assumed for case Ib. For all of the above cases expressions were derived for the sum of flows of substrate and product ($J_s + J_p$) and the sum of substrate and product concentrations ($S + P$) at any point in the membrane. By assuming that local reaction rates obey first-order kinetics we derived explicit expressions for the separate flows and concentration profiles of substrate and product in the membrane. The over-all rate of the membrane enzymic reaction was also evaluated. In case Ic an estimate was made of the over-all rate of enzymic reaction for a more general case in which the local enzymic reaction obeys Michaelis–Menten kinetics.

CASE Ia. ONE-LAYER ENZYME–MEMBRANE UNDER ASYMMETRIC BOUNDARY CONDITIONS: $S_1 > S_2, P_1 > P_2$ (SEE FIGURE 1). The integration constants a and b appearing in eq 4 are given in the present case by

$$b = D_s'S_1 + D_p'P_1 \tag{7a}$$

$$a = D_s'(S_2 - S_1)/l + D_p'(P_2 - P_1)/l \tag{7b}$$

where l denotes the thickness of the membrane. The sum of flows of substrate and product ($J_s + J_p$) at any point of the enzymically active membrane in the stationary state is given by $-a$ (see eq 3); eq 7b thus shows that $J_s + J_p$ equals the sum of the diffusion flows of substrate and product in a membrane identical in all respects with the active one but devoid of enzymic activity. It should be noted, however, that whereas in an enzymically inactive membrane constant concentration gradients of substrate and product will prevail throughout the membrane, in the system under discussion, in an enzymically active membrane, the absolute value as well as the direction of substrate and product concentration gradient will vary along the membrane, both being determined by the external boundary conditions as well as by the local rates of enzymic reaction. In the special case when $D_s' = D_p'$ the sum of substrate and product concentrations ($S + P$) at each point of the enzymically active membrane equals the respective sum in a similar membrane which is devoid of enzymic activity.

Substitution for f(S) in eq 1 by the first-order reaction rate equation, eq 5, yields a second-order differential equation, the solution of which is

$$S = A \exp(\alpha x) + B \exp(-\alpha x) \tag{8}$$

where α is defined by eq 9

$$\alpha = (k/D_s')^{1/2} \tag{9}$$

and A and B are the two integration constants, which can be evaluated by eq 8 with the boundary conditions specified for the case under discussion.

$$A = \frac{S_2 - S_1 \exp(-\alpha l)}{\exp(\alpha l) - \exp(-\alpha l)}$$

$$B = \frac{-S_2 + S_1 \exp(\alpha l)}{\exp(\alpha l) - \exp(-\alpha l)} \tag{10}$$

The explicit dependence of the local substrate concentration in the membrane upon α, x, and l, is thus given by

$$S = \frac{S_1 \sinh \alpha(l - x) + S_2 \sinh (\alpha x)}{\sinh (\alpha l)} \tag{11}$$

The flow of substrate at any point x is determined by the corresponding substrate concentration gradient

$$\frac{dS}{dx} = \frac{\alpha[S_2 \cosh (\alpha x) - S_1 \cosh \alpha(l - x)]}{\sinh (\alpha l)} \tag{12}$$

The substrate concentration gradient is negative and

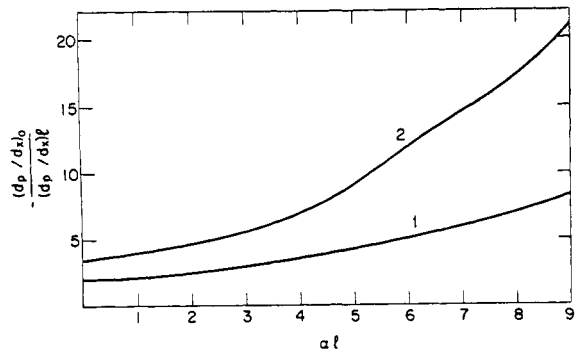

FIGURE 3: Variation with αl of the ratio of flow of product from an enzyme membrane into compartment 1, $-D_p'\cdot(dP)/(dx)_0$, to the flow of product into compartment 2, $D_p'(dP/dx)_l$. Curve 1: calculated values derived for a one-layer enzyme–membrane exposed to the boundary conditions specified in case Ib (see eq 21). Curve 2: calculated values derived for a two-layer enzyme–membrane exposed to the boundary conditions specified in case II (see eq 46).

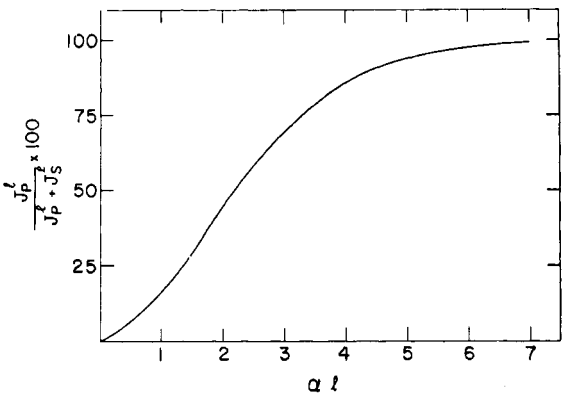

FIGURE 4: Variation with αl of the fraction of the flow of product into compartment 2 (J_p^l) out of the total flow of product and substrate ($J_p^l + J_s^l$) into the same compartment. The calculated values given were derived from eq 22 for a one-layer enzyme–membrane exposed to the asymmetric boundary conditions specified for case Ib.

the direction of flow of substrate is positive at $x = 0$, since $S_1 > S_2$. The direction of flow of substrate at $x = l$, however, is determined by the value of αl. When $S_2 \cosh(\alpha l) < S_1$ the direction of flow of substrate is positive, but when $S_2 \cosh(\alpha l) > S_1$ the direction of substrate flow is negative. The ratio of substrate flow at $x = 0$ to that at $x = l$ is symmetric with respect to S_1 and S_2 and equals 1 when $S_1 = S_2$.

The over-all rate of substrate consumption, or product formation, per centimeter squared, of membrane (V) is given by

$$V = J_s^0 - J_s = \frac{\alpha D_s'(S_1 + S_2)[\cosh(\alpha l) - 1]}{\sinh(\alpha l)} \quad (13)$$

where J_s^0 and J_s are the flows of substrate at $x = 0$ and $x = l$, respectively.

The concentration of product (P) and the concentration gradient dP/dx, prevailing at any given point of the membrane, can be readily derived from eq 3, 4, 7, 11, and 12.

$$P = -\frac{D_s'}{D_p'}\left[\frac{S_1 \sinh \alpha(l - x) + S_2 \sinh(\alpha x)}{\sinh(\alpha l)}\right] + \frac{[D_s'(S_2 - S_1) + D_p'(P_2 - P_1)]x}{D_p' l} + \frac{D_s' S_1}{D_p'} + P_1 \quad (14)$$

$$\frac{dP}{dx} = -\frac{\alpha D_s'}{D_p'}\left[\frac{S_2 \cosh(\alpha x) - S_1 \cosh \alpha(l - x)}{\sinh(\alpha l)}\right] + \frac{D_s'(S_2 - S_1) + D_p'(P_2 - P_1)}{D_p' l} \quad (15)$$

CASE Ib. ONE-LAYER ENZYME–MEMBRANE UNDER BOUNDARY CONDITIONS: $S_1 \neq 0$, $S_2 = P_1 = P_2 = 0$. The sum of flows of substrate and product at any point of the membrane is given in this case by eq 16 derived from eq 7b and 3. The flow of product at $x = l$ in an enzymically active membrane thus cannot exceed the flow of substrate in an identical membrane which is devoid of enzymic activity. In the system under investigation substrate flows unidirectionally, *via* the membrane, from compartment 1 toward compartment 2; the flow of product, on the other hand, varies in direction, product being liberated from the membrane into both compartments.

$$J_s + J_p = -D_s' S_1/l \quad (16)$$

Inserting the new boundary conditions into eq 11, 12, 14, and 15 one obtains for S, dS/dx, P, and dP/dx, the expressions

$$S = \frac{S_1 \sinh \alpha(l - x)}{\sinh(\alpha l)} \quad (17)$$

$$\frac{dS}{dx} = -\frac{\alpha S_1 \cosh \alpha(l - x)}{\sinh(\alpha l)} \quad (18)$$

$$P = -\frac{D_s' S_1}{D_p'}\left[\frac{\sinh \alpha(l - x)}{\sinh(\alpha l)} + \frac{x}{l} - 1\right] \quad (19)$$

$$\frac{dP}{dx} = \frac{D_s' S_1}{D_p'}\left[\frac{\alpha \cosh \alpha(l - x)}{\sinh(\alpha l)} - \frac{1}{l}\right] \quad (20)$$

