Kinetic Behavior of Enzymes in Artificial Membranes. Inhibition and Reversibility Effects†

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ABSTRACT: The artificial binding of enzymes into artificial membranes makes possible a study of the interaction between membrane structure and enzyme kinetics within a well-defined context. Artificial proteic membranes bearing immobilized enzymes are produced by using a co-cross-linking method. The influence of competitive and noncompetitive inhibitors on the kinetic behavior of enzymes in a membrane is described. The effect of the diffusion limitations on a reversible enzyme system is observed. The studies are performed from both experimental and theoretical point of view. Some of the results obtained with artificial enzyme membranes may be used in the elucidation of the action of enzymes contained in biological membranes.

In several systems (*e.g.*, respiratory chain, fatty acid synthesis, etc.) the environment exerts a major influence on enzyme kinetics by new local conditions unpredictable from experiments performed in a homogeneous solution.

Enzyme immobilization in an artificial membrane allows for the study of the influence of the structure on enzyme behavior in a well-defined context (Thomas and Caplan, 1974). In such studies, two factors play an important part in enzyme modulation: (a) the chemical composition and physical state of the carrier itself (hydrophobic or hydrophilic properties, nature and density of fixed charges); (b) the local concentration distribution of the reactants in the carrier. The last effect is the result of a balance between the flow of matter and enzyme reactions.

The influence of the structure on enzyme behavior can be studied in any insoluble phase bearing immobilized enzymes. However the membrane is the easiest form from both experimental and theoretical point of view.

In this way, Nims (1968), Pasynski *et al.* (1964), and Blumenthal *et al.* (1967) have described associations between enzyme activities in solution and in an inert membrane, such as cellophane, to produce transport models, to study the interaction between diffusion and metabolism. Obviously, these models cannot show the resulting intramembrane phenomena.

Goldman *et al.* (1965, 1968a,b, 1971) emphasized the limiting effect of substrate accessibility and of product elimination by using collodion–papain membranes. A pH-shift effect was shown in various conditions. Thomas *et al.* (1972a) gave an analysis of the effects of the diffusion constraints on glucose oxidase kinetics cross-linked in a proteic membrane. Mattiasson and Mosbach (1971) have shown the interest of using an association of enzymes bound to particles as models of the effect of active-site vicinity on the behavior of sequential multienzyme systems. Several other papers dealt with the kinetic properties of the enzymes inside miscellaneous carriers (Kasche *et al.*, 1971; Sundaram and Laidler, 1972; Blaedel and Bogulaski, 1972; Kobayashi and Laidler, 1973). Among the above-mentioned models, those which give rise to a kinetic analysis were driven using the steady-state assumption, with irreversible monoenzyme systems in the absence of effectors. Kinetic analysis was mainly adapted to zero- or first-order reactions and not for the frequently observed michaelian reactions.

The co-cross-linking method developed by the authors (Broun *et al.*, 1973) allows the obtainment of an homogeneous distribution of sites and the immobilization of multienzyme systems inside some artificial membranes (Broun *et al.*, 1972; Lecoq *et al.*, 1974).

Numerical methods of analysis and simulation on computer (Kernevez, 1972a) take into account all experimental conditions from first- to zero-order reactions, as well as transient states.

Using these opportunities, a systematic study of enzyme behavior in artificial membranes is currently being conducted. This paper deals with some examples of systems where enzyme activity is ruled alternatively; *i.e.*, by the presence of various types of inhibitors and by the presence of a product in a reversible reaction.

Material and Methods

Membranes Production. Two previously described methods were used (Broun *et al.*, 1973; Thomas *et al.*, 1972a). In the first one, the enzyme molecules diffused inside a preformed matrix and are then cross-linked by a bifunctional agent. The second one was a co-cross-linking method, the membrane being produced with a mixture of the enzyme and of the inactive protein carrier so that the active sites were homogeneously distributed inside the matrix.

Measurement of Fluxes and Experimental Determination of Diffusion Coefficients. In order to study diffusion and diffusion reaction through a membrane, diffusion cells were used in which the film under investigation separated two compartments, each one containing a substrate solution.

One type was made of quartz. Its useful diffusion surface was 0.49 cm^2. It permitted a direct and continuous assessment of the concentration of substrates or of products in both compartment in a spectrophotometer when a specific absorption (ultraviolet or visible) can be checked.

