Mimicked translocation of glucose and glucose 6-phosphate with artificial enzyme membranes

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An approach to the mechanism which may govern the behaviour of biological compartmentalized systems is presented. Artificial enzyme membranes with immobilized glucose oxidase, invertase or hexokinase were used to separate two compartments of a specially designed diffusion cell. Asymmetry in volume, hydrodynamic conditions and enzyme location was purposely chosen in order to create situations which could not be obtained with an enzyme free in solution, and was then used to tentatively mimic situations existing *in vivo*. Experiments were conducted and a translocation effect of H₂O₂, glucose and glucose 6-phosphate was obtained. A theoretical analysis taking into account the different identified parameters of the system was elaborated.

INTRODUCTION

The effect of microenvironment on immobilized enzyme behaviour and the effect of diffusional barriers on solute uptake in biological systems have been recognized for many years (Katchalski, 1970; Engasser & Horvath, 1976; Dietschy, 1978). Fractionation studies on whole cells or organelles have provided evidence that most of the so-called intracellular soluble enzymes do not really exist *in vivo* but exist as 'clusters', i.e. as membrane-bound enzymes or enzyme aggregates (Welch, 1977). It is now an accepted fact that a bound enzyme may display an activity different from that of a soluble enzyme. This is primarily because the diffusion of substrates and products to and from the membrane-bound enzyme is affected by the presence of a diffusion layer existing close to the enzymic membrane (Broun *et al.*, 1970). Furthermore, the distribution of the biocatalyst within the membrane may modulate the apparent bound-enzyme kinetics by mass transfer processes governing substrate access to the active site of the enzyme (De Simone & Caplan, 1973; Bunow & Caplan, 1984).

Another key factor which may play a prominent role in product distribution is the natural asymmetry of most of the biological compartments. So far, little attention has been paid to asymmetry in product build-up in a defined compartment and this occurrence has been underlined only recently in a few papers mentioning studies with enzyme membrane electrodes (Maïsterrena *et al.*, 1986b; Uto *et al.*, 1986).

Thus, several factors can affect the product distribution in a two-compartment system separated by a membrane-bound enzyme: (a) the existence of a diffusion layer in the immediate vicinity of the enzymic surface, (b) the distribution of the enzyme on one or two sides, or within the membrane, (c) the widely different volumes of compartments on both sides of the membrane, (d) the substrate flow coming from a defined compartment.

Most of the studies described which simulate metabolic transport processes with artificial membranes *in vitro* have been performed by considering only one or two of

these factors. So far, no such study has taken all of them into account.

Recently, using a specially designed diffusion cell with two compartments of widely different volumes separated by a membrane bearing an immobilized enzyme, we tried to assess more precisely the role of a diffusion layer on the product distribution in both compartments (Maïsterrena *et al.*, 1986a). Our results have shown that the diffusion layer surrounding the bioactive surface adjacent to the larger compartment leads to a product hyperconcentration effect in the smaller, opposite one. However, in that study, initial substrate concentrations were conveniently chosen to be identical on both sides of the enzymic membrane.

The goal of the work presented here is to analyse a more complex situation which can be considered to be closer to that found *in vivo*, by taking into account two new parameters, i.e. substrate diffusion from one compartment to the other through the enzymic membrane, and the distribution of enzyme molecules on one or both sides of the membrane.

A theoretical analysis is elaborated, and experimental data obtained with immobilized glucose oxidase (EC 1.1.3.4) and invertase (EC 3.2.1.26) attesting to the validity of the model are provided. In addition, hexokinase (EC 2.7.1.1) was used as a model enzyme to mimic a glucose 6-phosphate translocation process.

MATERIALS AND METHODS

Membranes

Two types of membranes were used in the work presented here. Collagen membranes (Type F61) were a gift from the Centre Technique du Cuir, Lyon, France. Their thickness after enzyme binding ranged between 120 and 160 µm depending on the collagen preparations, and was determined with a 1234 IC Millitron device with a MR 1301-type probe supplied by Mahr Feinprüf, Göttingen, Germany.