The ratio of flow of product at $x = 0$, J_p^0, to that at $x = l$, J_p^l, can be derived from eq 20.

$$J_p^0/J_p^l = \left(\frac{dP}{dx}\right)_0 \Big/ \left(\frac{dP}{dx}\right)_l = \frac{\alpha l \cosh(\alpha l) - \sinh(\alpha l)}{\alpha l - \sinh(\alpha l)} \quad (21)$$

Equation 21 shows that the ratio J_p^0/J_p^l is determined entirely by the activity of the enzyme embedded in the membrane, and by the physical parameters of the latter (D_s' and l), but is independent of the concentration of substrate in compartment 1 (S_1). The dependence of J_p^0/J_p^l on αl is given in Figure 3. When $\alpha l \to 0$ $|J_p^0/J_p^l| \to 2$, *i.e.*, the flow of product into compartment 1 is twice its flow

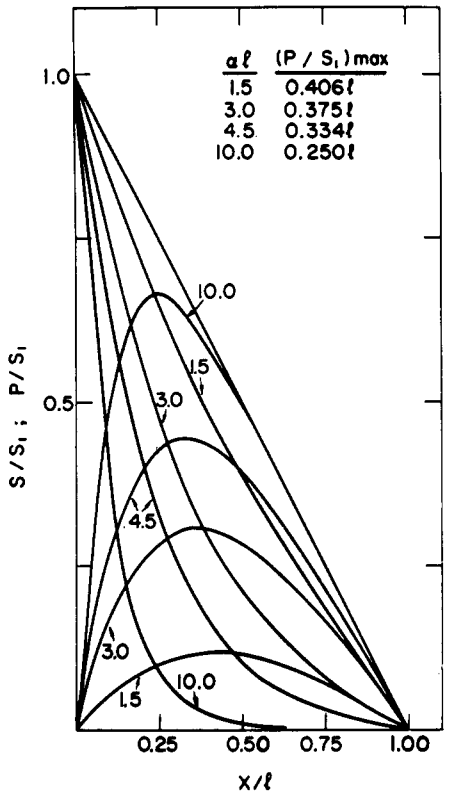

FIGURE 5: Calculated concentration profiles for substrate and product in a one-layer enzyme–membrane exposed to the asymmetric boundary conditions specified for case Ib. The local concentration of substrate (S) was calculated by making use of eq 17; the local concentration of product (P) was calculated with the aid of eq 19 assuming $D_s' = D_p'$. The arbitrary αl values chosen for the different curves presented are specified on the curves. The local concentrations of substrate and product within the membrane are expressed as fractions of the concentration of substrate (S_1) at $x = 0$.

into compartment 2 even when the enzymic activity of the membrane is very low. When αl is large ($\alpha l \geqslant 4$), $|J_p^0/J_p^l| = \alpha l - 1$, *i.e.*, the flow of product into compartment 1 markedly exceeds that into compartment 2. Thus when a solution of substrate is enclosed in a membrane bag of high enzymic activity, practically all of the product formed enzymically will flow into and accumulate in the bag.

An analysis of the flow of material into compartment 2 yields eq 22 which is graphically represented in Figure 4.

$$J_p^l/(J_p^l + J_s^l) = \frac{\sinh(\alpha l) - \alpha l}{\sinh(\alpha l)} \qquad (22)$$

The calculated data given show that practically all of the flux of material into compartment 2 consists of product when $\alpha l \geqslant 7$. The latter condition can be attained either by increasing α or by increasing the thickness of the membrane (l).

The total reaction rate (V) of a membrane with enzymic activity exposed to the boundary conditions under discussion is given by the difference between the flows of substrate at $x = 0$ and $x = l$, or by the sum of flows of

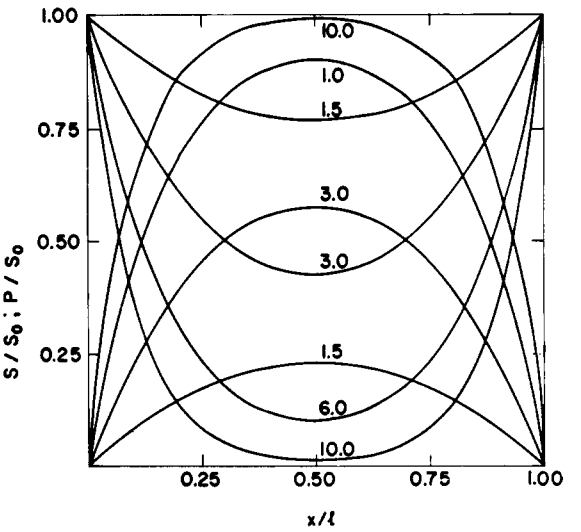

FIGURE 6: Calculated concentration profiles for substrate and product in a one-layer enzyme–membrane exposed to the symmetric boundary conditions specified for case Ic. The local concentration of substrate (S) was calculated with the aid of eq 26 assuming $D_s' = D_p'$ and $P_0 = 0$. The arbitrary αl values chosen for the different curves presented are specified on the curves. The local concentrations of substrate and product within the membrane are expressed as fractions of the concentration of substrate at the external solution (S_0).

product out of the membrane at its outer edges

$$V = J_p^l - J_p^0 = J_s^0 - J_s^l = \frac{\alpha S_1 D_s'[\cosh(\alpha l) - 1]}{\sinh(\alpha l)} \qquad (23)$$

The substrate and product concentration profiles in an enzymically active membrane, at the boundary conditions specified for case Ib, are given in Figure 5. In calculating the curves given for different αl values it was assumed that the diffusion coefficients of substrate and product are the same, *i.e.*, $D_s' = D_p'$. The profiles given for the substrate concentration reveal that at any given value of x, S decreases markedly on increasing αl. It may thus be implied that on increasing αl the thickness of the enzyme layer participating in the catalytic reaction decreases, whereas the thickness of the layer devoid of substrate increases. The product concentration profile passes through a maximum value whose coordinates are determined by αl. An increase in αl leads to a concomitant increase in the value of P_{max} and a decrease in the value of x at which P_{max} appears.

In this connection it is pertinent to recall the findings of Doscher and Richards (1963) who observed that the catalytic activity toward pyrimidine nucleoside 2′,3′-cyclic phosphates of crystalline suspensions of ribonuclease-S is markedly lower than expected. This was explained by the assumption that because of the high enzyme activity only a thin layer near the surface of the enzyme crystal has the opportunity to react with substrate whereas the rest of the crystal is devoid of substrate.

CASE Ic. ONE-LAYER ENZYME–MEMBRANE EXPOSED TO THE SYMMETRIC BOUNDARY CONDITIONS: $S_1 = S_2 = S_0$,

$P_1 = P_2 = P_0$. The sum of flows of substrate and product ($J_s + J_p$) at any point of the enzymically active membrane equals zero in the present case, since the constant a appearing in eq 7b equals zero. The flows J_s and J_p thus have the same absolute value but are opposite in direction. Because of the symmetric boundary conditions $J_s = 0$ and $J_p = 0$ at the midpoint of the membrane, *i.e.*, at $x = l/2$.

Inserting the above specified boundary conditions into eq 11 and 12, one obtains for S and (dS/dx) the expressions

$$S = \frac{S_0[\sinh(\alpha x) + \sinh \alpha(l - x)]}{\sinh(\alpha l)} \quad (24)$$

$$\frac{dS}{dx} = \frac{\alpha S_0[\cosh(\alpha x) - \cosh \alpha(l - x)]}{\sinh(\alpha l)} \quad (25)$$

Figure 6 gives the calculated concentration profiles of substrate and product in an enzymically active membrane for different αl values, assuming $D_s' = D_p'$ and $P_0 = 0$. The values of S were calculated with the aid of eq 24, whereas those of P were derived from the relation given in eq 26. The latter equation was obtained from eq 4 for the case under discussion.

$$S + P = S_0 \quad (26)$$

The profiles given show that at the midpoint of the membrane substrate concentration approaches zero, whereas product concentration approaches S_0, as αl increases from 1.5 to 10. The concentration of substrate decreases gradually toward the inner parts of the membrane for any given value of αl, and reaches minimum at $x/l = 0.5$. The calculations further show that for membranes with high enzymic activity, when $\alpha l > 6$, the volume fraction of the membrane which participates in the enzymic reaction decreased on increasing the specific activity (α) of the membrane. This is because the middle portion of membranes with high enzymic activity is devoid of substrate, and because the width of the middle portions increases on increasing α.