The other types of diffusion cells were made of Altuglass. Their diffusion surfaces were respectively 50 and 2 cm^2 and their respective capacities of 250 and 50 ml.

Mixing was performed by magnetic stirring or gas bubbling. All devices were thermostatically controlled.

Fluxes were measured from time-dependent evolution of substrate (or product) concentrations.

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By using Fick's first law, mean effective experimental diffusion coefficients were calculated from the steady-state fluxes in diffusion experiments (without reaction): $\bar{D}^* = Je/\Delta c$ with J = instantaneous flux, Δc = difference in substrate concentration across of the membrane, and e = membrane thickness.

By measuring the in-going flux for $S \gg K_m$, a determination of the maximal activity V_m was obtained.

Enzyme Activity Measurements. GLUCOSE-6-PHOSPHATE ISOMERASE ACTIVITY (Sigma, St. Louis, from yeast). The enzyme activity was obtained by the glucose 6-phosphate or fructose 6-phosphate concentration measurements. Glucose 6-phosphate concentration was measured with Hyvarinen and Nikila's method (1962) and fructose 6-phosphate concentration by Roe's method (1934). Kinetic measurements were performed in a Tris-HCl buffer (0.4 M, pH 7.6) in the presence of 10^{-2} M $MgCl_2$.

URICASE ACTIVITY (Sigma, St. Louis, from *Candida utilis*): Uric acid is transformed into allantoin by uricase in the presence of oxygen. Enzyme activity was obtained by a spectrophotometric measurement of the substrate consumption at 293 nm. Kinetic measurements were performed at pH 9.3 in 0.2 M borate buffer.

LACTATE DEHYDROGENASE ACTIVITY (Sigma, St. Louis, from beef heart). The enzyme was studied with pyruvate as a substrate and tartrate as an inhibitor and reduced NAD as a cosubstrate. These kinetics were obtained from spectrophotometric measurements of the cofactor concentration evolution at 340 nm. Experiments were performed at pH 7.5, buffer 0.05 M (P_i).

INSOLUBLE ENZYME. Immersed particles and membranes were tested in a continuously stirred tank reactor (Lilly *et al.*, 1966). In both cases, the solution flows through a spectrophotometer cuvet.

Theoretical Section

Equations for the different enzyme membrane systems are given in this section. They were solved numerically on a computer (Kernevez, 1972a,b). Numerical results will be described in the next section with Experimental Results.

Irreversible Monoenzyme Membrane. Equations are given with the Michaelian assumption. The classical Michaelian kinetics cannot be used with heterogeneous systems for no unique substrate concentration value is valid inside the whole of the membrane. Nevertheless the Michaelian expression of the enzyme activity is valid *for an elementary volume.*

Let us consider a membrane of thickness e between two compartments with concentrations of substrate S_1 and S_2. $x = 0$ is chosen for the interface between membrane and compartment 1, where x refers to the distance from this origin in a direction perpendicular to the membrane surface.

For each x inside the membrane, $\partial[S]/\partial t$ is linked to the enzyme reaction and metabolite diffusion:

$$\partial[S]/\partial t = (\partial[S]/\partial t)_{diffusion} + (\partial[S]/\partial t)_{reaction} \quad (1)$$

$$(\partial[S]/\partial t)_{reaction} = -k_2 E_0[S]/(K_m + [S]) \quad (2)$$

and according to Fick's second law

$$(\partial[S]/\partial t)_{diffusion} = D_S(\partial^2[S]/\partial x^2) \quad (3)$$

where D_S is substrate diffusion coefficient (L^2T^{-1}).

The system is ruled by the equation

$$\partial[S]/\partial t = D_S\{(\partial^2[S]/\partial x^2) - [k_2 E_0[S]/(K_m + [S])]\} \quad (4)$$

and the product equation is

$$\partial[P]/\partial t = D_P\{(\partial^2[P]/\partial x^2) + [k_2 E_0[S]/(K_m + [S])]\} \quad (5)$$

where [P] = product concentration and D_P = diffusion coefficient of P.

Let us replace x by $x' = x/e$ and t by $t' = t/(e^2/D_S)$, x' and t' are dimensionless.