Biodyne ImmunoAffinity membranes were from

Symbols used: these are defined in Table 1.

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Pall, Glen Cove, NY 11542, U.S.A. These polyamide-activated membranes (120 μm thickness, 0.2 μm size cut-off) were initially devoted to antibody and antigen immobilization.

Enzymes

Glucose oxidase (EC 1.1.3.4), Grade I, freeze-dried powder from *Aspergillus niger* (250 $\text{U} \cdot \text{mg}^{-1}$), and invertase (EC 3.2.1.26), lyophilized powder from yeast (300 $\text{U} \cdot \text{mg}^{-1}$) were purchased from Boehringer.

Hexokinase (EC 2.7.1.1), Type F 300, lyophilized powder from baker's yeast (450 $\text{U} \cdot \text{mg}^{-1}$), and glucose 6-phosphate dehydrogenase (EC 1.1.1.49), type VII from yeast (300 $\text{U} \cdot \text{mg}^{-1}$) were purchased from Sigma.

Other reagents of the highest grade available were supplied by Prolabo, Paris, France and Sigma, St. Louis, MO, U.S.A.

Enzyme immobilization

For the collagen membranes, discs of 5 cm-diam. were cut out of the dry membrane and activated by the acylazide procedure previously developed in our laboratory (Coulet *et al.*, 1974). These membranes, because of their low cut-off, allowed the coupling of enzymes on only one side of the membrane as previously reported (Maïsterrena *et al.*, 1985).

For Pall Biotryne ImmunoAffinity membranes, one of the three methods recommended by the supplier for antibody and antigen immobilization was adapted to enzyme coupling (Assolant-Vinet & Coulet, 1986). The procedure was performed at room temperature by immersion of a preactivated disc of 5 cm-diam. cut out of the dry membrane for 2 h in 10 ml of a stirred enzyme solution at 0.2 $\text{mg} \cdot \text{ml}^{-1}$ in 0.1 M-phosphate buffer, pH 7.

In all cases, the enzymic discs were washed after coupling in 1 M-KCl for 30 min and stored in appropriate buffers, depending on the nature of the enzymic membrane.

Diffusion cell

A previously-designed asymmetric diffusion cell was used. This cell, thermostatically controlled at 25 °C, consists of two compartments, I and II, of 9 and 350 cm^3 respectively, separated by the enzymic membrane. In Compartment I, a high stirring rate makes the unstirred layer negligible, whereas in Compartment II, a low stirring rate allows the creation of a diffusion layer of thickness δ , as previously reported (Maïsterrena *et al.*, 1985).

Measurements of product generation

Glucose oxidase immobilized on collagen membrane. The two compartments of the diffusion cell were filled with 0.2 M-sodium acetate buffer/0.2 M-KCl/0.02% (w/v) NaN_3 , pH 5.5, at 25 °C. Glucose at the desired concentration was added to compartment II, and the appearance of H_2O_2 generated by the following reaction:

was monitored with time on samples withdrawn from both compartments by using a PRGE-type polarograph from Solea-Tacussel, Villeurbanne, France, as previously described (Maïsterrena *et al.*, 1986a).

Invertase immobilized on Pall Biotryne ImmunoAffinity membrane. The two compartments of the diffusion cell were filled with 0.2 M-sodium acetate buffer/0.2 M-KCl/0.02% (w/v) NaN_3 , pH 4.5, at 25 °C. Sucrose at the desired concentration was added to Compartment II.

Samples of glucose generated by the following reaction:

were withdrawn from both compartments and incubated at 50 °C for 2 h, allowing glucose mutarotation into $\beta\text{-D-glucose}$. The determination of $\beta\text{-D-glucose}$ was carried out using a PRGE-type polarograph equipped with a glucose electrode (Thévenot *et al.*, 1979).