The over-all rate of reaction of the enzyme membrane (V) in the system Ic equals, in the stationary state, the sum of flows of substrate into the membrane at both its outer edges. Since the membrane is symmetric $J_s^0 = -J_s^l$ and V is given by

$$V = 2J_s^0 = \frac{2\alpha S_0 D_s'[\cosh(\alpha l) - 1]}{\sinh(\alpha l)} \quad (27)$$

The over-all rate in this case equals twice that derived for case Ib (see eq 23). This is so because the concentration profile of the symmetric case Ic is a superposition of two substrate concentration profiles of the asymmetric case Ib, the one prevailing in a system in which the substrate is located only in compartment 1, the other prevailing in a system in which the same substrate concentration is located only in compartment 2. At high αl values ($\alpha l \gg 4$), eq 27 reduces to

$$V = 2D_s'\alpha S_0 \quad (28)$$

The over-all reaction rate of a membrane with high enzymic activity is thus independent of the thickness of the enzyme layer, and is directly proportional to the external concentration of substrate.

At this stage it seems worthwhile to compare the over-all rate of reaction of an enzyme–membrane with that of an equal amount of soluble enzyme, both acting on substrate at concentration S_0. The reaction rate for the soluble enzyme (V_0) is

$$V_0 = klS_0 \quad (29)$$

The ratio V/V_0, denoted by f, is obtained from eq 27 and 29

$$f = V/V_0 = \frac{2[\cosh(\alpha l) - 1]}{\alpha l \sinh(\alpha l)} \quad (30)$$

The calculated dependence of f upon αl for a wide range of αl values is given in Figure 7. The calculated values presented show that f decreases markedly on increasing αl. At very low αl values ($f \to 1$) the bound enzyme of the membrane shows the same activity as an equal amount of soluble enzyme.

A similar treatment was used to characterize heterogeneous catalysis by inorganic and organic porous catalysts which proceeds by first-order kinetics (Thiele, 1939; Wheeler, 1951; Helfferich, 1962). The "degree of catalyst utilization," a variable analogous to f, was found to depend upon the "Thiele modulus," a variable analogous to α.

Above we have calculated the over-all rate of reaction of an enzymically active membrane in which the local enzymic activity can be described by a first-order reaction with respect to substrate, *i.e.*, $f(S) = kS$. Let us turn to a more general case in which $f(S)$ is given by the Michaelis–Menten equation (Michaelis and Menten, 1913; Laidler, 1958), *i.e.*, $f(S) = k_{cat}E_0S/(K_m(app) + S)$. For the stationary state eq 1 gives

$$D_s'\frac{d^2S}{dx^2} = \frac{k_{cat}E_0S}{K_m(app) + S} \quad (31)$$

Denoting dS/dx by Y so that $d^2S/dx^2 = dY/dx = YdY/dS$, one can rewrite eq 31 in the form

$$YdY = \frac{CSdS}{2(K_m(app) + S)} \quad (32)$$

where $C = 2k_{cat}E_0/D_s'$. Because of symmetry requirements the concentration gradient of substrate at the midpoint of the membrane equals zero, $(dS/dx) = Y_{l/2} = 0$. Integration of eq 32 between any given point (x) in the membrane and the midpoint ($l/2$) thus yields for the concentration gradient

$$\frac{dS}{dx} = \left\{C\left[(S - S') + K_m(app) \ln \frac{K_m(app) + S'}{K_m(app) + S}\right]\right\}^{1/2} \quad (33)$$

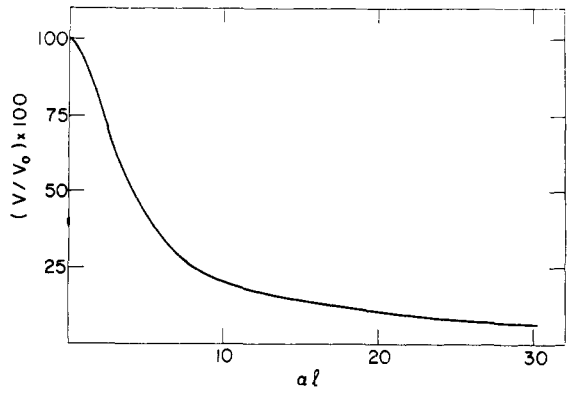

FIGURE 7: Variation with αl of the ratio of the rate of enzymic reaction (V) of a one-layer enzyme–membrane exposed to the boundary conditions specified for case Ic, to the rate of enzymic reaction (V_0) of a soluble native enzyme, in an amount equal to that present within the membrane, when exposed to similar conditions. The curve presented was calculated with the aid of eq 30.

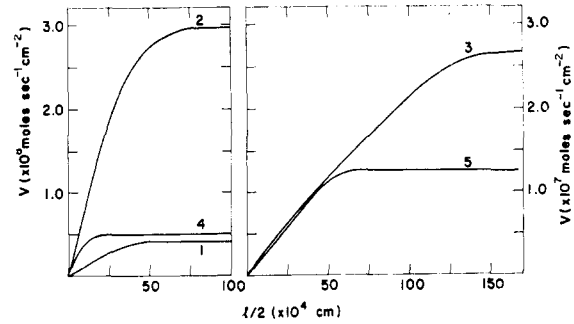

FIGURE 8: The over-all rate of hydrolysis of benzyl-L-argininamide (V) by a one-layer papain–membrane as a function of its thickness. V was calculated with the aid of eq 35, assuming k_{cat} = 8.7 sec^{-1}, K_m(app) = 0.032 M (Whitaker and Bender, 1965), and concentration of enzyme within the membrane, E_0, = 1.4 mM (see Table II). The values of S' were calculated with the aid of eq 34. Curves 1, 2, and 3 were derived for the external concentration of benzyl-L-argininamide (S_0) of 0.002, 0.016, and 0.320 M, respectively, assuming D_s' = 3 × 10^{-6} cm^2 sec^{-1}. Curves 4 and 5 were calculated for values of S_0 of 0.008 and 0.640 M, respectively, assuming D_s' = 3 × 10^{-7} cm^2 sec^{-1}.

where S' is the substrate concentration at $x = l/2$. The explicit dependence of substrate concentration S upon x cannot be derived readily from eq 33. One can obtain, however, from the numerical integration of eq 34, derived from eq 33, the relation between S' and $l/2$ for any given value of S_0.

$$C^{-1/2}\int_{S_0}^{S'}\left[S - S' + K_m(\text{app})\ln\frac{K_m(\text{app}) + S'}{K_m(\text{app}) + S}\right]^{-1/2} ds = l/2 \quad (34)$$

Numerical evaluation of S' for any given membrane of thickness l, immersed in a solution of substrate at a concentration S_0, allows calculation of the over-all rate of enzymic reaction.

$$V = -2D_s'\left(\frac{dS}{dx}\right)_0 = -\left\{8D_s'k_{cat}E_0 \times \left[S_0 - S' + K_m(\text{app})\ln\frac{K_m(\text{app}) + S'}{K_m(\text{app}) + S_0}\right]\right\}^{1/2} \quad (35)$$

To illustrate the dependence of V upon S_0 and l, V was calculated from eq 35 for a papain–membrane acting on benzoyl-L-argininamide. The parameters D_s', l, and E_0, which characterize the papain–membrane, are those of the papain–collodion membranes described previously (Goldman *et al.*, 1968). The catalytic parameters k_{cat} and K_m(app) for the papain–benzoyl-L-argininamide system were taken from the literature (Whitaker and Bender, 1965).

The data presented in Figure 8 for papain–membranes differing in their thickness, l, show that for any given value of S_0, V reaches a limiting maximum value on increasing l. This is obviously due to the fact that the concentration of substrate drops to zero at the inner layers of the enzyme–membranes. Curves 1, 2, and 3 show that for the concentrations of substrate S_0 = 0.002, 0.016, and 0.32 M, limiting values of V are attained at the corresponding values of $l/2$ = 50, 75, and 170 μ. A decrease in the apparent diffusion coefficient of the substrate in the membrane (D_s') by a factor of 10 (compare curves 1 and 4, and 3 and 5) lowers the over-all rate of membrane activity and decreases the thickness of the enzyme–membrane layer which actually participates in the catalytic reaction.

The variation of the over-all rate of reaction (V) with substrate concentration (S_0) for two hypothetical papain membranes of thickness 100 and 200 μ, acting on benzoyl-L-argininamide, is given in Figure 9. The corresponding rates of reaction of native papain are given for comparison. The rates given were normalized with respect to the rate calculated for the native enzyme and for both of the membranes at a substrate concentration of $S_0 = 10K_m$(app). It should be noted that whereas native papain reaches maximum activity (V_{max}) at $S_0 = 10K_m$(app) = 0.32 M, both membranes reach V_{max} only at higher substrate concentration. Maximum activity of an enzyme–membrane might be expected at substrate concentrations (S_0) at which the concentration of substrate at the midpoint of the membrane (S') approaches $10K_m$(app). The calculated data presented in Figure 9 show clearly that the Michaelis constant of an enzyme embedded in a membrane cannot be derived from the regular plot of V *vs.* S_0.