K_m is chosen as a concentration unit. $s = [S]/K_m$ and $p = [P]/K_m$ are substituted respectively to [S] and [P]. s and p are dimensionless.

Equation 4 can be expressed with reduced variables

$$\partial s/\partial t' - \partial^2 s/\partial x'^2 + \sigma[s/(1 + s)] = 0 \quad (6)$$

where

$$\sigma = (V_m/K_m)(e^2/D_S)$$

In the same way p concentration equation can be written as

$$\partial p/\partial t' - (D_P/D_S)(\partial^2 p/\partial x'^2) - \sigma[s/(1 + s)] = 0 \quad (7)$$

BOUNDARY CONDITIONS FOR AN IMMERSED MEMBRANE. Let us write that the substrate concentration variation in compartment 1 is equal to the flux through the interface between this compartment and the membrane. V is the bulk solution volume and Ω is the area of the membrane-solution interface (the variables are given in the reduced system).

$$V(d/dt')s(0,t') = \Omega(\partial s/\partial x')(0,t') \quad (8)$$

$$(d/dt')s(0,t') = \alpha(\partial s/\partial x')(0,t') \quad (9)$$

where

$$\alpha = (\Omega/V)2 =$$

$$\text{membrane area} \times 2/\text{volume of the bulk solution} \quad (10)$$

Boundary conditions are

$$\partial s/\partial t' + \partial s/\partial \nu = 0 \quad (11)$$

initial value of s is s_1, where

$$\partial/\partial \nu = \begin{cases} -\partial/\partial x' & \text{for } x' = 0 \\ +\partial/\partial x' & \text{for } x' = 1 \end{cases} \quad (12)$$

The boundary conditions for p are similar, but in this case α is replaced by $(D_P/D_S)\alpha$.

If the system is studied over a long period of time, it can be demonstrated that the system evolution is ruled by the following equations for the same boundary conditions (Kernevez, 1972a,b).

$$-\partial^2 s/\partial x'^2 + \sigma[s/(1 + s)] = 0 \quad (13)$$

$$\partial s/\partial t' + \alpha(\partial s/\partial \nu) = 0 \quad (14)$$

Reversible Monoenzyme System. Reaction scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P \quad (15)$$

With the quasi-stationary assumption on ES concentration, the reaction rate is

$$\left(\frac{\partial[S]}{\partial t}\right)_{reaction} = -V_m \frac{[S] - a[P]}{K_m + [S] + b[P]} \quad (16)$$

$$a = (k_{-1}/k_1)(k_{-2}/k_2) \quad (17)$$

$$b = k_{-2}/k_1$$

With the dimensionless variables, the equation becomes:

$$\frac{\partial s}{\partial t'} - \frac{\partial^2 s}{\partial x^2} + \sigma \frac{s - ap}{1 + s + bp} = 0 \quad (18)$$

Boundary and initial conditions are the same as in the irreversible system.

Monoenzyme System with Inhibitors or Activators. COMPETITIVE INHIBITION. By a way similar to the above-described one, it is possible to write equations

$$\frac{\partial s}{\partial t} - \frac{\partial^2 s}{\partial x^2} + \sigma \frac{s}{1 + (K_m/K_i)i} = 0 \quad (19)$$

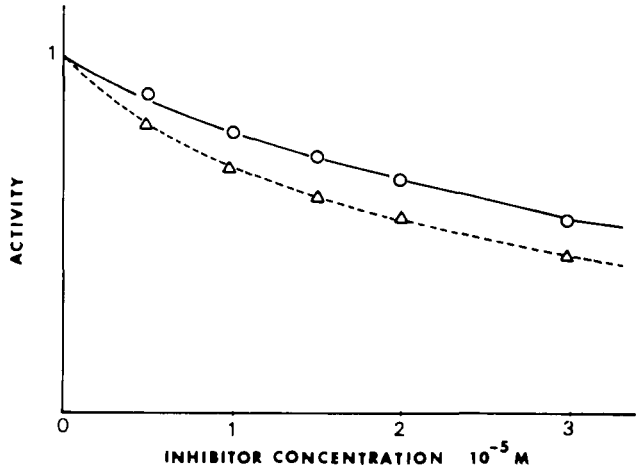

FIGURE 1: Experimental uricase activity as a function of the xanthine concentration for a constant substrate (urate) concentration ($2K_m$). Enzyme in solution (- - -) and inside a membrane (—). The enzyme activity without inhibitor is the unit for the activity rate in both cases.

