Kinetic Behavior of Alkaline Phosphatase–Collodion Membranes*

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ABSTRACT: A theoretical analysis of the role of the Nernst diffusion layer in determining the apparent kinetic behavior of an enzyme membrane is given. It is shown that the overall rate of reaction, V, is determined by the concentration of substrate at the membrane–solution interface, S_0, and the catalytic and physical parameters of the enzyme–membrane. The value of S_0 is smaller than that of the concentration of substrate within the bulk of the solution, S_b, and is determined by the thickness of the diffusion layer as well as the catalytic parameters of the enzyme–membrane–substrate system.

Quantitative relations between V and S_0 were derived for enzyme–membrane catalysis in the absence and presence of product inhibition. Two enzyme–membrane–substrate systems were analyzed: an alkaline phosphatase membrane acting on *p*-nitrophenyl phosphate and a papain membrane acting on benzoyl-L-argininamide. The effect of diffusion layers 10- and 50-μ thick was shown to depend primarily on the values of V and that of the apparent Michaelis constant, $K_{m(app)}$, for the enzyme–substrate couple. The contribution of the diffusion layer in determining the kinetic behavior of enzyme–membranes was demonstrated experimentally with alkaline phosphatase–collodion membranes. Three-layer alkaline phosphatase–collodion membranes, in which the enzyme layers were 1.5-, 3.0-, and 9.0-μ thick, were prepared by controlling the time of enzyme adsorption by a collodion membrane 210-μ thick. The adsorbed enzyme was less stable at elevated temperatures (60 and 80°) than the native enzyme, but could be stored at 4°, for at least 14 days without detectable loss in activity. The pH–activity profile of the alkaline phosphatase membranes, within the range of pH 8–11, using *p*-nitrophenyl phosphate as substrate, at low and high ionic strength, but in the absence of buffer, deviated from that recorded for the native enzyme. The deviation could be accounted for by assuming a local pH within the membrane of 1–2 pH units lower than that of the external solution. The above deviation was cancelled in the presence of 0.4 M borate buffer. A plot of the experimental values of V *vs.* S_b for the three alkaline phosphatase–collodion membranes prepared, showed that the $K_{m'(app)}$ values obtained (assuming $K_{m'(app)} = S_b$ at $V/V_{max} = 0.5$) are higher by a factor of 10 than those calculated for the corresponding membranes taking product inhibition into account and ignoring the presence of a diffusion layer. The apparent Michaelis constants determined experimentally were 25- to 350-fold that of the native enzyme. The above deviations could be accounted for by assuming values of 42–66 μ for the thickness of the diffusion layer adhering to the enzyme–membranes employed.

Systems consisting of enzymes embedded in synthetic membranes might serve as useful models for the study of the effect of the individual parameters of a microenvironment on the mode of action of enzymes embedded in native membranes. So far only the properties and mode of action of several papain–collodion membranes have been thoroughly investigated (Goldman *et al.*, 1965, 1968a,b). The pH–activity profiles of the papain membranes prepared, using various low molecular weight synthetic substrates, differed markedly from each other, and from the corresponding pH–activity curves of the native enzyme. These differences could be accounted for by assuming a microenvironment within the domain of the membrane which differs from that of 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.

A theoretical analysis of the kinetic characteristics of an enzyme–membrane, in which the embedded enzyme is homogeneously distributed, was worked out (Goldman *et al.*, 1968a). This study enabled the derivation of the magnitude and direction of the flows of substrate and product within the membrane at given boundary conditions. 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, it was possible to correlate the overall 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. Some of the theoretical results were confirmed experimentally for various papain–collodion membranes.

In the present communication we describe the preparation and properties of several alkaline phosphatase membranes. These membranes were characterized with respect to their structure, stability, and pH–activity profiles. Their kinetic behavior was found to be determined, in addition to the parameters investigated previously (Goldman *et al.*, 1968a), by the presence of an unstirred layer at the membrane solution interface, and by the effect of product inhibition. A theoretical analysis of the role of each of these factors in determining the kinetic behavior of an enzyme–membrane was carried out.

Role of the Nerst Diffusion Layer in Determining the Apparent Kinetic Behavior of Enzyme–Membranes. 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 of the membrane phase 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

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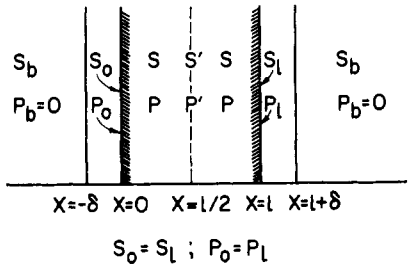

FIGURE 1: Scheme describing an enzyme membrane of thickness l, with two adhering diffusion layers of thickness δ, separating two infinite compartments containing the same concentration of substrate and devoid of product.

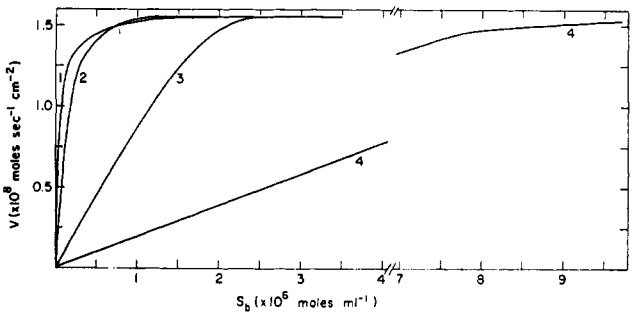

FIGURE 2: The effect of an unstirred layer on the kinetic behavior of alkaline phosphatase membranes. Curve 1 represents the Michaelis–Menten kinetics of the native enzyme (21 μg of alkaline phosphatase/ml). Curve 2 describes the behavior of a membrane 3-μ thick assuming that the concentration of substrate at the membrane–solution interface, S_0, equals that in the bulk of the solution, S_b. The curve was calculated with the aid of eq 3 and 4. Curves 3 and 4 give the behavior of enzyme membranes to which unstirred layers 10- and 50-μ thick, respectively, adhere. For the calculation of S_b, the corresponding values of S_0 and V from curve 2 were inserted into eq 6. The numerical values assigned to the various parameters appearing in the above equations were chosen to fit the system investigated experimentally: alkaline phosphatase–collodion membranes acting on *p*-nitrophenyl phosphate: k_{cat} = 63 sec^{-1} (Trentham and Gutfreund, 1968); $K_{m(app)}$ = 3.4 × 10^{-5} M; D_s' = 2.3 × 10^{-6} cm^2 sec^{-1}; D_s = 5 × 10^{-6} cm^2 sec^{-1}; E_0 = 0.82 mM (see Results and Discussion).