CASE II. TWO-LAYER ENZYME–MEMBRANE EXPOSED TO ASYMMETRIC BOUNDARY CONDITIONS: $S_1 \neq 0$, $S_2 = P_1 = P_2 = 0$. An estimate of the sum of flows of substrate and product at $x = l + d$ (see Figure 2) requires the determination of the relation between S_1 and the concentrations of substrate and product at $x = l$, S_l, and P_l, respectively. The sum of flows of substrate and product ($J_s + J_p$) at $x = l$ is given by the left-hand side of eq 36 derived from eq 3 and eq 4. $J_s + J_p$ at $x = l$ equals the sum of flows of substrate and product at $x = l + d$, the latter being given by the right-hand side of eq 36, where D_s'' and D_p'' denote the apparent diffusion coefficients of substrate and product in the inert layer.

$$[D_s'(S_1 - S_l) - D_p'P_l]/l = (D_s''S_l + D_p''P_l)/d \quad (36)$$

Equation 36 can be rewritten in the following form demonstrating the correlation between S_l, P_l, and S_1.

$$S_l(D_s'/l + D_s''/d) + P_l(D_p'/l + D_p''/d) = D_s'S_1/l \quad (37)$$

In an enzymically inactive two-layer membrane similar to the one under discussion, eq 38 holds at the stationary state, where S^* is the substrate concentration at $x = l$.

$$-D_s'(S^* - S_1)/l = D_s''S^*/d \quad (38)$$

$$S^* = D_s'S_1/l(D_s'/l + D_s''/d) \quad (38a)$$

Comparison of eq 37 with eq 38a shows that

$$S^* = S_l + P_l \quad (39)$$

when $D_s' = D_p'$ and $D'' = D_p''$. Consequently, one may expect that the sum of flows of substrate and product in an enzymically active two-layer membrane will equal the flow of substrate in a corresponding enzymically inactive system.

If the local activity of the enzyme in the two-layer enzyme–membrane obeys first-order kinetics, the concentration gradient of substrate at any point of the enzyme layer can be derived from eq 12 by substitution of S_l for S_2. The flow of substrate (J_s^l) at $x = l$ is given by eq 40 in which a substrate gradient of S_l/d was assumed to prevail in the inert part of the two-layer enzyme membrane.

$$J_s = -D_s'\left(\frac{dS}{dx}\right)_l = D_s''S_l/d \quad (40)$$

From eq 12 and 40 one obtains eq 41a and 41b for the concentration gradients of substrate at the outer edges of the enzyme layer.

$$\left(\frac{dS}{dx}\right)_l = \frac{-\alpha S_1}{(\alpha d D_s'/D_s'')\cosh(\alpha l) + \sinh(\alpha l)} \quad (41a)$$

$$\left(\frac{dS}{dx}\right)_0 = \frac{-\alpha S_1[(\alpha d D_s'/D_s'')\sinh(\alpha l) + \cosh(\alpha l)]}{(\alpha d D_s'/D_s'')\cosh(\alpha l) + \sinh(\alpha l)} \quad (41b)$$

The last two equations lead to an expression for the over-all rate of reaction of the enzymically active two-layer membrane

$$V = D_s'\left(\frac{dS}{dx}\right)_l - D_s'\left(\frac{dS}{dx}\right)_0 = \frac{\alpha S_1 D_s'[(\alpha d D_s'/D_s'')\sinh(\alpha l) + \cosh(\alpha l) - 1]}{(\alpha d D_s'/D_s'')\cosh(\alpha l) + \sinh(\alpha l)} \quad (42)$$

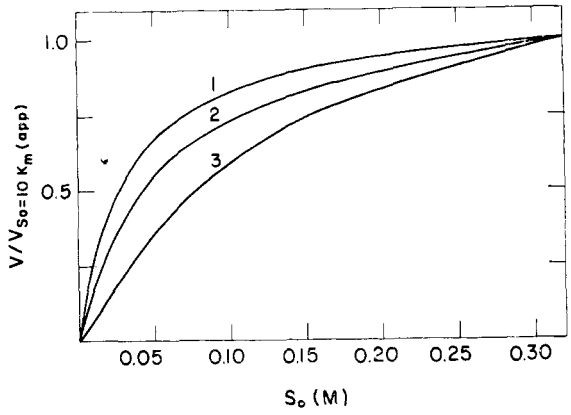

FIGURE 9: Rates of hydrolysis of benzyl-L-argininamide (V) by native papain (curve 1), or by one-layer papain–membranes 100 and 200 μ thick (curves 2 and 3, respectively), as a function of external substrate concentration (S_0). S' and V were calculated with the aid of eq 34 and 35, respectively. V for soluble papain was calculated from the Michaelis–Menten equation. The constants used for the calculated data are given in the legend for Figure 7. D_s' was taken as 3×10^{-6} cm^2 sec^{-1}. The calculated results were normalized by using the corresponding rates of hydrolysis at $S_0 = 10K_m$(app) as reference.

When the enzymic activity of the membrane is low, *i.e.*, when $\alpha l \ll 4$ and $D_s'/l = D''_s/d$, the over-all rate of reaction of the two-layer enzyme–membrane is higher than the corresponding value for a one-layer enzyme–membrane of thickness l. For example, when $\alpha l = 1$, V of the two-layer enzyme–membrane exceeds the over-all rate of the corresponding one-layer enzyme–membrane by a factor of 1.4. This is explained by the fact that the over-all concentration of substrate in the enzymic section of a two-layer enzyme–membrane is higher than its over-all concentration in a similar one-layer membrane, since $S_l \neq 0$ in the former case, whereas $S_l = 0$ in the latter case (see case Ib). The inert layer thus seems to enhance in the present case the enzymic activity of the enzyme layer. For high values of αl, *i.e.*, when $\alpha l \gg 4$, eq 42 reduces to eq 43, and the over-all rate of reaction is first order with respect to substrate and is independent of the thickness of the enzyme layer.

$$V = \alpha D_s'S_1 \quad (43)$$

The same result was obtained for the over-all rate of reaction of a one-layer enzyme membrane in which $\alpha l \gg 4$ (see eq 23 derived for case Ib).

The concentration gradient of product at any point of the enzyme layer can be derived from eq 15 by substitution of P_l for P_2. The flow of product (J_p^l) at $x = l$ is given by eq 44 in which a product gradient of P_l/d was assumed to prevail in the inert part of the two-layer enzyme–membrane.

$$J_p = -D_p'\left(\frac{dP}{dx}\right)_l = D_p''P_l/d \quad (44)$$

From eq 15 and 44 one obtains eq 45a and 45b for the concentration gradients of product at the outer edges of the enzyme layer.

$$\left(\frac{dP}{dx}\right)_l = \frac{\alpha S_1}{(\alpha d D_s'/D_s'')\cosh(\alpha l) + \sinh(\alpha l)} - \frac{S_1}{l[(D_s'd/D_s''l) + 1]} \quad (45a)$$

$$\left(\frac{dP}{dx}\right)_0 = \frac{\alpha S_1[(\alpha d D_s'/D_s'')\sinh(\alpha l) + \cosh(\alpha l)]}{(\alpha d D_s'/D_s'')\cosh(\alpha l) + \sinh(\alpha l)} - \frac{S_1}{l[(D_s'd/D_s''l) + 1]} \quad (45b)$$

From eq 45a and 45b the ratio of the flow of product at $x = 0$ to that at $x = l$, in a two-layer enzyme–membrane in which $D_p'/l = D_p''/d$, is

$$J_p^0/J_p^l = \frac{[2(\alpha l)^2 - 1]\sinh(\alpha l) + \alpha l\cosh(\alpha l)}{2\alpha l - \sinh(\alpha l) - \alpha l\cosh(\alpha l)} \quad (46)$$

The calculated values for J_p^0/J_p as a function of αl are given in Figure 3. $|J_p^0/J_p^l|$ approaches a value of 3.5 as $\alpha l \to 0$. Curve 2 of Figure 3 shows that in a two-layer enzyme–membrane of high enzymic activity ($\alpha l > 6$) most of the product flows into compartment 1. The inert layer thus acts as a barrier for the product produced within the enzymically active layer of the membrane.