(for the substrate), with $i = [I]/K_m$, K_i is the inhibition constant.

$$\frac{\partial i}{\partial t} - \frac{D_I}{D_S}\frac{\partial^2 i}{\partial x^2} = 0 \qquad (20)$$

(for the inhibitor)

NONCOMPETITIVE INHIBITION

$$\frac{\partial s}{\partial t} - \frac{\partial^2 s}{\partial x^2} + \sigma\frac{1}{1 + (K_m/K_i)i}\frac{s}{1 + s} = 0 \qquad (21)$$

(for the substrate)

$$\frac{\partial i}{\partial t} - \frac{D_I}{D_S}\frac{\partial^2 i}{\partial x^2} = 0 \qquad (22)$$

(for the inhibitor)

ACTIVATOR

$$\frac{\partial s}{\partial t} - \frac{\partial^2 s}{\partial x^2} + \sigma\frac{a}{1 + a}\frac{s}{1 + s} = 0 \qquad (23)$$

(for the substrate), with $a = [A]/K_m$.

$$\frac{\partial a}{\partial t} - \frac{D_A}{D_S}\frac{\partial^2 a}{\partial x^2} = 0 \qquad (24)$$

(for the activator)

Initial and boundary conditions for the different substances were described in eq 11-14.

Experimental Results and Numerical Simulation

Irreversible Monoenzyme Systems with Competitive and Noncompetitive Inhibitors. COMPETITIVE INHIBITOR. The effect of xanthine on the kinetic behavior of urate oxidase was studied with the enzyme in solution and immobilized in an artificial membrane. The enzyme immobilization was produced with an activity yield of 5% of the initial activity.

(i) The experimental reaction rate was studied for free and immobilized uricase as a function of the xanthine concentration in the bulk solution with a constant uric acid concentration ($2K_m$). This relationship appears in Figure 1. The activity is given as a fraction of the activity without inhibitor

Each reaction rate value is obtained from the initial slope of the optical density *vs.* time curves recorded with the device described in the Material and Methods section.

Uricase activity is less sensitive to the inhibition inside the membrane. This result can be explained by the rules controlling heterogeneous enzyme kinetics. The slower the diffusion

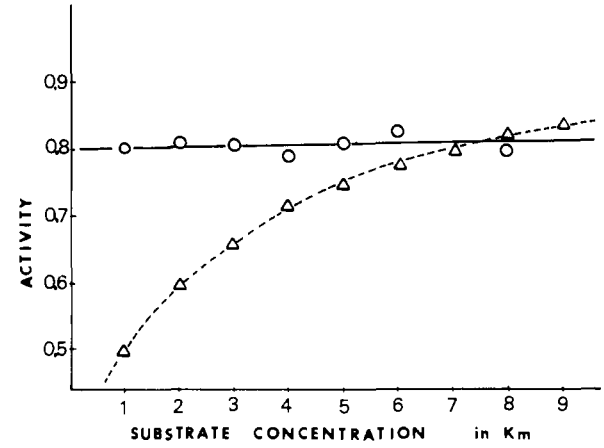

FIGURE 2: Experimental uricase activity as a function of uric acid concentration for a constant xanthine concentration ($2K_i$). Enzyme in solution (- - -) and inside a membrane (—).

and the quicker the reaction rate, the greater the diffusion limitations. Any modification of the reaction rate gives rise to an effect on the diffusion limitations. Therefore the effect of the inhibitor is compensated by the decrease in the diffusion limitation. It is important to note that the decrease of the inhibition efficiency is not due to a poor diffusivity of the inhibitor: its diffusion coefficient value is 10^{-3} cm^2 hr^{-1}. According to the simulation, the resulting external and internal concentration are equal after a few seconds.