P are the local concentrations of substrate and product in the membrane. The local concentrations of substrate and product do not vary with time because 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. Using Fick's law to describe the diffusion of substrate and product, the relationship between the enzyme reaction, assumed to obey Michaelis–Menten kinetics, and the diffusion process at the stationary state, can be summarized by eq 1 and 2. In these equations E_0 denotes enzyme

$$D_s' \frac{d^2S}{dx^2} - \frac{k_{cat}E_0S}{K_{m(app)} + S} = 0 \quad (1)$$

$$D_p' \frac{d^2P}{dx^2} + \frac{k_{cat}E_0S}{K_{m(app)} + S} = 0 \quad (2)$$

concentration within the membrane, k_{cat} is the turnover number, $K_{m(app)}$ is the apparent Michaelis constant, and D_s' and D_p' are the apparent diffusion coefficients of substrate and product in the membrane, respectively. Henceforth, it will be assumed that the two diffusion coefficients (D_s' and D_p') are independent of substrate and product concentration. Equations 1 and 2 were solved for a given set of boundary conditions to yield explicit expressions for the overall rate of substrate consumption, and for the concentration profiles of substrate and product within the membrane (Goldman *et al.*, 1968a).

In the theoretical analysis presented it has been assumed that the concentration of substrate at the outer surfaces of an enzyme membrane of thickness l, *i.e.*, at $x = 0$ and $x = l$, is equal to that of the corresponding adjacent bulk solutions. The effect of an unstirred diffusion layer invoked as early as 1904 by Nernst (Nernst, 1904) to explain some of the characteristics of heterogeneous catalysis was assumed to be negligible. Such diffusion layers are well known from hydrodynamic studies, and their thickness was shown to depend on the conditions of shaking or stirring (Ginzburg and Katchalsky, 1963; Helfferich, 1962). The existence of an unstirred layer at the interface between an enzyme–membrane and an outer substrate solution (see Figure 1) leads to the formation of a substrate concentration gradient across the layer. The concentration of substrate at the outer surfaces of the membrane, S_0, is thus a function of the thickness of the diffusion layer, as well as the catalytic parameters of the enzyme–membrane–substrate system.

In previous publication (Goldman *et al.*, 1968a) it has been shown that in the absence of a diffusion layer, the overall reaction rate of an enzyme–membrane, V, immersed in solution of different substrate concentrations, S_0, is given by eq 3, where S' denotes the substrate concentration at $x = l/2$.

$$V = -\left\{8D_s'k_{cat}E_0\left[S_0 - S' + K_{m(app)} \ln \frac{K_{m(app)} + S'}{K_{m(app)} + S_0}\right]\right\}^{1/2} \quad (3)$$

The value of S' corresponding to any given set of values of S_0 and $l/2$ can be evaluated by a numerical intergration of eq 4, where $C = 2k_{cat}E_0/D_s'$. Equations 3 and 4 will describe

$$C^{-1/2}\int_{S_0}^{S'}\left[S - S' + K_{m(app)} \ln \frac{K_{m(app)} + S'}{K_{m(app)} + S}\right]^{1/2} dS = l/2 \quad (4)$$

the overall rate of reaction also in the presence of a diffusion layer (see Figure 1) provided that one interprets S_0 as the concentration of substrate at the two membrane–solution interfaces, *i.e.*, at $x = 0$ and $x = l$. It is obvious that because of the presence of a diffusion layer, the concentration of substrate in the bulk solution, S_b, will in general exceed S_0.

The net flow of substrate into the membrane *via* the two membrane–solution interfaces, $2J_s$, equals, at the stationary state, the overall rate of substrate consumption within the enzyme membrane, V. The flow of substrate through the diffusion layer under these conditions is given by eq 5, where

$$J_s = -D_s(S_0 - S_b)/\delta \quad (5)$$

D_s is the diffusion coefficient of substrate in the solution, and δ is the thickness of the diffusion layer (see Figure 1). Thus

$$V = 2J_s = 2D_s(S_b - S_0)/\delta \quad (6)$$

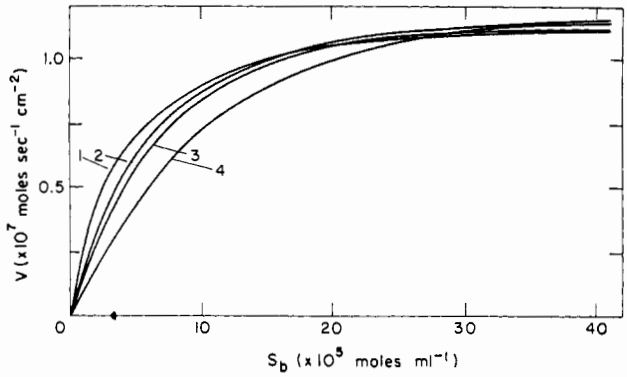

FIGURE 3: The effect of the unstirred layer on the kinetic behavior of papain membranes. Curve 1 represents the Michaelis–Menten kinetics of the native enzyme (272 μg/ml). Curve 2 describes the behavior of a membrane 100-μ thick assuming that the concentration of substrate at the membrane–solution interface, S_0, equals that of the bulk of the solution, S_b. The curve was calculated by means of eq 3 and 4. Curves 3 and 4 give the behavior of enzyme membranes to which unstirred layers of the 10- and 50-μ thick layers adhere. For the calculation of S_b, the corresponding values of S_0 and V for curve 2 were inserted into eq 6. The numerical values assigned to the various parameters appearing in the above equations were chosen to fit the papain–collodion membranes Bz-L-ArgNH$_2$ systems studied: k_{cat} = 8.7 sec^{-1}; $K_{m(app)}$ = 0.032 M (Whitaker and Bender, 1965); E_0 = 1.4 mM; D_s' = 3 × 10^{-6} cm^2 sec^{-1}; D_s = 5 × 10^{-6} cm^2 sec^{-1} (Goldman *et al.*, 1968a,b).