Hexokinase immobilized on Pall Biotryne ImmunoAffinity membrane. The two compartments of the diffusion cell were filled with 0.2 M-triethanolamine buffer/2 mM-ATP/2 mM- MgCl_2 , pH 7.6, at 25 °C. Glucose at the desired concentration was added to Compartment II. Samples of glucose 6-phosphate generated by the following reaction:

were withdrawn from both compartments and assayed in the presence of 0.2 M-triethanolamine buffer/glucose 6-phosphate dehydrogenase (0.4 $\text{U} \cdot \text{ml}^{-1}$)/0.2 mM- NADP^+ /5 mM- MgCl_2 , pH 7.6, at 25 °C. The increase in absorbance at 340 nm associated with the reduction of NADP was measured, allowing D-glucose 6-phosphate determination after calibration (Lang & Michal, 1974).

RESULTS

Enzyme bound on to the surface of only one side of a porous membrane

The system illustrated in Fig. 1 (inset) mainly consists of a permeable artificial membrane separating two compartments of widely different volumes ($V_{\text{II}} > V_{\text{I}}$) (for definitions of all symbols used, refer to Table 1). A one-substrate enzyme is bound on to the surface of only one side (Side II) facing a large compartment (Compartment II) in which a diffusion layer exists in the vicinity of the biocatalyst. In the smaller and opposite Compartment I, it is assumed that the unstirred layer is negligible. Such conditions were experimentally obtained as previously reported (Maïsterrena *et al.*, 1985).

In order to analyse such a system, and to obtain at every time point an expression of the product appearance in both compartments (P_{I} and P_{II}) as a function of the substrate concentration in Compartment II (S_{II}), the following assumptions were made: (a) The initial concentration of products in Compartment I and Compartment II is zero, i.e. $P_{\text{I}} = P_{\text{II}} = 0$. (b) The initial concentration of substrate in Compartment I is zero, i.e. $S_{\text{I}} = 0$. (c) Substrate concentration in Compartment II, S_{II} , is kept constant. (d) The flows of substrate and product are perpendicular to the surface of the membrane so that their distribution in the system conforms to Fick's first law. (e) At steady-state, the amount of product in the diffusion layer and in the membrane is negligible compared with the total amount existing in Compartments I and II.

At steady-state, and under first-order kinetics conditions, the substrate distribution in the system can be expressed as follows:

Fig. 1. Product concentration pattern obtained versus time under first-order kinetics with respect to glucose (2.5 mM). Fitting between theoretical and experimental data when using an asymmetrically immobilized glucose oxidase collagen membrane

The situation considered here is given in the inset. The enzyme is grafted only on one side of the membrane (thickness = d) adjacent to Compartment II (E_{II}) in which a diffusion layer at the enzymic membrane/solution interface of thickness δ exists. It is assumed that S_{II} , which is the initial substrate concentration in Compartment II, remains constant versus time during the experiment. S_0 and S_1 are the substrate concentration values appearing at the enzyme level and in Compartment I respectively. P_0 , P_I and P_{II} are the product concentration at the enzyme level and in Compartment I and Compartment II respectively. The curved arrows indicate substrate or product mass transfer; the straight arrow indicates the enzymic reaction. The volumes of Compartment I and Compartment II are respectively $V_I = 9 \text{ cm}^3$ and $V_{II} = 350 \text{ cm}^3$. In Compartment I the stirring rate ($450 \text{ rev} \cdot \text{min}^{-1}$) makes the unstirred layer negligible, while in Compartment II the stirring rate ($38 \text{ rev} \cdot \text{min}^{-1}$) leads to a diffusion layer value (δ) equal to $320 \mu\text{m}$. The reaction is initiated by adding glucose in Compartment II in order to obtain a glucose concentration (S_{II}) of 2.5 mM. The open (○) and closed (●) circles are the data for Compartment I and Compartment II respectively. The continuous (—) and broken (-----) lines are theoretical curves obtained by using eqns. (7) and (8) respectively with the parameters given in the text. For symbols, see Table 1.

$$\frac{1}{R_{\delta,s}}(S_{II} - S_{0,t}) = k_2 S_{0,t} + \frac{1}{R_{m,s}}(S_{0,t} - S_{1,t}) \quad (1)$$