Enzyme Membranes Which Catalyze Reactions in Which Acid Is Produced. In the previous section it has been tacitly assumed that the inner pH of the membrane is constant, and equals the pH of the external solutions in compartments 1 and 2. In the following section a theoretical analysis will be given for an enzyme–membrane catalyzing a reaction in which acid is formed. It will be shown that, in such a case, the pH varies across the membrane, and the over-all rate of reaction is determined not only by the parameters discussed above but also by the local hydrogen ion concentration at each infinitesimal layer of membrane. A general derivation of the over-all rate of reaction of an enzyme–membrane producing hydrogen ions as a result of the enzymic reaction is difficult because of the different dependence upon pH of the characteristic catalytic constants of the various enzymes. In the following we choose to study theoretically the mode of action of a papain–membrane acting on benzoyl-L-arginine ethyl ester hydrochloride. The treatment given can be applied to other enzyme membranes as well. The papain–membrane–benzoyl-L-arginine ethyl ester system was chosen for the following reasons. (a) The variation with pH of the kinetic parameters of the hydrolysis of benzoyl-L-arginine ethyl ester by papain has been thoroughly investigated (Whitaker and Bender, 1965). (b) A set of papain–membranes is available for which the parameters D_s', D_p', and E_0 have been evaluated. (c) In papain–collodion membranes acting on benzoyl-L-arginine ethyl ester the pH of the membranes is considerably lower than that of the external solution (Goldman *et al.*, 1965, 1968).

For the sake of simplicity only a case similar to case Ic will be discussed. For this case it will be assumed (a) that the papain–membrane–benzoyl-L-arginine ethyl ester system is at a stationary state; (b) that the catalytic parameters of the enzyme embedded in the membrane are the same as those of the native enzyme; (c) that the external pH in compartments 1 and 2 is held constant at pH 7.0; and (d) that for each mole of ester hydrolyzed, 1 mole of a fully dissociated acid is formed. The stoichiometry of the hydrolysis is given by

$$\text{benzoyl-L-arginine ethyl ester} \xrightarrow{\text{papain}} BA^{\pm} + H^+ + Cl^- + C_2H_5OH$$

where $BA^{\pm}$ denotes benzoylarginine; (e) there is no product inhibition in the pH range prevailing in the membrane. In view of these assumptions we shall identify the concentration of product (P) with the concentration of hydrogen ions (H^+), and the flow of product (J_p) with the flow of hydrogen ions (J_{H^+}).

The dependence on pH of the catalytic parameters of the hydrolysis of benzoyl-L-arginine ethyl ester by papain is given according to Whitaker and Bender (1965) by eq 47 in which k_2 denotes the rate constant for acylation of the enzyme, and K_1 and K_2 are the two ionization constants of the ionizable groups of the enzyme–substrate complex participating in this step. $k_2(\lim)$ is the maximum value of k_2 reached at neutral pH and K_s is the association constant of the enzyme–substrate complex. At neutral or acid pH values $K_2/(H^+)$ can be neglected in comparison to $1 + (H^+)/K_1$. $f(S)$ thus assumes the following form

$$\frac{k_{cat}}{K_m(app)} = \frac{k_2}{K_s} = \frac{k_2(\lim)}{(1 + (H^+)/K_1 + K_2/(H^+))K_s} \quad (47)$$

$$f(S) = \frac{k_2(\lim)E_0S}{(1 + P/K_1)[1 + S/K_m(app)]K_s} \quad (48)$$

The second derivative of P with x as a function of P and S_0 is obtained on inserting eq 48 into eq 2 and substituting for S the corresponding value of P ($P = D_s'(S_0 - S)/D_p'$) derived from eq 4 assuming $P_0 = 0$. This assumption is justified since the hydrogen ion concentration, even at the outer edges of the membrane, is considerably higher than that in the substrate solution.

$$\frac{d^2P}{dx^2} = \frac{M[(D_s'S_0/D_p') - P]}{(K_1 + P)(N + D_p'P)} \quad (49)$$

In eq 49 the constants $M = k_2(\lim)E_0K_1K_m(app)/K_s$ and $N = -D_s'(K_m(app) + S_0)$. Denoting dP/dx by Z, so that $d^2P/dx^2 = dZ/dx = Z(dZ/dP)$, eq 49 can be rewritten in the form

$$Z dZ = \frac{M[(D_s'S_0/D_p') - P]dP}{(K_1 + P)(N + D_p'P)} \quad (50)$$

Integration of the left-hand side of eq 50 with respect to Z, between Z at any given point x and Z at the midpoint of the membrane ($l/2$), where $Z_{l/2} = 0$, and integration of the right-hand side of the equation with respect to P, within the limits of P at x to P' at $l/2$, gives

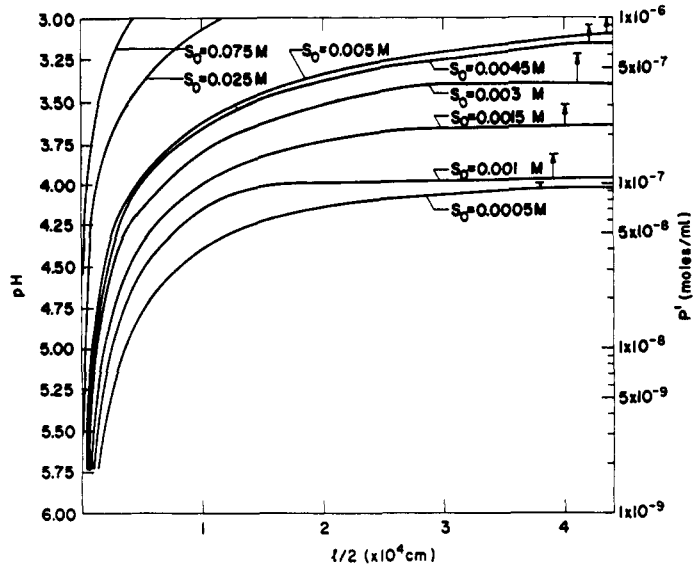

FIGURE 10: Calculated values for the concentration of product (P') at the midpoint of a one-layer papain–membrane acting on benzoyl-L-arginine ethyl ester as a function of the thickness of the membrane, l. The different values of P' were calculated with the aid of eq 52 for several external concentrations of substrate (S_0), assuming K_s = 0.055 M, K_1 = 5.13 × 10^{-5} M, k_2(lim) = 65 sec^{-1}, K_m(app) = 0.014 M (Whitaker and Bender, 1965), E_0 = 1.45 mM, D_s' = 3 × 10^{-6} cm^2 sec^{-1}, and D_p' = 15 × 10^{-6} cm^2 sec^{-1} (Goldman *et al.*, 1968). The arrow heads denote the values of P'_{max} defined in eq 53. A logarithmic scale was used for the ordinate. The ordinate on the right gives the values of P', whereas that on the left gives the corresponding values in pH units.

$$\frac{dP}{dx} = \left\{\frac{2M}{K_1D_p' - N}\left[\frac{D_s'K_m(app)}{D_p'}\ln\frac{N + D_p'P'}{N + D_p'P} + \left(\frac{D_s'S_0}{D_p'} + K_1\right)\ln\frac{K_1 + P'}{K_1 + P}\right]\right\}^{1/2} \quad (51)$$

In order to obtain an explicit expression for P as a function of x, one has to integrate eq 51. Since a complicated expression might be expected, we have limited our derivation to a numerical integration of eq 51 in the limits of $x = 0$ and $x = l/2$, in order to obtain the relation between P' and $l/2$. The values of $l/2$ given in Figure 10 were computed for the corresponding arbitrarily chosen values of P' with the aid of eq 52. It should be noted that

$$\left(\frac{2M}{K_1D_p' - N}\right)^{-1/2}\int_{P_0}^{P'}\left[\frac{D_s'K_m(app)}{D_p'}\ln\frac{N + D_p'P'}{N + D_p'P} + \left(\frac{D_s'S_0}{D_p'} + K_1\right)\ln\frac{K_1 + P'}{K_1 + P}\right]^{-1/2}dP = l/2 \quad (52)$$

the values of P' in eq 51 may vary within the limits of P_0 to P'(max). P'(max) corresponds to $S' = 0$, and is given by eq 53 which has been derived from eq 4 using the appropriate boundary conditions.

$$P'(max) = D_s'S_0/D_p' \quad (53)$$

The variation of P' with $l/2$ for various values of S_0 is illustrated in Figure 10. The curves presented show that at the constant external substrate concentrations specified, the inner pH of the hypothetical papain–membranes drops markedly as the thickness of the membrane (l) is increased from 0 to 1 to 2 μ. Further increase in l leads only to a moderate decrease in pH. The leveling off of the inner pH with l is the result of the decrease in substrate concentration in the inner layers of the membrane, as well as the concomitant drop in enzymic activity as the result of the low pH. It should be noted that the lowest pH values reached, at any given S_0, correspond to P'(max) which is directly proportional to S_0. Because of the relatively low pH values within the domain of the various hypothetical papain–membranes acting on benzoyl-L-arginine ethyl ester, relatively low over-all enzymic activities of the membrane should be expected, even when the inner concentration of substrate does not limit the rate of reaction.