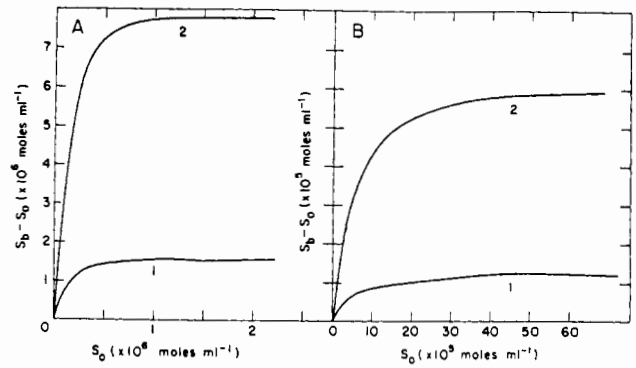

FIGURE 4: The dependence of the substrate concentration difference across the unstirred layer, $S_b - S_0$, on substrate concentration at the membrane solution interface, S_0. (A) Alkaline phosphatase membranes using *p*-nitrophenyl phosphate as substrate. (B) Papain membranes using Bz-L-ArgNH$_2$ as substrate. Curves 1 and 2 represent membranes with unstirred layers 10- and 50-μ thick, respectively. $S_b - S_0$ was calculated by means of eq 6 inserting the corresponding values of V and S_0 obtained from eq 3 and 4. The various parameters used for the calculation are given in the legends for Figures 2 and 3.

For the calculation of the reaction rate for a given bulk concentration S_b and known enzyme-kinetic parameters, it is convenient to compute V initially as a function of S_0, from eq 3 and 4. The desired relation between S_b and V is then obtained from eq 6, introducing the corresponding values of V and S_0. These calculations were carried out for two enzyme membranes of widely differing enzyme parameters. Figure 2 shows the influence of the unstirred layer on the activity of an alkaline phosphatase membrane 3-μ thick. Curve 1 describes the rate of reaction catalyzed by the native enzyme, whereas curve 2 gives the activity of the same amount of enzyme when embedded in a membrane, assuming ideal stirring at the membrane–solution interface, *i.e.*, $S_b = S_0$. Curves 3 and 4 show the drastic change in the kinetic behavior to be expected as a result of the presence of an unstirred layer.

The results of a similar calculation for a papain membrane 100-μ thick are shown in Figure 3. Curves 3 and 4 were calculated assuming unstirred layers 10- and 50-μ thick, respectively. The unstirred layers chosen in this case are the same as those given in Figure 2 for the alkaline phosphatase membrane. It should be noted that the unstirred layers specified have relatively little effect on the kinetic behavior of the papain membrane in spite of the fact that the papain membrane chosen shows a maximal activity which is approximately seven to eightfold higher than that of the alkaline phosphatase membrane. It is obvious that practically no effect of the unstirred layer on the kinetic behavior could be expected for papain membranes of an activity similar to that of the alkaline phosphatase membrane specified above. The difference in the behavior of both membranes should be attributed to the marked difference in the apparent K_m values for both enzymes, $K_{m(app)}$ = 0.032 M for papain using Bz-L-ArgNH$_2$[1] as substrate, and $K_{m(app)}$ = 3.4 × 10^{-5} M for alkaline phosphatase using *p*-nitrophenyl phosphate as substrate.

A plot of $S_b - S_0$ *vs.* S_0 for the two enzyme–membranes discussed possessing different unstirred layers is shown in Figure 4. It should be noted that in all cases $S_b - S_0$ reaches a constant value at substrate concentrations S_0 at which the membrane attains maximal activity.

In a previous article (Goldman *et al.*, 1968a), expressions were derived for the kinetic behavior of papain–collodion membranes ignoring the difference between the concentration of substrate in the bulk of the solution and that at the membrane solution interface. That this assumption is justified for the experimental conditions employed has been shown above. In general, however, one might expect that the presence of an unstirred layer will markedly affect the kinetic behavior of enzyme membranes possessing high catalytic activity and low $K_{m(app)}$ values.

Effect of Product Inhibition on Enzyme–Membrane Kinetics. Alkaline phosphatase–membranes were chosen in the present study to illustrate the role of the diffusion layer in determining the overall kinetic behavior of enzyme–membranes. Phosphate ions liberated during the hydrolysis of alkaline phosphatase substrates are known to act as competitive enzyme inhibitors (Garen and Levinthal, 1960). Product inhibition had thus to be taken into account in the analysis of the kinetic behavior of the enzyme–membranes investigated. In the following, the effect of product inhibition on the overall rate of an enzyme membrane is discussed in some detail.

The rate of substrate consumption by a native enzyme in the presence of a competitive inhibitor is given by eq 7 (Laidler, 1958), where I is the concentration of inhibitor,

$$V = \frac{k_{cat}E_0S}{K_{m(app)}\left(1 + \frac{I}{K_I}\right) + S} \qquad (7)$$

and K_I is the enzyme–inhibitor dissociation constant. The same equation describes the local reaction rate within the enzyme membrane. At the stationary state the local concen-

[1] Abbreviation used is: Bz-L-ArgNH$_2$, benzoyl-L-argininamide.