(ii) Uricase activity was studied for the free and immobilized enzyme as a function of the substrate concentration with a constant inhibitor concentration ($2K_i$). This relationship (Figure 2) is given because it is interesting not only to know the effectiveness of the inhibition after immobilization but also the degree of competitivity of the inhibition. In Figure 2 the classical competitive behavior is shown for the enzyme in solution. For the immobilized enzyme, the effect of the variation of the ratio between the substrate and inhibitor concentrations is quite negligible. It is possible to explain this behavior by taking into account diffusion phenomena. The effect of the inhibitor is compensated by the decrease in diffusion limitations as before, when the substrate concentration is small enough in comparison with K_m, for by definition, diffusion limitation effects are nil for zero-order kinetics. When the substrate concentration is increasing from zero to higher values, the kinetic order is decreasing from one to zero and the drop of the diffusion limitation effect compensates the decrease of the competitive inhibition efficiency. Consequently, the enzyme inhibition in a membrane is less dependent or the ratio between substrate and inhibitor concentrations than free enzyme. The effect of the inhibitor tends to become of a noncompetitive type.

(iii) Experiments described in (i) and (ii) were performed for a limited range of parameter values. The qualitative observations can be generalized for a larger range of substrate and inhibitor concentrations by simulation on computer.

Previously described equations were computed by using the numerical values given on the Figure 3.

The calculated reaction rate is given as a function of the inhibitor concentration for a series of substrate concentrations with a Michaelian enzyme in solution (Figure 3a) and in a membrane (Figure 3b). For the membrane system the curve series is more focused than in solution, and obviously the inhibition tends to be noncompetitive.

NONCOMPETITIVE INHIBITOR. The effect of tartrate on the kinetic behavior of lactic dehydrogenase was studied, the enzyme being in solution and immobilized in an artificial mem-

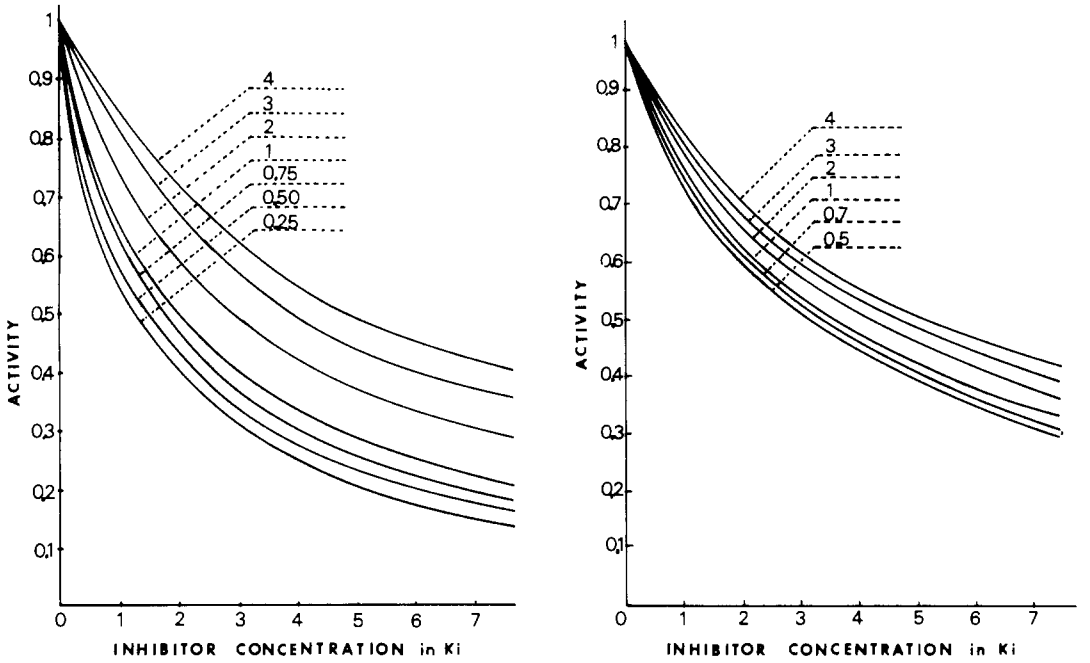

FIGURE 3: Calculated reaction rate values as a function of the competitive inhibitor concentration for different substrate concentrations: (a, left) unbound enzyme in solution; (b, right) enzyme homogeneously bound into a membrane with the parameters. Values from the experimental systems are for maximum enzyme activity, $V_m = 1.5 \times 10^{-5}$ mol cm^{-3} hr^{-1}; for uric acid diffusion coefficient, $D_S = 2.8 \times 10^{-4}$ cm^2 hr^{-1}; for Michaelis constant, $K_m = 2.7 \times 10^{-8}$ mol cm^{-3}; for inhibition constant, $K_i = 1.5 \times 10^{-8}$ mol cm^{-3}; for substrate concentration, $[S_1] = 2 \times 10^{-8}$ mol cm^{-3}.

brane. The enzyme immobilization was produced with an activity yield of 5% of the initial activity.