The treatment given above holds strictly for a papain–membrane acting on benzoyl-L-arginine ethyl ester only when the inner pH of the membrane does not drop below pH 4.0. At pH values below 4.0 one cannot assume that the amount of hydrogen ions liberated by the enzymic reaction equals the amount of product, benzoylarginine, formed since the pK of the α-carboxyl group of the latter is 3.24 (Whitaker and Bender, 1965). Benzoylarginine is a buffer in the pH range of 3.24 ± 1 and one should therefore expect within the membrane smaller pH gradients and higher over-all reaction rates than those calculated above. A further complication stems from the fact that benzoylarginine in its unionized carboxyl form acts as a competitive inhibitor of papain (Sluyterman, 1964). In the case of a hypothetical papain–membrane immersed in a limited bath containing benzoyl-L-arginine ethyl ester, the local pH of the membrane will steadily increase as a result of the consumption of substrate in the outer solution (see Figure 10). Because of the characteristic dependence upon pH of the kinetic parameters of papain one might expect an increase with time in the specific activity of the enzyme embedded in the membrane. The rate of reaction of a papain–membrane acting on benzoyl-L-arginine ethyl ester will thus be less sensitive to the decrease in external substrate concentration than native papain acting under similar conditions.

Hydrogen ions are liberated or consumed in many enzymic reactions. For example, H^+ is generated in enzymic oxidation reactions in which NAD or NADP participate as cofactors, in the hydrolysis of carboxylic esters, thiol esters, peptide bonds, acid anhydrides, as well as in phosphorylation reactions by ATP. Since many of these reactions occur *in vivo* in membranes or on insoluble particles, one may expect local changes in pH as a

result of many local enzymic reactions. It was shown (see Figure 10) that even in relatively slow reactions one might expect a difference of 2–3 pH units across an enzyme membrane 200–300 Å thick. It thus seems plausible that marked pH variations occur in biological membranes. Such variations may play an important role in determining the permeability and transport across cell membranes (for review, see Heinz, 1967), as well as the rate of reaction of membrane-bound enzymes (Bass and Mcilroy, 1968).

Silman and Karlin (1967) have observed that a subcellular fraction containing membrane-bound acetylcholineesterase shows an anomalous pH dependence relative to soluble acetylcholineesterase when assayed in the absence of buffer in the pH-Stat. When assayed in the presence of buffer the membrane preparation was apparently activated and the pH dependence became similar to that of the soluble enzyme. The anomalous pH dependence was explained as due to local pH changes in the vicinity of the membrane-bound enzyme consequent to the hydrolysis of substrate. Based on these findings as well as those previously reported on the establishment of an acid pH within the domain of a papain–collodion membrane acting on benzoyl-L-arginine ethyl ester solution of neutral pH (Goldman *et al.*, 1965), Podleski and Changeux (1967) suggested an explanation for the depolarization by acetylcholine of the residual resting potential of M cells. Their explanation is based on the assumption that (a) the depolarization of the M cells by acetylcholine is initiated by local change of pH at the membrane level after hydrolysis of acetylcholine by the membrane-bound acetylcholineesterase; (b) the change in pH is caused by the accumulation of hydrogen ions within diffusion barriers surrounding the acetylcholineesterase molecules integrated into the membrane structure.

Experimental Section

Materials

Papain (three-times crystallized) was obtained from Worthington Biochemical Corp., Freehold, N. J. Collodion nitrocellulose (type HA 35E, lot 2-1108) was obtained from Du Pont. Benzoyl-L-arginine ethyl ester, benzoyl-L-argininamide, and acetyl-L-glutamic acid diamide were obtained from Yeda Research and Development Co., Rehovoth, Israel. Benzidine-2,2′-disulfonic acid was prepared according to the literature (Nikolenko, 1961).

Papain–Membranes. Three papain–collodion membranes were used in the experiments designed for the determination of the effect of the thickness of the enzyme–membrane on the over-all enzymic reaction. The matrix porous collodion membrane used in the preparation of all three papain–membranes was made by casting a collodion solution on rotating tubes according to the procedure of Carr and Sollner (1944) and of Gregor and Sollner (1946). The casting solution consisted of nitrocellulose (4%) in a mixture of ethanol, ether, and water (48:50:2, v/v). The porous collodion membranes, containing 90% water, were impregnated at 4° with well-stirred solutions of crystalline papain, and the adsorbed enzyme was cross-linked with bisdiazobenzidine-2,2′-disulfonic acid. The papain solutions were 0.05 M in sodium acetate buffer (pH 4.0) and 0.15 M in NaCl. The impregnation solution (50 ml) for membrane 1 (area 9.7 cm^2, average thickness 470 μ) contained 34.3 mg of crystalline papain. Membrane 1 adsorbed a maximum amount of 30.8 mg of papain within 70 hr. The membrane's adsorption capacity for papain (ρ) is thus 67.5 mg/cm^3 (enzyme concentration 3.1×10^{-3} M). The numerical value given for ρ was used in the evaluation of the thickness of the enzyme layers in membranes 2 and 3. Membranes 2 and 3 of area 10 cm^2 and average thickness 400 μ were immersed in impregnation solutions (50 ml) containing 10.6 and 3.4 mg of papain, respectively, until all of the papain was exhausted from the external solution. Enzyme-impregnated membrane 2 was found to consist of three layers: two outer papain layers each 78 μ thick, and an inner collodion layer 244 μ thick. Membrane 3 consisted also of three layers: two outer papain layers each 24.5 μ thick, and an inner collodion layer 350 μ thick. The papain-impregnated membranes were washed with water and put into 0.1 M sodium phosphate buffer (pH 7.6, 2 ml for each centimeters squared of membrane) containing approximately 10 moles of bisdiazobenzidine-2,2′-disulfonic acid/mole of enzyme adsorbed (*i.e.*, 525 μg of cross-linking reagent/cm^2 of membrane 1, 175 $\mu g/cm^2$ of membrane 2, and 60 $\mu g/cm^2$ of membrane 3). For further details concerning the structure and properties of one- and three-layer papain–membranes, see previous communication (Goldman *et al.*, 1968).

Treatment of membrane 2 with methanol according to the procedure described (Goldman *et al.*, 1968) yielded a one-layer membrane (membrane 4) used in the study of the enzymic hydrolysis of benzoyl-L-arginine ethyl ester. A two-layer papain–membrane (membrane 5) (Goldman *et al.*, 1968) (400 μ thick) containing 800 μg of papain/cm^2 in the enzyme layer (120 μ thick) was also prepared.

Assays Used in the Determination of the Rate of Enzymic Hydrolysis of Various Substrates. The enzymic hydrolysis of benzoyl-L-arginine ethyl ester was followed with a pH-Stat (automatic titrator Model TTT1C, and Titrigraph type SBR 2C, Radiometer, Copenhagen); 0.1 N NaOH was used as titrant. The rates of hydrolysis of benzoyl-L-argininamide and acetyl-L-glutamic acid diamide were determined by the Conway microdiffusion method (Conway, 1939). The outer compartment of the standard assay plate contained 0.01 M iodoacetic acid.

Experimental Results

Enzymic Activity of Papain–Collodion Membranes as a Function of αl under Boundary Conditions as in Case Ic. In case Ic it was shown theoretically that the over-all rate of reaction of an enzyme–membrane exposed to symmetric boundary conditions is determined uniquely by the parameter αl, when the local activity of the membrane is described by a reaction first order with respect to substrate. α is a function of the kinetic parameters of

TABLE I: Calculated and Experimental Data for the Activity of Papain–Collodion Membranes with Acetyl-L-glutamic Acid Diamide as Substrate.