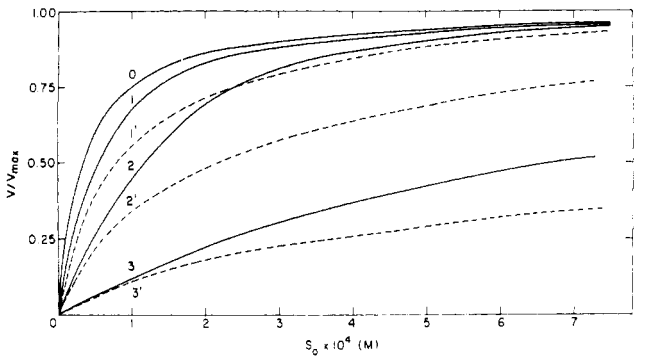

FIGURE 5: Calculated normalized rates of hydrolysis of *p*-nitrophenyl phosphate, V/V_{max}, by three alkaline phosphatase membranes of different thickness as a function of substrate concentration at the membrane–solution interface, S_0. Curves 1, 2, and 3 describe the behavior of membranes of thickness 1.5, 3, and 9 μ, respectively, ignoring product inhibition. Curves 1′, 2′, and 3′ describe the behavior of three membranes with the same parameters, taking product inhibition into account. Curve 0 gives V/V_{max} as a function of S_0 for the native enzyme. Curves 1, 2, and 3 were calculated with the aid of eq 3 and 4; curves 1′, 2′, and 3′ using eq 10 and 11. Curve 0 was derived from the Michaelis–Menten equation. The numerical values assigned to the various parameters appearing in the above equations are given in the legend to Figure 2. In addition it was assumed that $K_I = 0.32K_{m(app)}$ (Garen and Levinthal, 1960) and that $D_p' = 2D_s'$.

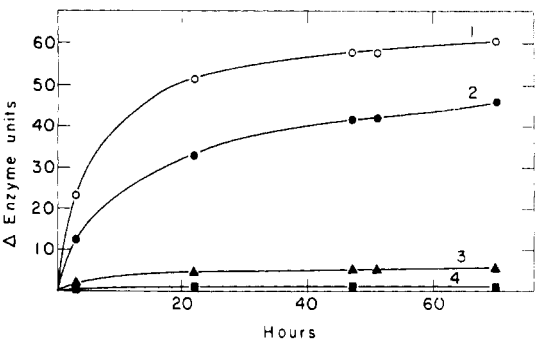

FIGURE 6: Time course of adsorption of alkaline phosphatase on collodion membranes. Membrane disks of 1.2-cm diameter and 210-μ thick were immersed into four enzyme solutions of different enzyme concentration, and enzyme adsorption carried out with stirring at 4°. The amount of enzyme adsorbed was followed by measuring the decrease in the enzymic activity of the different solutions employed. In the experiments described by curves 1, 2, 3, and 4, the initial amounts of enzyme, in enzyme units, per 2.5 ml of of 0.1 M Tris buffer, pH 8, were 182, 60.5, 5.3, and 1.1, respectively.

trations of substrate and product (inhibitor) within any given volume element of the membrane do not vary with time. The relation between the local concentrations of substrate and product for a system in which $P_0 = 0$ is given by eq 8 derived from eq 1 and 2 (see Goldman *et al.*, 1968a).

$$D_s'S + D_p'P = D_s'S_0 \tag{8}$$

Substitution in eq 1 of the term describing the enzymic reaction rate by eq 7, and of $I = P$ by the appropriate expression derived from eq 8 gives

$$D_s' \frac{d^2S}{dx^2} = \frac{D_p'k_{cat}E_0K_IS}{K_{m(app)}(D_p'K_I + D_s'S_0) + (K_ID_p' - K_{m(app)}D_s')S} \tag{9}$$

Integration of eq 9 by the procedure described in our previous article (Goldman *et al.*, 1968a, see eq 31–35) yields

$$V = -2D_s'\left\{\frac{2C}{B^2}\left[B(S_0 - S') + A \ln \frac{A + BS'}{A + BS_0}\right]\right\}^{1/2} \tag{10}$$

$$\left(\frac{2C}{B^2}\right)^{-1/2}\int_{S_0}^{S'}\left[B(S - S') + A \ln \frac{A + BS'}{A + BS}\right]^{-1/2} dS = l/2 \tag{11}$$

where $A = K_{m(app)}(D_p'K_I + D_s'S_0)$; $B = K_ID_p' - K_{m(app)} \times D_s'$; and $C = D_p'k_{cat}E_0K_I/D_s'$. Equations 10 and 11 give the overall rate of reaction of an enzyme membrane affected by product inhibition, when exposed to the symmetric boundary conditions; $S = S_0$ at $x = 0$ and at $x = l$ and $P = 0$ at $x = 0$ and at $x = l$. These equations resemble eq 3 and 4 which give the overall rate of reaction of an enzyme membrane not inhibited by the product, exposed to the same boundary conditions. In systems in which the diffusion layer might be neglected, S_0 in eq 10 and 11 equals the concentration of substrate in the bulk solution, S_b; whereas in systems in which the diffusion layer effects the overall rate of reaction, S_0 stands for the concentration of substrate at the membrane–solution interface, and as a rule $S_0 < S_b$.

A graphical presentation of the overall rate of enzyme reaction, V, as a function of S_0, for three alkaline phosphatase membranes of thickness 1.5, 3.0, and 9.0 μ, is given in Figure 5. Curves 1, 2, and 3 show that as the thickness of the enzyme membrane increases, higher concentrations of substrate are required to reach maximal overall reaction rates (V_{max}). Product inhibition leads to a further increase in the value of S_0 required to reach V_{max}.

Experimental Section

Materials and Methods. Chromatographically purified alkaline phosphatase, BAPC, lot 82A (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, *Escherichia coli*) was obtained as a suspension (10 mg/ml) in 65% saturated ammonium sulfate, from Worthington Biochemical Corp., Freehold, N. J. Assay of enzymic activity according to Malamy and Horecker (1964) yielded a specific activity of 70–85% of that recorded for the pure enzyme.

Disodium *p*-nitrophenyl phosphate, Sigma 104 phosphatase substrate, and tris(hydroxymethyl)aminomethane (Trizma base) were obtained from Sigma Chemical Co. Collodion nitrocellulose (type HA-35E, lot 2-1108) was obtained from Du Pont.