$R_{\delta,s}$ is the unstirred layer's resistance to substrate reaching the enzymic surface, i.e. $\delta/(D_s A)$, where δ is the thickness of the boundary layer, D_s the substrate diffusivity and A the membrane area. $R_{m,s}$ is the membrane resistance to the substrate reaching Compartment I, i.e. $d/(D_s A_w)$, where d represents the membrane thickness and A_w is the effective membrane pore area. S_{II} , $S_{1,t}$ and $S_{0,t}$ are the substrate concentrations in Compartment II, in Compartment I and at the enzyme level respectively. k_2 is the first order rate constant for the membrane-bound enzyme facing Compartment II.

As substrate appearance in Compartment I can be expressed as follows:

$$\frac{dS_I}{dt} = \frac{1}{V_I R_{m,s}}(S_{0,t} - S_{1,t}) \quad (2)$$

Table 1. Definition of symbols used and their subscripts

(a) Symbol	Definition	Units
A	Membrane area	cm^2
A_w	Effective membrane pore area	cm^2
D	Diffusion coefficient	$\text{cm}^2 \cdot \text{min}^{-1}$
d	Membrane thickness	cm
J	Product flux toward compartment	$\text{mm} \cdot \text{min}^{-1} \cdot \text{cm}^3$
K_m	Michaelis constant	mM
k_1, k_2	First-order rate constants for the membrane-bound enzymes I and II	$\text{cm}^3 \cdot \text{min}^{-1}$
P	Product concentration	mM
R	Diffusion resistance	$\text{min} \cdot \text{cm}^{-3}$
r_T	Overall apparent enzyme activity	$\text{mmol} \cdot \text{min}^{-1}$
S	Substrate concentration	mM
t	Time	min
V	Volume compartment	cm^3
V_T	Total liquid volume of both compartments	cm^3

(b) Greek letter	Definition	Units
δ	Boundary layer thickness	cm
μ	Dimensionless external diffusion modulus for substrate in Compartment II ($k_2 R_{\delta,s}$)	—

(c) Subscript	Definition
0	Membrane level
I	Relative to Compartment I
II	Relative to Compartment II
δ	Relative to the boundary layer
m	Relative to the membrane
p	Relative to the product
s	Relative to the substrate

where V_I is the volume of Compartment I, we can introduce $S_{0,t}$ obtained from eqn. (1) into eqn. (2), in order to obtain, after integration, the change in concentration of S_I with time. When substituting this $S_{1,t}$ value into eqn. (1), we obtain the change in concentration $S_{0,t}$:

$$S_{0,t} = \frac{S_{II}}{B} \left\{ R_{m,s} + \frac{R_{\delta,s}}{(\mu + 1)} \left[1 - \exp\left(-\frac{(\mu + 1)}{V_I B} t\right) \right] \right\} \quad (3)$$

where $B = [(\mu + 1) R_{m,s} + R_{\delta,s}]$, with μ representing the dimensionless external diffusion modulus for substrate, i.e. $k_2 R_{\delta,s}$.

At steady-state and at any time, the total product quantity generated in the system can be expressed as follows:

$$V_I P_{I,t} + V_{II} P_{II,t} = k_2 \int_0^t S_{0,t} dt \quad (4)$$

where the new parameters introduced, V_{II} , $P_{I,t}$ and $P_{II,t}$, are respectively the volume of Compartment II and the product concentrations in Compartments I and II. When substituting the expression of $S_{0,t}$, given by eqn. (3) into eqn. (4), we can obtain the change in concentration $P_{II,t}$ versus $P_{I,t}$.