(i) The reaction rate was studied for free and immobilized enzyme as a function of the inhibitor concentration with a constant substrate concentration ($2K_m$). This relationship appears on Figure 4. The activity is given as a function of the activity without inhibition.

As in the previous case, the LDH activity is less sensitive to inhibition after immobilization. This phenomenon can be explained the same way as previously for uricase activity.

(ii) Lactic dehydrogenase activity was also studied for free and immobilized enzyme as a function of the substrate concentration with a constant inhibitor concentration ($2K_i$). This relationship is given on Figure 5. These results show that in the membrane, the inhibition effect is no longer independent from the substrate concentration.

(iii) In order to explain and extend these experimental results, the system was simulated on a computer by taking into account diffusion and reactions phenomena. The simulation results are given on Figure 6. The calculated reaction rate as a function of the inhibitor concentration is given for several values of substrate concentrations. There is only one curve in solution but a series for the membrane enzyme. A qualitative modification of the enzyme behavior is evident from this figure.

All the enzyme membrane curves are above the solution curve—immobilized enzyme is less sensitive to the inhibition effect, as it appeared from the experimental results.

Reversible Monoenzyme System. Reversibility is the simplest mechanism for feedback limitation of a product accumulation. For this study, the product is formed inside the membrane structure.

The study of a reversible system also provides the opportunity of demonstrating some kinetic effects of an intramembrane phenomenon.

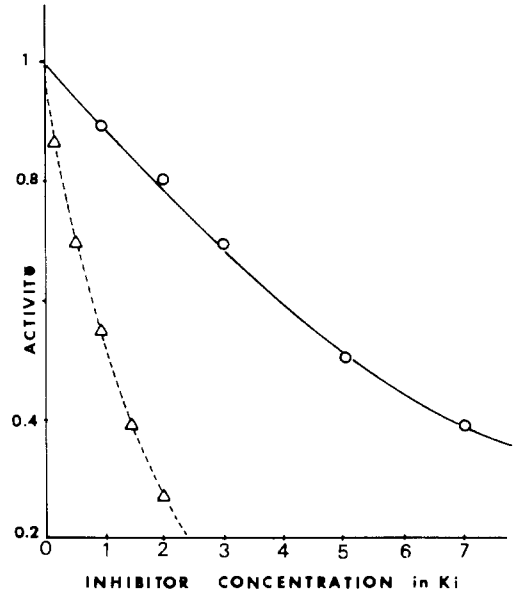

FIGURE 4: Experimental lactic dehydrogenase activity as a function of the tartrate concentration for a constant pyruvate concentration ($2K_m$). Enzyme in solution (- - -) and in a membrane (—).

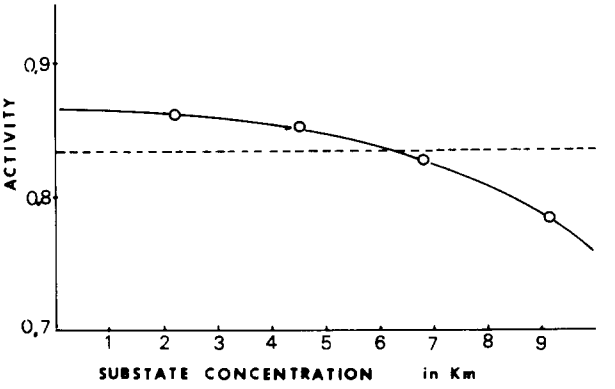

FIGURE 5: Experimental lactic dehydrogenase activity as a function of the pyruvate concentration for a constant tartrate concentration ($2K_i$). Enzyme in solution (- - -) and in a membrane (—).