Collodion Membranes. Porous collodion sheets were prepared by the following modification of the methods of Carr and Sollner (1944) and Gregor and Sollner (1946). A solution of collodion, consisting of 4% nitrocellulose (w/v) in a mixture of ethanol–ether–water (48:50:2, v/v) was poured over a smooth glass plate (25 × 25 cm) and spread evenly with a stainless steel rod raised 1.2 mm from the surface. The solvent was allowed to evaporate for 25 min and the plate immersed in distilled water at room temperature. The collodion membrane detached from the glass plate within an hour. Collodion films thus prepared possessed an average thickness of 210 μ. Disks of 0.59 cm² were cut out from the collodion film and used in most of the experiments to be described. Disks of

1.13 cm^2 were used in the alkaline–phosphatase adsorption experiments presented in Figure 6.

Adsorption Procedure. The alkaline phosphatase collodion membranes used in most of the experiments were prepared as follows. Disks of collodion of an area of 0.59 cm^2 were immersed in a 1-ml solution of 0.1 M or 1.0 M Tris buffer, pH 8, containing 50 μg of alkaline phosphatase. Stirring was effected magnetically using a small Teflon-coated stirrer. Enzyme adsorption was carried out at 2°, for the time interval specified in the text. For the determination of the course of enzyme adsorption by the collodion membranes the conditions specified in the legend to Figure 6 were employed.

Assay of Alkaline Phosphatase. The standard assay solution (1 ml) was 5 mM in *p*-nitrophenyl phosphate and 1 M in Tris buffer, pH 8. The reaction was started by the addition of alkaline phosphatase (10 μl of a solution containing 50 μg of enzyme per ml), and the liberation of *p*-nitrophenol at 25° was followed by measuring the adsorption at 410 mμ with a Zeiss PM QII spectrophotometer. The molar extinction coefficient of *p*-nitrophenol under these conditions is 1.62×10^4. A unit of enzyme was defined as the quantity required to liberate 1 μmole of *p*-nitrophenol per min.

For the determination of the pH–activity profile of alkaline phosphatase in the absence of buffer, the following procedure was adopted. The reaction mixture was brought to the required pH by a pH-Stat using 1 N NaOH or 1 N HCl as a titrant. The pH was kept constant during the assay by means of an automatic titrator, pH-Stat (Model TTT 1C). No significant pH changes occurred within the first 5 min of reaction. The course of *p*-nitrophenyl phosphate hydrolysis was followed spectrophotometrically as above. Since the molar extinction coefficient of *p*-nitrophenol changes with pH, the suitable corrections were made.

Assay of the Enzymic Activity of Alkaline Phosphatase–Collodion Membranes. The standard assay mixture (10 ml) was 0.05 M in *p*-nitrophenyl phosphate and 1 M in Tris buffer, pH 8. The assay was carried out in a thermostated cell (25°) and was started by the addition of the membrane to the mixture. The assay mixture was stirred magnetically using a small Teflon-coated magnetic bar. Aliquots (1 ml) were withdrawn at 1- to 2-min intervals, read spectrophotometrically and returned to the reaction mixture. When the assay was carried out in the absence of buffer, the required pH was attained with a pH-Stat and the reaction was followed as described above.

Results and Discussion

Adsorption of Alkaline Phosphatase onto a Collodion Membrane. The course of adsorption of alkaline phosphatase onto a collodion membrane, 210-μ thick, as a function of the concentration of the enzyme in the adsorption solution, is shown in Figure 6. Curve 1 represents the adsorption kinetics from a solution containing a high excess of enzyme. Under these conditions saturation of the membrane was attained within 40–50 hr. The saturated membrane was found to contain 1.7 mg of enzyme per 1.13 cm^2, corresponding to an adsorption capacity for alkaline phosphatase of 70 ± 5 mg/cm^3, or to an enzyme concentration E_0 of 0.82 mM. Curve 2 represents the course of adsorption of enzyme by the collodion membrane from a solution containing initially an amount of enzyme adequate for its saturation. Approximately 75% of the enzyme was absorbed within a time interval of 70 hr. The course of enzyme adsorption by similar collodion membranes from solutions containing only a fraction of the amount of enzyme required for their saturation is given in curves 3 and 4. Most of the enzyme (95–99%) was adsorbed within 20 hr. This implies that the adsorbed enzyme is tightly bound onto the collodion matrix.

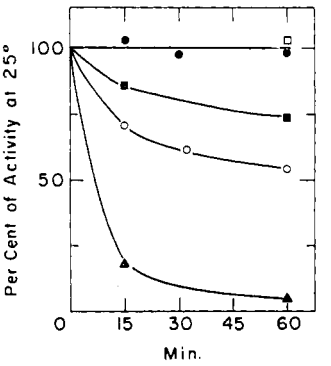

FIGURE 7: Thermal stability of alkaline phosphatase–collodion membranes and of the native enzyme in solution. Collodion disks (0.59 cm^2) were impregnated with enzyme by exposure for 30 min at 2° to a 1-ml buffer solution (1 M Tris-HCl, pH 8–0.01 M $MgCl_2$) containing 50 μg of alkaline phosphatase. After a 30-min wash in 4 ml of the buffer, each membrane was assayed at 25° using *p*-nitrophenyl phosphate as substrate (3 ml of 0.1 M *p*-nitrophenyl phosphate in the buffer solution), an activity of about 0.25 μmole min^{-1} was recorded. Each membrane was washed thoroughly and then kept in 1.5 ml of buffer at the temperature specified for the time indicated on the absissa. The enzyme activity of each of the membranes was reassayed as described above and the per cent of activity left after the heat treatment was plotted. Heating at 25 (□–□); at 60° (■–■); at 80° (▲–▲). The thermal stability of native alkaline phosphatase was determined as follows. A stock solution consisting of crystalline alkaline phosphatase dissolved in the above Tris buffer, containing about 100 μg of enzyme per ml (1 ml of this solution hydrolyzed 4 μmoles of *p*-nitrophenyl phosphate per min^{-1}) was prepared. Aliquots (1 ml) were heated for an hour at 60 or 80°. At the times specified, an aliquot of 5 μl was withdrawn and assayed at 25°. The assay solution (1 ml) consisted of 1 mM *p*-nitrophenyl phosphate in the Tris buffer solution: (●–●) and (○–○) correspond to results obtained for the soluble enzyme at 60 and 80°, respectively.