In order to express P_I and P_{II} as a function of S_{II} , the product distribution in the system must be considered. At any time and at steady-state, the product distribution corresponds to the following equation:

$$k_2 S_{0,t} = \frac{1}{R_{m,p}} (P_{0,t} - P_{I,t}) + \frac{1}{R_{\delta,p}} (P_{0,t} - P_{II,t}) \quad (5)$$

where $R_{m,p}$ is the membrane resistance to the product reaching Compartment I, i.e. $d/(D_p A_w)$ where D_p is the product diffusivity, $R_{\delta,p}$ is the diffusion layer's resistance to product reaching Compartment II, i.e. $\delta/(D_p A)$, and $P_{0,t}$ is the product concentration at the enzyme level. Introducing now the $S_{0,t}$ value given by eqn. (3) and the $P_{II,t}$ value into eqn. (5), we obtain an expression of $P_{0,t}$ versus $P_{I,t}$.

On the other hand, the appearance of product in Compartment I can be expressed as follows:

$$\frac{dP_I}{dt} = \frac{1}{V_I R_{m,p}} (P_{0,t} - P_{I,t}) \quad (6)$$

When substituting the $P_{0,t}$ value obtained into eqn. (6), the change in concentration P_I with time is given after integration by the following equations:

$$\begin{aligned} P_{I,t} = & \frac{k_2 S_{II}}{(\mu + 1) V_T} t \\ & + \left\{ \frac{\mu S_{II} [V_I B - (\mu + 1) V_{II} R_{\delta,p}]}{(\mu + 1)^2 [V_T B - (\mu + 1) V_{II} (R_{m,p} + R_{\delta,p})]} \right. \\ & \times \left[\exp - \left(\frac{\mu + 1}{V_I B} t \right) - \exp - \left(\frac{V_T}{V_I V_{II} (R_{m,p} + R_{\delta,p})} t \right) \right] \Bigg\} \\ & + \left\{ \frac{k_2 S_{II} [(\mu + 1) V_{II} (V_I R_{m,p} - V_{II} R_{\delta,p}) + V_T V_I R_{\delta,s}]}{(\mu + 1)^2 V_T^2} \right. \\ & \times \left[\exp - \left(\frac{V_T}{V_I V_{II} (R_{m,p} + R_{\delta,p})} t \right) - 1 \right] \Bigg\} \quad (7) \end{aligned}$$

In the same way, the change in concentration $P_{II,t}$ with time can be obtained:

$$\begin{aligned} P_{II,t} = & \frac{k_2 S_{II}}{(\mu + 1) V_T} t \\ & + \left\{ \frac{\mu S_{II} [V_I B - (\mu + 1) V_{II} R_{m,p}]}{(\mu + 1)^2 [V_T B - (\mu + 1) V_{II} (R_{m,p} + R_{\delta,p})]} \right. \\ & \times \left[\exp - \left(\frac{\mu + 1}{V_I B} t \right) - \exp - \left(\frac{V_T}{V_I V_{II} (R_{m,p} + R_{\delta,p})} t \right) \right] \Bigg\} \\ & + \left\{ \frac{k_2 S_{II} V_I [(\mu + 1) (V_{II} R_{\delta,p} - V_I R_{m,p}) + V_T R_{\delta,s}]}{(\mu + 1)^2 V_T^2} \right. \\ & \times \left[\exp - \left(\frac{V_T}{V_I V_{II} (R_{m,p} + R_{\delta,p})} t \right) - 1 \right] \Bigg\} \quad (8) \end{aligned}$$

First, different parameters were determined experimentally in order to test the validity of the corresponding mathematical analysis [eqns (7) and (8)]. Experiments were performed by using a flat and porous collagen membrane bearing glucose oxidase immobilized on only one side of the membrane. It must be emphasized that at

the temperature of the experiment (25 °C), the concentration of the co-substrate O_2 can be considered as constant and equal to 0.2 mM (Hitchman, 1978). In Compartment I, a high stirring rate (450 rev·min⁻¹) obtained with a special impeller makes the unstirred layer negligible, while a low stirring rate in Compartment II allows the creation of a diffusion layer of thickness δ adjacent to the enzymic surface (Maïsterrena *et al.*, 1985).

In a first set of experiments (results not shown), using a low and identical initial substrate concentration of glucose (2.5 mM) in both compartments, the thickness of the diffusion layer δ and the first order rate constant k_2 of enzyme immobilized on side II were calculated as shown below.