Membrane[a]	Thickness of Papain Layer (l) ($\times 10^4$ cm)	αl[b] Derived from Amt of Papain Adsorbed (sec cm^{-1})	Reaction Rate (V)[c] (μmole min^{-1} cm^{-2})	μg of Act. cm^{-2}[d]	αl[e] Derived from Enzymic Act. (sec cm^{-1})	f[f]
1	470	1.70	0.185–0.254	1390–1910	1.16	0.90
2	156	0.56	0.057–0.068	427–514	0.39	0.99
3	49	0.18	0.011–0.016	83–118	0.12	1.00

[a] Experimental. [b] Calculated by use of eq 6 and 9 for the values of E_0 = 3.1 mM (concentration of adsorbed inactive enzyme), $D_s' = 3 \times 10^{-6}$ cm^2 sec^{-1}, K_m(app) = 0.432 M, and k_{cat} = 0.55 sec^{-1} (Blumenthal *et al.*, 1967). [c] Reaction mixture (3 ml) was 0.25 M in acetyl-L-glutamic acid diamide, 0.01 M in cysteine, and 0.004 M in EDTA and contained 1–2 cm^2 of the corresponding papain–membrane. Reaction was carried out at pH 7 at a temperature of 37°. The rate of hydrolysis was followed by the Conway (1939) method. [d] Reaction rate expressed in micrograms of native papain exposed to the same reaction mixture. Native papain (1000 μg) liberated 0.133 μmole of ammonium ions/min. [e] E_0 = 1.45 mM, an average value calculated from column 5 using the relation $E_0 = (\mu g \text{ of activity/cm}^2)/(Ml \times 1000)$; M = 20,700 (Glazer and Smith, 1961). [f] Calculated by use of eq 30.

the enzyme–substrate complex

$$\alpha = \left(\frac{k_{cat}E_0}{K_m(\text{app})D_s'}\right)^{1/2}$$

whereas l is the thickness of the membrane. The validity of the conclusions drawn in case Ic could thus be tested with the aid of three papain–collodion membranes of different thickness acting on the two substrates, acetyl-L-glutamic acid diamide and benzoyl-L-argininamide, which differ markedly in α.

The three papain–collodion membranes were a one-layer papain–membrane 470 μ thick (membrane 1, Table I); a three-layer papain–membrane ~400 μ thick, consisting of two enzyme layers 78 μ each, separated by a collodion layer (membrane 2, Table I); and a three-layer papain–membrane resembling the above three-layer membrane, but with enzyme layers 24.5 μ thick (membrane 3, Table I). The kinetic behavior of a three-layer papain–membrane, under the experimental conditions specified, is expected to be the same as that of a one-layer papain–membrane, the thickness of which is the sum of the corresponding two enzyme layers. We can thus attribute the results obtained with the three enzyme–membranes used to one-layer papain–membranes of 470, 156, and 49 μ.

The specific adsorption capacity for papain protein of the collodion matrix used in the preparation of the three membranes described was 3.1 μmoles/cm^3 (see Experimental Section and Goldman *et al.*, 1968). Because of partial enzyme inactivation as a result of adsorption and cross-linking one should expect a concentration of active enzyme (E_0) which is lower than the concentration of the protein adsorbed. The theoretical analysis of case Ic has shown that for an enzyme–membrane–substrate system for which $\alpha l < 1$ the over-all rate of enzymic reaction (V) equals the rate V_0 of the same amount of enzyme when in solution (f = 1, Figure 7), and is therefore proportional to the concentration of active enzyme, *i.e.*, $E_0 = VK_m(\text{app})/lS_0k_{cat}$. The use of a substrate in an enzyme–membrane system for which $\alpha l < 1$ thus enables the calculation of the concentration of active enzyme in the membrane. The calculated values of αl for membranes 1, 2, and 3, with acetyl-L-glutamic acid diamide as substrate, and with an assumed apparent maximum concentration of papain within the membrane (E_0) = 3.1 μmoles/cm^3, are given in column 3 of Table I. The values given for membranes 2 and 3 are markedly lower than 1.0. In this case the true concentration of active papain may be derived from their activity on acetyl-L-glutamic acid diamide. A calculation of this type has shown that the enzyme adsorbed in membranes 2 and 3 retains approximately 50% of its catalytic activity. Because of this reduction factor the real αl for membrane 1 is 1.16 (see column 6, Table I). All three membranes used thus show an activity toward acetyl-L-glutamic acid diamide similar to that of the corresponding amounts of native papain in solution. The activity per centimeters squared of the three membranes toward acetyl-L-glutamic acid diamide increases linearly with l, as expected (see column 4 of Table I).

Benzoyl-L-argininamide is a much better substrate for papain than acetyl-L-glutamic acid diamide. The calculated α values for membranes 1, 2, and 3 for this substrate are thus markedly higher than 1.0 (see column 3 of Table II). For membranes 1 and 2 $\alpha l > 4$; both membranes thus show similar activities toward benzoyl-L-argininamide, despite the difference in their thickness. Membrane 3, however, which has an αl of 1.77, hydrolyzes benzoyl-L-argininamide at a lower rate than membranes 2 and 3 (see column 6 of Table II). The ratio of the measured over-all rate of reaction to the calculated value of f (see eq 30) is proportional to l (column 7 of

TABLE II: Calculated and Experimental Data for the Activity of Papain–Collodion Membranes with Benzoyl-L-argininamide as Substrate.

Membrane[a]	Thickness of the Papain Layer (l) ($\times 10^4$ cm)	αl[b]	$(S_{l/2}/S_0) \times 100$[c]	f[d]	Reaction Rate (V)[e] (μmoles min^{-1} cm^{-2})	V/f
1	470	17.00	0.04	0.12	0.315–0.400	2.98
2	156	5.65	12.00	0.35	0.320–0.345	0.95
3	49	1.77	70.00	0.80	0.211–0.248	0.29

[a] See Experimental Section. [b] Calculated by use of eq 6 and 9 for the values of E_0 = 1.45 mM (for active enzyme concentration, see Table I), $D_a' = 3 \times 10^{-6}$ cm^2 sec^{-1} (an average value determined experimentally), $k_{cat} = 8.7$ sec^{-1}, and K_m(app) = 0.032 M (Whitaker and Bender, 1965). [c] Calculated by use of eq 24. [d] Calculated by use of eq 30. [e] Reaction mixture (10 ml) was 2 mM in benzoyl-L-argininamide, 5 mM in cysteine, 2 mM in EDTA, and 0.05 M in phosphate buffer (pH 6.15) and contained 1 cm^2 of the corresponding membrane. The reaction was carried out at 25° and was followed by the Conway (1939) method.

Table II). This is the result of the equality $V/f = V_0 \sim E_0 l$.

Hydrolysis of Benzoyl-L-Arginine Ethyl Ester by Papain–Collodion Membranes under Symmetric Boundary Conditions. The over-all rate of hydrolysis of benzoyl-L-arginine ethyl ester (V) at two different substrate concentrations, S_0 = 0.025 and 0.075 M, by the three papain–collodion membranes 1, 2, and 3, is given in Table III. The data presented show that V increases only slightly on increasing l from 49 to 470 μ. This is because most of the activity of the papain–membranes toward benzoyl-L-arginine ethyl ester resides in the outer layers, whereas the inner layers are actually devoid of activity because of the low pH prevailing in them (see Figure 10).

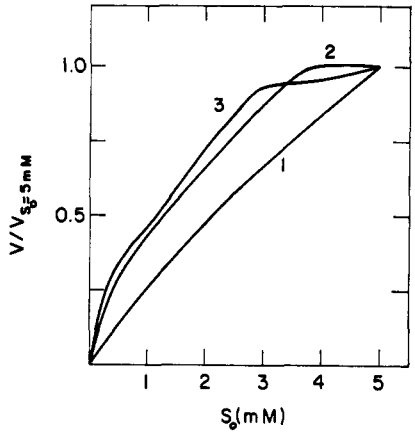

FIGURE 11: The experimental course of hydrolysis of benzoyl-L-argine ethyl ester by the papain–collodion membranes and by native papain. Curve 1 gives the experimental dependence of the reaction rate (V) upon the external concentration of substrate (S_0) for native papain. The same curve was derived from the Michaelis–Menten equation. Curves 2 and 3 give the dependence of V upon S_0 for a three-layer papain–membrane (membrane 2), and for a one-layer papain–membrane (membrane 4), respectively. The reaction mixture (5 ml, pH 7.0) was initially 5 mM in benzoyl-L-argine ethyl ester, 5 mM in cysteine, and 2mM in EDTA and contained ~1 cm^2 of the corresponding membrane, or 180 μg of native papain. The rate of hydrolysis was followed pH-statically at 30°. The results were normalized by using the corresponding rates of hydrolysis at S_0 = 5 mM as reference.

The "feed-back phenomenon" predicted from the theoretical and numerical analysis of the hydrolysis of benzoyl-L-arginine ethyl ester by papain–membranes was demonstrated experimentally in a one-layer and a three-layer papain–membrane. Curves 2 and 3 of Figure 11 show that the over-all rate of hydrolysis of benzoyl-L-arginine ethyl ester by the papain membranes, as a function of substrate concentration, differs from the substrate dependence exhibited by the soluble enzyme.