In order to determine the distribution of enzyme within the collodion matrix membranes 1 and 3, obtained after exposure to enzyme for 70 hr, were treated with glutaraldehyde (Quiocho and Richards, 1964) and embedded in paraffin; sections 5-μ thick, were stained with hematoxylene–eosin. In accord with expectation complete saturation with enzyme was observed in membrane 1. Membrane 3, on the other hand, showed a three-layer pattern, the enzyme being located in two thin and well-defined layers at the outer surfaces.

It is of interest to note that the course of alkaline phosphatase adsorption by the collodion membranes employed closely resembles that of papain (Goldman *et al.*, 1968b). Furthermore, the capacity of the collodion membranes for both enzymes is rather similar, *i.e.*, 70 ± 5 mg/cm^3 for alkaline phosphatase and 67.5 mg/cm^3 for papain.

Stability. A comparison between the thermal stability of native alkaline phosphatase and that of the enzyme adsorbed on a collodion membrane is given in Figure 7. The data presented show that at elevated temperatures (60 and 80°) the adsorbed enzyme is less stable than the native one. The enzyme–membranes as well as the native enzyme were exposed to 1 M Tris buffer, pH 8, 0.01 M in $MgCl_2$, conditions at which the native enzyme shows high thermal stability (Garen and Levinthal, 1960). Assay for enzymic activity of the buffer solutions in which the enzyme–membranes were immersed revealed that under the experimental conditions employed

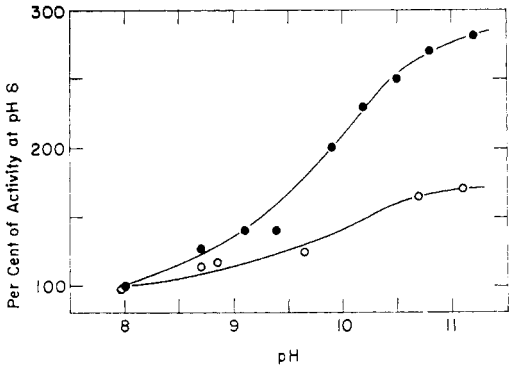

FIGURE 8: pH–activity profiles for an alkaline phosphatase–collodion membrane (●–●) and for native alkaline phosphatase (○–○) using *p*-nitrophenyl phosphate as substrate. Each of the membrane disks (0.59 cm²) used was impregnated with enzyme by exposure for 30 min at 2° to 1 ml of buffer solution (0.01 M Tris, pH 8), containing 50 μg of enzyme. After an extensive wash (at pH 8) each membrane was assayed in a series of solutions (10 ml each) 5×10^{-2} M in *p*-nitrophenyl phosphate, brought to the pH specified. Each membrane was first assayed at pH 8 and then at two additional pH values. Aliquots of 100 μl were withdrawn, mixed with 1 ml of 1 M Tris buffer, pH 8, and the optical density at 410 mμ recorded. The enzyme membranes showed at pH 8 an activity corresponding to the hydrolysis of 58 mμmoles of *p*-nitrophenyl phosphate min^{-1}. The native enzyme was assayed in 5×10^{-2} M *p*-nitrophenyl phosphate solutions (3 ml) brought to the pH values specified. The increase in optical density of the reaction mixture at 410 mμ was directly followed. The reaction rate at pH 8 of 0.5 μg ml^{-1} of enzyme corresponded to the hydrolysis of 6.8 mμmoles min^{-1} ml^{-1}. All enzymic reactions were tested at 25° and expressed as per cent of the corresponding activities at pH 8.

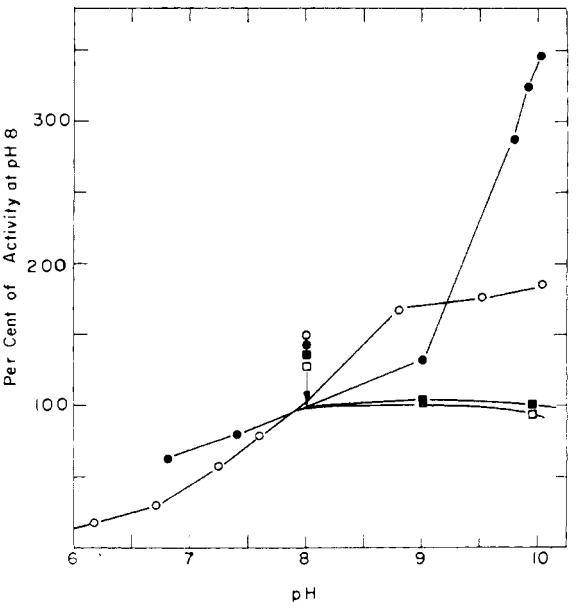

FIGURE 9: pH–activity profiles for an alkaline phosphatase collodion –membrane and for native alkaline phosphatase acting on *p*-nitrophenyl phosphate as substrate in a solution consisting of 1 M NaCl in the presence or absence of borate buffer. Each of the membrane discs used (0.59 cm²) was impregnated with enzyme for 30 min at 2° in 1 ml of buffer solution (0.01 M Tris, pH 8–1 M NaCl) containing 50 μg of alkaline phosphatase. After an extensive wash in 1 M NaCl, pH 8, each membrane was assayed at 25° in a series of solutions of 10 ml each, 5×10^{-2} M in *p*-nitrophenyl phosphate and 1 M in NaCl, at the pH specified (●–●), or in solutions containing in addition 0.4 M borate buffer of the pH specified (■–■). Each membrane was first assayed at pH 8 and then at two additional pH values. Aliquots of 100 μl were withdrawn and mixed with 1 ml of 1 M Tris buffer, pH 8, and the optical density at 410 mμ recorded. Membrane activity at pH 8 corresponded to the hydrolysis of 64 mμmoles of *p*-nitrophenyl phosphate min^{-1} (●–●) and of (■–■) 233 mμmoles of *p*-nitrophenyl phosphate min^{-1}. The assay mixture for the native enzyme (1 ml) was 5×10^{-2} M in *p*-nitrophenyl phosphate, and 1 M in NaCl (○–○), or in addition of 0.4 M borate buffer (□–□). The reaction was started by the addition of approximately 0.5 μg of enzyme (10 μl) and was followed for 4 min. At pH values below 8.0, 0.2 ml of 1 M Tris buffer, pH 8, was added after 4 min of assay and the opticai density recorded within 10 sec of mixing. Rate of reaction at pH 8 (25°) corresponded to the hydrolysis of approximately 10 mμmoles of *p*-nitrophenyl phosphate min^{-1} for the two specified conditions. All enzymic activities were expressed as per cent of the corresponding activities at pH 8.

less than 5% of the adsorbed enzyme leaked out into the buffer solution.