Determination of δ . The concentrations of P_I and P_{II} were measured in each compartment, and J_I and J_{II} , which are the fluxes of product toward Compartments I and II, were determined at different times. By solving the following equations:

$$J_I = \frac{1}{R_{m,p}} (P_{0,t} - P_{I,t}) \quad (9)$$

$$J_{II} = \frac{1}{R_{\delta,p}} (P_{0,t} - P_{II,t}) \quad (10)$$

$P_{0,t}$ and $R_{\delta,p}$ were calculated. Once $R_{\delta,p}$ was known, δ was then determined.

Determination of k_2 . From the same set of experiments, it was also possible to determine the first-order rate constant k_2 . When using an identical and low initial substrate concentration (2.5 mM) with respect to the K_m value of glucose oxidase (40 mM), the overall enzyme activity r_T is constant versus time and can be related to the first-order rate constant k_2 :

$$k_2 = \frac{r_T}{(S_{II} - r_T R_{\delta,s})} \quad (11)$$

k_2 can be calculated knowing $R_{\delta,s}$, S_{II} and r_T (experimentally determined) and is equal to $(V_I P_{I,t} + V_{II} P_{II,t})/t$. For the other parameters used in eqns. (7) and (8), the following values, previously determined, (Maïsterrena *et al.*, 1986a) were used: the membrane porosity A_w/A (20.5%), the product diffusivity ($D_{H_2O_2} = 1.75 \times 10^{-3} \text{ cm}^2 \cdot \text{min}^{-1}$), the substrate diffusivity ($D_{\text{glucose}} = 4.04 \times 10^{-4} \text{ cm}^2 \cdot \text{min}^{-1}$) and the membrane thickness ($d = 120 \mu\text{m}$).

The case described in the inset to Fig. 1 was then tested experimentally under first-order kinetics. Glucose was only added in Compartment II. A constant stirring rate (38 rev·min⁻¹) leads to a diffusion layer of 320 μm in this compartment and a k_2 value of $4.5 \times 10^{-2} \text{ cm}^3 \cdot \text{min}^{-1}$ was used. The theoretical curves were calculated by using eqns. (7) and (8) with the previously-given parameters. A good fit is obtained, as shown in Fig. 1.

We must emphasize that when the enzymic reaction follows zero-order kinetics (results not shown), the substrate gradient has no influence on the maximal enzymic rate (Engasser & Horváth, 1976), and the sequences of the reactions allowing the calculation of $P_{I,t}$ and $P_{II,t}$ are the same as those described previously (Maïsterrena *et al.*, 1986a).

Enzyme bound to the whole surface of a porous membrane

In the case considered in Fig. 2 (inset), the same enzyme is bound on both sides of the membrane ($E_I = E_{II}$). All the assumptions previously mentioned in the previous section were maintained.

Under first-order kinetics, the substrate appearance in Compartment I can be expressed as follows:

$$\frac{dS_I}{dt} = \frac{1}{V_I R_{m,s}} (S_{0,t} - S_{I,t}) - \frac{k_1}{V_I} S_{I,t} \quad (12)$$

where k_1 is the first-order rate constant for the membrane-bound enzyme facing Compartment I.

$S_{0,t}$ and $S_{I,t}$ can be expressed as a function of S_{II} as follows. Introducing the $S_{0,t}$ value obtained from eqn. (1) into eqn. (12), the change in concentration S_I with time after integration can be obtained. Substituting this $S_{I,t}$ value into eqn. (1), we then obtain the change in concentration of $S_{0,t}$ versus S_{II} .

At steady-state and at any time, the total amount of product generated by the enzyme on sides I and II can be expressed as follows:

$$V_I P_{I,t} + V_{II} P_{II,t} = k_2 \int_0^t S_{0,t} dt + k_1 \int_0^t S_{I,t} dt \quad (13)$$

When substituting the expression of $S_{0,t}$ and $S_{I,t}$ into eqn. (13) and solving the equation, we obtain the change in concentration of $P_{II,t}$ versus $P_{I,t}$. Then, introducing this $P_{II,t}$ value into eqn. (5), we obtain an expression of $P_{0,t}$ versus $P_{I,t}$.