TABLE III: Over-All Rate of Hydrolysis of Benzoyl-L-arginine Ethyl Ester by Papain–Collodion Membranes.

Membrane[a]	Thickness of Papain Layer (l) ($\times 10^4$ cm)	Reaction Rate[b] (μmoles min^{-1})	
		S_0 = 0.025 M	S_0 = 0.075 M
1	470	1.55 ± 0.2	2.2 ± 0.1
2	156	1.2 ± 0.2	2.0 ± 0.1
3	49	0.8 ± 0.1	1.0 ± 0.1

[a] See Experimental Section. [b] Reaction mixture (5 ml) was S_0 in benzoyl-L-arginine ethyl ester, 5 mM in cysteine, and 2 mM in EDTA and contained 1 cm^2 of the corresponding membrane. The enzymic hydrolysis was carried out at 30° and was followed pH-statically at pH 7. When the papain membrane was substituted by 50 μg of native papain the following rates of hydrolysis were recorded: 1.18 μmoles/min when assayed with 0.025 M benzoyl-L-arginine ethyl ester, 1.82 μmoles/min when assayed with 0.075 M benzoyl-L-arginine ethyl ester.

TABLE IV: Flows of Substrate and Product in a Two-Layer Papain–Collodion Membrane Exposed to the Asymmetric Boundary Conditions Specified for Case II.

Membrane[a]	Flow of Arginine Derivatives[b] (moles min^{-1} cm^{-2})
Inactive[c]	0.29 ± 0.01 (benzoyl-L-arginine ethyl ester)
Inactive[d]	0.29 ± 0.01 (benzoyl-L-arginine)
Active[a,c,e]	0.30 ± 0.01 (benzoyl-L-arginine ethyl ester + benzoyl-L-arginine)

[a] A two-layer papain–collodion membrane (membrane 5, 3.9 cm^2, ~400 μ thick, containing 800 μg of papain/cm^2) was used. [b] The membrane was mounted between the two compartments of a diffusion cell kept at 24 ± 1°. Both compartments (containing 12 ml of solution each) were 0.1 N in phosphate buffer (pH 7). Compartment 1 was in contact with the papain layer and contained 0.1 N benzoyl-L-arginine ethyl ester or 0.1 N benzoyl-L-arginine; compartment 2 was devoid of substrate and product at the beginning of the experiment. Aliquots of 50 μl were withdrawn at 8-min intervals from both compartments and were analyzed for arginine residue by the modified Sakaguchi method (Bhattacharya *et al.*, 1958). The same molar extinction coefficients were found for benzoyl-L-arginine ethyl ester and benzoyl-L-arginine under the experimental conditions employed. [c] Compartment 1 contained benzoyl-L-arginine ethyl ester (0.1 N) at the beginning of the experiment. [d] Compartment 1 contained benzoyl-L-arginine (0.1 N) at the beginning of the experiment. [e] Activation was attained by the addition of cysteine (final concentration 5 mM) and EDTA (final concentration 2 mM) to both compartments.

In the course of hydrolysis of benzoyl-L-arginine ethyl ester, a decrease in external substrate concentration from 5 to 3 mM leads to a decrease of 30% in the activity of native papain, but to a decrease of only 5–10% in the activity of the papain–membranes.

Flows of Substrate and Product in a Two-Layer Papain–Collodion Membrane under Boundary Conditions of Case II. In the theoretical analysis of case II (see Figure 2) it was predicted that the sum of flows of substrate and product into compartment 2 in an enzymically active two-layer membrane equals the flow of substrate into the same compartment in a corresponding enzymically inactive system, when $D_s' = D_p'$ and $D_s'' = D_p''$ (see conclusion drawn from eq 39). The same rates of flow across the two-layer membrane (membrane 5) were found for the substrate benzoyl-L-arginine ethyl ester and the product benzolyarginine (see Table IV). Both compounds thus seem to possess similar diffusion coefficients in the papain layer as well as in the collodion layer. That this is the case was confirmed independently by the measurement of the rates of flow of benzoyl-L-arginine ethyl ester and benzoylarginine across a one-layer papain–membrane and a collodion membrane void of enzyme. The rate of flow of substrate and product across membrane 5 into compartment 2 (0.30 ± 0.01 mole min^{-1} cm^{-2}), under the experimental conditions used (see Table IV), was found to equal the rate of flow of substrate (0.29 ± 0.01 mole min^{-1} cm^{-2}) through the same two-layer membrane prior to its activation. In this system only product (benzoylarginine) is liberated into compartment 2, and the rate of flow of product into compartment 1 exceeds markedly the rate of flow of product into compartment 2. Separation between benzoyl-L-arginine ethyl ester and benzoylarginine, in the aliquots withdrawn for analysis, was attained by high-voltage paper electrophoresis (45 min) in pyridine–acetate buffer (pH 3.5). The spots were detected by the Sakaguchi reagent (Bhattacharya *et al.*, 1958).

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The Stereochemistry of Enzymatic Transamination*

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ABSTRACT: An approach to the determination of the complete stereochemistry of enzymatic transamination is described. Stereospecificity in the enzymatic labilization of one of the 4-methylene protons of pyridoxamine has been demonstrated in the transamination of pyridoxamine catalyzed by apoglutamate–oxaloacetate transaminase.

Both enantiomers of the 4-(CHD-NH_2) pyridoxamine have been prepared. These compounds show the expected kinetic isotope effects in the enzymatic transamination. This effect provides a convenient way to compare the symmetries of monodeuteriopyridoxamine samples derived from different enzymes. It is suggested that the symmetry of the hydrogen labilized at the pyridoxamine 4-methylene group may be related to the symmetry of the amino acid substrate. A tentative assignment of the absolute symmetry of the monodeuteriopyridoxamines is made.

The large family of enzymes utilizing pyridoxal phosphate as cofactor catalyze a great variety of transformations of amino acids (Braunstein, 1963).

In all cases the mode of action of the cofactor can be understood in terms of the original mechanism of Braunstein and Schemyakin (1953) and Snell (Metzler *et al.*, 1954).[1] In this formulation all of the enzymatic reactions involve a common intermediate, the cofactor amino acid Schiff base. The properties of reaction, substrate, and stereospecificity are then imposed on this intermediate by the apoenzyme. Given the relative simplicity of this Schiff base intermediate and the limited numbers of conformations it can assume, one may hope to achieve a real understanding of the basis for reaction and stereospecificity in this group of enzymes. In an earlier paper we suggested that reaction specificity in pyridoxal phosphate enzymes must involve enzymatic control of the amino acid C_α–N bond conformation (Dunathan, 1966). In this paper we begin to define the precise stereochemistry of enzymatic transamination.

The fundamental step of transamination is the tautomerism or 1,3-prototropic shift shown in Figure 1. This simple reaction must take place within the confines of only a few stereochemical variables. These can be listed simply: (1) the configuration at the amino acid α-carbon (C_α); (2) the configuration of the proton added to the pyridoxal carbon (C_p); (3) the conformation about the C_α–N single bond; (4) the conformation of the C_p=N double bond; and (5) the stereochemistry of the proton transfer (see Figure 1). In any real enzymatic transamination, the configuration of the amino acid is known, the conformation about the C_p=N is almost certainly "*trans*," and the C_α–N conformation is restricted to one of the two in which the C_α–H bond lies in a plane perpendicular to the plane of the cofactor π system (Dunathan, 1966).

The only real unknowns are the configuration of the proton added to the pyridoxal carbon, the choice of the C_α–N conformation, and the stereochemistry of proton transfer. This transfer will be called *cis* if the CH bond breaking and making both take place on the same side of the π system plane or *trans* if on opposite sides.

These five variables have an "algebraic" relationship to each other such that knowledge of any four of the five will define the fifth. Definition of all five will in turn define a large part of the geometry of the active site and will restrict considerably the mechanistic possibilities for the prototropic shift.

In stating these unknowns we have assumed enzymatic stereospecificity in adding and removing a proton at the pyridoxamine 4-methylene carbon. This is certainly to be expected considering the number of examples of enzymatic discrimination between the protons of a X–CH_2–Y grouping (Rose, 1966).

In this paper we describe proof of this stereospecificity at pyridoxamine and the isolation of the two monodeuteriopyridoxamine enantiomers. We have made tentative assignment of the absolute configuration of these compounds.

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[1] The requirement for pyridoxal phosphate in phosphorylase (Fisher *et al.*, 1958) cannot be explained by the Snell–Braunstein mechanism.