Enzyme–membranes possessing an activity corresponding to the hydrolysis of 0.38 μmole of *p*-nitrophenyl phosphate per min per cm² retained full activity for at least 14 days when stored in 0.1 M Tris buffer, pH 8, at a temperature of 4°.

pH–Activity Profile. The pH–activity profiles of alkaline phosphatase and of an alkaline phosphatase–collodion membrane, in the pH range 8–11 at relatively low ionic strength, is given in Figure 8. The solutions used to assay enzymic activity consisted of 5×10^{-2} M *p*-nitrophenyl phosphate and were void of other salts. The activities are expressed as per cent of the corresponding activities at pH 8. The curves presented show that whereas the activity of the native enzyme at pH 11 is 1.7-fold that at pH 8, the activity of the adsorbed enzyme at pH 11 is 2.8-fold that at pH 8. This difference might be attributed to the establishment within the membrane, as a result of the enzymic reaction, of a local pH which is lower than that of the outer solution.

The pK_a of *p*-nitrophenol is 7.15; the three pK_a values of phosphoric acid are 2.16, 7.16, and 12.4. Hydrolysis of *p*-nitrophenyl phosphate in the pH range 8–11 should thus liberate two hydrogen ions per molecule of substrate. The enzymic hydrolysis of *p*-nitrophenyl phosphate by an alkaline phosphatase membrane leads, therefore, in analogy to the hydrolysis of benzoyl-L-arginine ethyl ester by a papain–collodion membrane (Goldman *et al.*, 1968b), to a microenvironment within the domain of the membrane of lower pH than in the outer solution. The sigmoid pH–activity curve of native alkaline phosphatase can be fitted by a titration curve with a p*K* of 7.1–7.7, depending on the ionic strength (Lazdunski and Lazdunski, 1967). Relatively small shifts in pH within the membrane, at the range pH 7–8, might, therefore, markedly distort its pH–activity profile. Assuming that at the ionic strength employed the acidic dissociation constant, p*K*, of the basic group participating in enzyme activity is 7.3 (Lazdunski and Lazdunski, 1967) one should expect for native alkaline phosphatase at pH 8 an activity corresponding to approximately 80% of V_{max} to be reached at pH values above pH 9. However, if at an outer pH of 8 the pH within the enzyme–membrane is pH 7, the activity of the membrane should amount to only 40% of the V_{max} to be attained at pH values above 10.

The pH–activity profiles of an alkaline phosphatase–collodion membrane in the presence of 1 M NaCl, using *p*-nitrophenyl phosphate as substrate, and of the native enzyme acting under the same conditions, are given in Figure 9. Sodium chloride is known to enhance the activity of alkaline phosphatase (Wilson *et al.*, 1964); adsorbed enzyme acting at high ionic strength should thus release more acid per unit time than when acting at low ionic strength. A greater differ-

TABLE I: Structure and Activity of Three Alkaline Phosphatase Collodion–Membranes.

Membrane	Time of Adsorption[a] (hr)	Decrease of Enzyme Activity from the Adsorption Solution (μmoles min^{-1})	Amount of Enzyme Adsorbed[b] (μg/membrane)	Thickness of Enzyme Layer (l),[c] μ	Maximum Reaction Rate (V_{max}) of the Membrane[d] (μmoles min^{-1})
1	0.25	0.22	6.5	1.6	0.13
2	1	0.35	11.0	2.6	0.30
3	20	1.21	36.5	8.8	1.05

[a] A disk of collodion membrane of 0.59 cm^2 and 210-μ thick was immersed in 1 ml of 1 M Tris buffer, pH 8.0, containing 50 μg of alkaline phosphatase. The total enzyme activity of the solution employed at standard assay conditions corresponded to the hydrolysis of 1.67 μmoles of *p*-nitrophenyl phosphate per min. The adsorption of enzyme by the membrane was allowed to proceed at 2° for time intervals specified. [b] Calculated from initial enzymic activity and the activity left in solution at the time specified. No decrease in enzymic activity was detected in control experiments. [c] Calculated from the amount of enzyme adsorbed and the capacity of the membrane for enzyme adsorption (ρ = 70 mg/cm^3). [d] The values of V_{max} for membranes 1 and 2 were determined using 0.08 M *p*-nitrophenyl phosphate and that for membrane 3 using 0.25 M *p*-nitrophenyl phosphate.

ence between the pH–activity profile of the enzyme membrane and that of the native enzyme should therefore be expected under the conditions specified in the legend for Figure 9 than for that to be expected for the conditions specified in the legend for Figure 8. The experimental data given in Figure 9 are in accord with this conclusion.

The use of buffers of high buffering capacity eliminates differences in hydrogen ion concentration between the enzyme membrane phase and the external solution. The pH–activity profile of the alkaline phosphatase membrane in the presence of buffer thus closely resembles that of the native enzyme (see Figure 9). In this context it should be noted that the absolute rate of activity of the enzyme membrane at pH 10, in the presence of 1 M NaCl, but in the absence of buffer, is approximately the same as that recorded in the presence of buffer in the pH range 8–10.