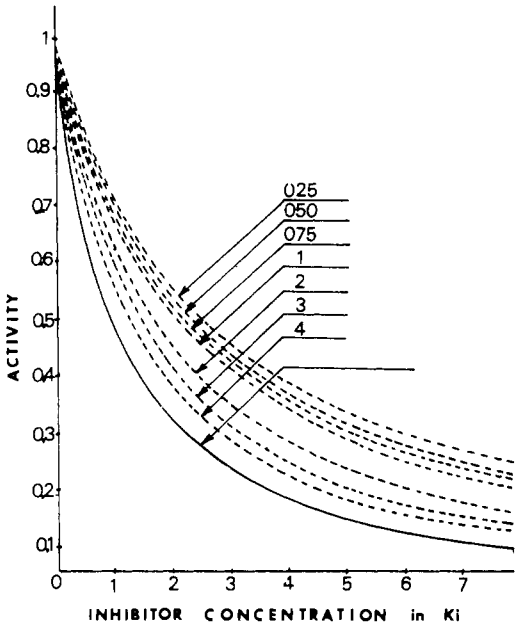

FIGURE 6: Calculated reaction rate as a function of a noncompetitive inhibitor concentration for different concentrations of substrate. Enzyme in solution (—) and inside a membrane (- - -) with the parameter values from the experimental system: for Michaelis constant, K_m = 6.4 × 10^{-6} mol cm^{-3}; for tartrate inhibition constant, K_i = 4.2 × 10^{-5} mol cm^{-3}; for maximum activity by volume unit, V_m = 5 × 10^{-4} mol cm^{-3} hr^{-1}; diffusion coefficient, D = 10^{-3} cm^2 hr^{-1}.

This problem was experimentally analyzed with a glucose-6-phosphate isomerase membrane which catalyses the reversible conversion of fructose 6-phosphate into glucose 6-phosphate.

Substrate evolution in the bulk solution for free enzyme and for an immersed membrane is given in Figure 7. Maximum activities are the same in both cases. In the membrane, the feedback of reversibility is increased by the diffusion limitations.

The substrate is consumed inside the membrane and its local concentration is lower than in the bulk solution. Similarly the product is produced inside the membrane and its local concentration is higher than in the bulk solution. These two simultaneous phenomena bring the enzyme system in the membrane more quickly to a substrate:product concentration ratio close to the equilibrium one. An extension of the results obtained with this system was also made possible by simulation on computer. Experimental results are in good agreement with the simulation.

Calculated instantaneous concentration profiles for substrate and product in a membrane clearly show this phenomenon (Figure 8).

Discussion

An experimental artificial system can work as a valuable model as far as the context is well defined and if some basic principles ruling the behavior of this kind of system arise from the results. In order to mimic the biological membranes bearing linked enzymes, the simplest way is the use of enzymes grafted at a solid–liquid interface. The geometry in this case is well defined but the kinetic behavior is ruled in fact by the unstirred layers at the interface level and it is quite difficult to control and to quantify them experimentally. This trouble is quite negligible when the enzyme is homogeneously immobilized inside the insoluble phase and the kinetic laws satisfying the needs of the heterogeneous enzymology can be studied with both systems. Particles bearing enzymes have to be spherical and with only one size, in order to be well defined. The membrane shape with an uniform thickness is well defined and allows monodimensional calculations. Quite recently it was shown by electron microscopy and scanning electron microscopy (Barbotin and Thomas, 1974) that membranes described in this paper have an uniform thickness. The active and inactive proteins are homogeneously distributed and there are no hole and no pore until the proteic molecule level.

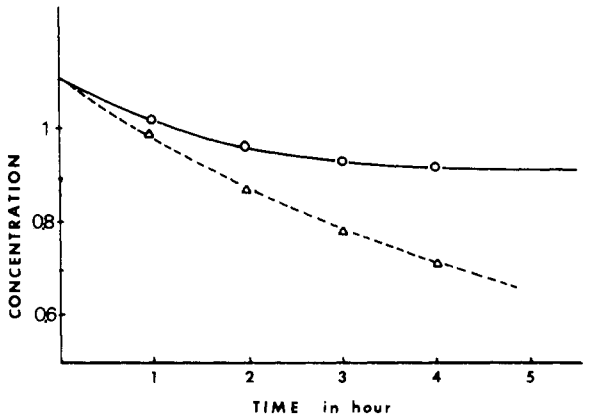

FIGURE 7: Reversible monoenzyme system (glucose-6-phosphate (G-6-P) isomerase): experimental and calculated fructose 6-phosphate (F-6-P) concentration in evolution for the enzyme in solution (- - -) and in an immersed membrane (—). The amounts of enzyme activity and the bulk solution volumes are the same in both cases.