On the other hand, the product appearance in Compartment I can be expressed as follows:

$$\frac{dP_I}{dt} = \frac{k_2}{V_I} S_{I,t} + \frac{1}{V_I R_{m,p}} (P_{0,t} - P_{I,t}) \quad (14)$$

On substituting into eqn. (14) the $P_{0,t}$ value and the $S_{I,t}$ value previously obtained, the change in concentration P_I with time is given after integration by the following equation:

$$\begin{aligned} P_{I,t} = S_{II} \left\{ \frac{k_2 (C R_{m,s} + R_{\delta,s}) + k_1 B}{B C V_T} \right\} t + \left\{ S_{II} \left(\frac{k_2 R_{\delta,s} [V_I B - C V_{II} R_{\delta,p}] + k_1 B [V_I B - C V_{II} (R_{m,p} + R_{\delta,p})]}{C^2 [V_T B - C V_{II} (R_{m,p} + R_{\delta,p})]} \right) \right. \\ \times \left[\exp - \left(\frac{C}{V_I B} t \right) - \exp - \left(\frac{V_T}{V_I V_{II} (R_{m,p} + R_{\delta,p})} t \right) \right] \left. + S_{II} \left\{ \frac{k_2 [C V_{II} (V_I R_{m,p} - V_{II} R_{\delta,p}) (C R_{m,s} + R_{\delta,s}) + V_T V_I R_{\delta,s} B]}{B C^2 V_T^2} \right. \right. \right. \\ \left. \left. + \frac{k_1 B [V_T V_I B - V_{II} C (R_{m,p} + R_{\delta,p})]}{B C^2 V_T^2} \right\} \left\{ \exp - \left(\frac{V_T}{V_I V_{II} (R_{m,p} + R_{\delta,p})} t \right) - 1 \right\} \right\} \quad (15) \end{aligned}$$

The change in $P_{II,t}$ with time can be also obtained in the same manner:

$$\begin{aligned} P_{II,t} = S_{II} \left\{ \frac{k_2 (C R_{m,s} + R_{\delta,s}) + k_1 B}{B C V_T} \right\} t + \left\{ S_{II} \left(\frac{k_2 R_{\delta,s} [V_I B - C V_I R_{m,p}] + V_I k_1 B^2}{C^2 [V_T B - C V_{II} (R_{m,p} + R_{\delta,p})]} \right) \right. \\ \times \left[\exp - \left(\frac{C}{V_I B} t \right) - \exp - \left(\frac{V_T}{V_I V_{II} (R_{m,p} + R_{\delta,p})} t \right) \right] \left. + S_{II} \left\{ \frac{k_2 V_I [C (V_{II} R_{\delta,p} - V_I R_{m,p}) (C R_{m,s} + R_{\delta,s}) + V_T R_{\delta,s} B]}{B C^2 V_T^2} \right. \right. \right. \\ \left. \left. + \frac{k_1 V_I B [V_{II} C (R_{m,p} + R_{\delta,p}) + V_T B]}{B C^2 V_T^2} \right\} \left\{ \exp - \left(\frac{V_T}{V_I V_{II} (R_{m,p} + R_{\delta,p})} t \right) - 1 \right\} \right\} \quad (16) \end{aligned}$$

where $C = (\mu + 1) + k_1 B$, and $B = [(\mu + 1) R_{m,s} + R_{\delta,s}]$.

It is to be noted that eqns. (15) and (16) can be simplified into eqns. (7) and (8) if there is no enzyme on the membrane side facing Compartment I, i.e. when $k_1 = 0$. Furthermore, the computerization of eqns. (15) and (16) when $k_2 = 0$ (results not shown) demonstrates that a product concentration effect can also be obtained in the small compartment adjacent to the enzymic surface.