Kinetic Behavior. Three alkaline phosphatase–collodion membranes differing in thickness of enzyme layer were prepared by varying the time of enzyme adsorption onto similar collodion disks from solutions of enzyme of the same initial concentration. Some of the characteristics of the enzyme membranes prepared are summarized in Table I. The data presented (see columns 3 and 6) show that the enzymic activity of membranes 2 and 3 correspond to approximately 90% of that to be expected from the amount of enzyme withdrawn from solution. The enzymic activity of membrane 1, containing the least amount of enzyme, corresponds to approximately 60% of that to be expected. The activity of membranes 1, 2, and 3 was determined at different substrate concentrations (see Figure 10). Assay of the enzymic activity in the presence of a large excess of *p*-nitrophenyl phosphate (S_b = 0.08 M for membranes 1 and 2, and S_b = 0.25 M for membrane 3) at the beginning and the end of a set of eight experiments (see Figure 10), revealed that the membranes lost 10–20% of their initial activity. This is most likely due to a small per cent of enzyme desorption from the membranes.

For the native enzyme, an apparent Michaelis constant of $K_{m(app)} = 3.4 \times 10^{-5}$ M was obtained. The V–S curves in an enzyme membrane do not conform to Michaelis–Menten kinetics, nevertheless it is useful to denote the concentration at $V/V_{max} = 0.5$ as $K_{m'(app)}$. A comparison of the experimentally determined values of V_{max} and $K_{m'(app)}$ for the three alkaline phosphatase membranes, with the values calculated for the corresponding hypothetical membranes is given in Table II. In deriving the values of V_{max} and of $K_{m'(app)}$ for the hypothetical membranes the characteristic kinetic parameters of alkaline phosphatase were assumed. These parameters are markedly effected by the experimental conditions of the enzyme assay (Wilson *et al.*, 1964; Lazdunski and Lazdunski, 1967). Furthermore, only approximate values of E_0 and of D_s' are available. A full agreement between the calculated values of V_{max} and those obtained experimentally can therefore hardly be expected. As a matter of fact, the calculated values of V_{max} for the three hypothetical membranes of enzyme thickness close to that of membranes 1, 2, and 3 exceed those of the corresponding experimental values only by a factor of about 2.

The data given in column 3 of Table II show that the $K_{m'(app)}$ of the three alkaline phosphatase–collodion membranes are greater by a factor of about 25, 100, and 350 than that recorded for the native enzyme. The values of $K_{m'(app)}$ given in column 6 were calculated from eq 3 and 4 using the kinetic parameters of the native enzyme (see curves 1, 2, and 3 of Figure 5). A further increase in $K_{m'(app)}$ is to be expected

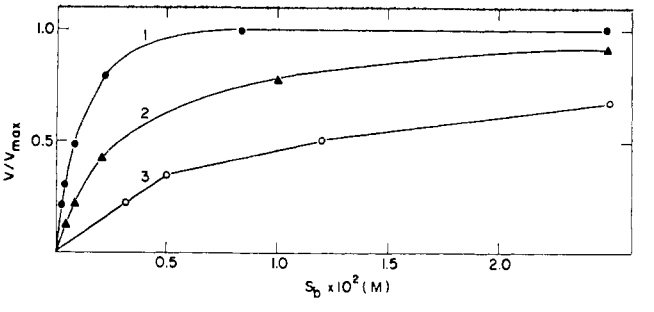

FIGURE 10: Rates of hydrolysis of *p*-nitrophenyl phosphate (V) by three alkaline phosphatase–collodion membranes as a function of external substrate concentration (S_b). Curves 1, 2, and 3 correspond to the three membranes described in Table I. Rates of hydrolysis were determined as described in the Experimental Section. Results were normalized by using the corresponding values of maximum reaction rates (V_{max}), given in the last column of Table I.

TABLE II: Experimental and Calculated Values of V_{max} and $K_{m'(app)}$ for Alkaline Phosphatase–Membranes.

Alkaline Phosphatase–Collodion Membranes			Hypothetical Alakline Phosphatase–Membranes			
Thickness of[a] Enzyme Layer (l) ($\times 10^4$ cm)	Maximum[a] Reaction Rate (V_{max}) (mμmole sec^{-1} cm^{-2})	$K_{m'(app)}$[b] ($\times 10^4$ M)	Thickness of Enzyme Layer (l) ($\times 10^4$ cm)	Maximum[c] Reaction Rate (V_{max}) (mμmole sec^{-1} cm^{-2})	$K_{m'(app)}$[d] Neglecting Product Inhibition ($\times 10^4$ M)	$K_{m'(app)}$[d] Product Inhibition Accounted for ($\times 10^4$ M)
1.6	3.7	8.5	1.5	7.8	0.5	0.8
2.6	8.5	29.0	3.0	15.6	1.2	2.2
8.8	29.7	120.0	9.0	46.9	6.8	18.0

[a] Derived from the data given in Table I. [b] Derived from the data presented in Figure 10. [c] Calculated by means of eq 3 and 4, using the parameters given in the legend for Figures 2 and 5. [d] Derived from the data presented in Figure 5.

from product inhibition. Column 7 gives $K_{m'(app)}$ allowing for this effect, according to eq 10 and 11 (see curves 1′, 2′, and 3′ of Figure 5). The difference between these values and the experimental ones may be attributed to an unstirred layer. Assuming that $K_{m'(app)}$ (experimental) $-K_{m'(app)}$ (calcd) is equal to the concentration difference across the unstirred layer at $V/V_{max} = 0.5$, one obtains for δ

$$\delta = 4D_s[\text{experimental } K_{m'(app)} - \text{calcd } K_{m'(app)}]/V_{max} \quad (12)$$

The numerical values of δ for membranes 1, 2, and 3 were obtained by inserting into eq 12 the corresponding numerical values of V_{max} and of $K_{m'(app)}$ obtained from experiment, and of the calculated values of $K_{m'(app)}$ in which product inhibition was taken into account (see last column of Table II). For D_s a value of 5×10^{-6} cm^2/sec was assumed. The estimated thickness of the unstirred layers thus obtained, $\delta = 42$–66 μ, is in the range to be expected (Helfferich, 1962; Ginzburg and Katchalsky, 1963).

The above considerations show that the difference, amounting to one to two orders of magnitude, between the experimental and the calculated $K_{m'(app)}$ values, is the result of the combined effect of concentration gradients within the membrane, product inhibition, and the presence of an unstirred layer adhering to the surface of the membrane.

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