In the described experimental conditions, the inhibition effects appeared to be less important when enzymes were bound into a membrane. This effect is not due to poor inhibitor permeabilities through the structure. Similar values were found for inhibitor and substrate diffusion coefficients.

The observed phenomenon is due to kinetic effect linked to the diffusion limitations—the lower the reaction rate, the lower the diffusion limitations. When an inhibitor diminishes the inherent enzyme activity a decrease of the diffusion limitation occurs. The structure gives a kinetic "buffer" effect on the action of any exogeneous effector. At the opposite a reversible system provides the opportunity of demonstrating some of the kinetic effects of an endogeneous phenomenon. In a membrane, the reversible monoenzyme system appeared more sensitive to a feedback effect of the product accumulation. The reaction product is produced in the structure itself and the local concen-

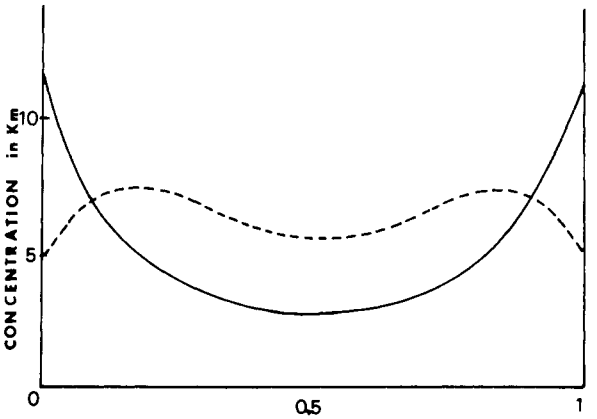

FIGURE 8: Instantaneous calculated concentration profiles of substrate (—) and product (- - -) for a reversible monoenzyme membrane. Computation was performed with the experimental parameters: for maximum G-6-P isomerase activity, V_m = 1.13 × 10^{-3} mol cm^{-3} hr^{-1}; for Michaelis constants, K_{m1} = 10^{-7} mol cm^{-3} (F-6-P–G-6-P), K_{m2} = 3.4 × 10^{-7} mol cm^{-3} (G-6-P–F-6-P); for equilibrium concentration ratio, F-6-P eq/G-6-P eq = 0.5; for diffusion coefficients for F-6-P, D_S = 0.4 × 10^{-3} cm^2 hr^{-1}; for G-6-P, D_P = 0.47 × 10^{-3} cm^2 hr^{-1}; for membrane thickness, e = 3.5 × 10^{-3} cm.

tration is higher than the bulk solution concentration. By taking into account both diffusion parameters and kinetic parameters of the free enzyme in a simulation on computer, calculated results obtained were similar to the experimental results. After the above observation the enzyme kinetic parameters used would not be modified after immobilization. This is in good agreement with previously described results (Thomas *et al.*, 1972a) dealing with an irreversible monoenzyme system without effector. The "true" K_m value was found unmodified after immobilization; at the opposite the apparent one was increased. The data provide evidence in favor of the assumption that the specific site of the enzyme is not modified after insolubilization. The loss of enzyme activity could be due to a complete inactivation of a fraction of the active sites. The results would be "all or none" for the enzyme activity, a part of the active site would be inactivated, the other one would not be modified at all.

This paper deals with the modulation by the structure of different kinds of enzyme systems known in classical Michaelian kinetics. But during the same time bienzyme systems with anisotropical or asymmetrical repartition of active sites can be produced (Thomas *et al.*, 1972b). These systems gave some vectorial effects as an active transport and the phenomenon is deeply linked to the metabolism–structure association (Broun *et al.*, 1972). During the last period, numerous papers have been devoted to the isolation of specific proteins from biological structures, especially binding proteins, permeation proteins, or receptors. It is quite difficult to explain the behavior of the proteins without the structure and their environment, especially in the case of proteins involved in membrane transport phenomena. It would be interesting to reintroduce them inside a well-defined structure, for a better understanding of their behavior *in situ*.

Acknowledgment

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