Immobilized invertase

First, in order to test this case and the corresponding mathematical analysis [eqns. (15) and (16)], different parameters were determined. Experiments were performed by using a Pall Biodyne ImmunoAffinity membrane of $0.2 \mu\text{m}$ size cut-off bearing invertase grafted on both sides of the membrane.

A first set of experiments (results not shown) with low identical substrate concentrations of sucrose (4 mM) in both compartments with respect to the K_m value of invertase (80 mM) was performed under fixed hydrodynamic conditions allowing, as mentioned above, the calculation of the δ value existing in Compartment II. The k_1 and k_2 values corresponding to the first-order rate constants of enzyme on faces I and II respectively were determined as follows.

After initiating the reaction, with $S_I = S_{II} = 4 \text{ mM}$, P_I and P_{II} were measured in each chamber, and J_I and J_{II} were determined for different times allowing the calculation of the overall enzyme activity, r_T . This activity is constant with time and can be expressed by the following equation:

$$r_T = k_2 S_{0,t} + k_1 S_{I,t} \quad (17)$$

Assuming a constant and equal value ($S_{I,t} = S_{II,t}$) during the first steps of the experiment, and also the same enzyme activity retained on both sides of the membrane, i.e. $k_1 = k_2$, it becomes, from (17):

$$r_T = k_1 \left(\frac{S_{II}}{(k_2 R_{\delta,s} + 1)} + S_{II} \right) \quad (18)$$

Fig. 2. Product concentration pattern obtained versus time under first-order kinetics with respect to sucrose (4 mM). Fitting between theoretical and experimental data when using an invertase symmetrical Pall Biotryne ImmunoAffinity membrane

The situation considered here is given in the inset. The same enzyme is grafted on both sides of the membrane. The curved arrows indicate substrate or product mass transfer; the straight arrows indicate the enzymic reactions. In Compartment I, the stirring rate was $450 \text{ rev} \cdot \text{min}^{-1}$; in Compartment II, it was $175 \text{ rev} \cdot \text{min}^{-1}$ leading to a diffusion layer (δ) value of $110 \mu\text{m}$. Symbols are as in Fig. 1. The continuous (—) and broken (-----) lines are theoretical curves obtained by using eqns. (15) and (16) respectively with the parameters given in the text. For symbols, see Table 1.

When solving eqn. (18), it becomes the following:

$$k_1 = k_2 = \frac{-(2S_{II} - R_{\delta,s} r_T) \pm \sqrt{(2S_{II} - R_{\delta,s} r_T)^2 + 4r_T S_{II} R_{\delta,s}}}{2S_{II} R_{\delta,s}} \quad (19)$$

knowing $R_{\delta,s}$, S_{II} and r_T , experimentally determined as $(V_I P_{I,t} + V_{II} P_{II,t})/t$, k_1 and k_2 can then be calculated. The other parameters determined or used were membrane porosity A_w/A (12.3%), membrane thickness ($120 \mu\text{m}$), sucrose diffusivity ($D_{\text{sucrose}} = 3.12 \times 10^{-4} \text{ cm}^2 \cdot \text{min}^{-1}$), glucose diffusivity ($D_{\text{glucose}} = 4.04 \times 10^{-4} \text{ cm}^2 \cdot \text{min}^{-1}$).

Knowing all the numerical values of the parameters in eqns. (15) and (16), the corresponding theoretical plots could be drawn (Fig. 2). The experimental conditions were: a low initial sucrose concentration (4 mM) in Compartment II, a constant stirring rate ($450 \text{ rev} \cdot \text{min}^{-1}$) in Compartment I, a constant stirring rate ($175 \text{ rev} \cdot \text{min}^{-1}$) in Compartment II giving a δ diffusion layer value of $110 \mu\text{m}$ and a first-order rate constant $k_1 = k_2 = 2.5 \times 10^{-2} \text{ cm}^3 \cdot \text{min}^{-1}$.

As shown in Fig. 2, a good correlation is obtained between the experimental data and the theoretical curves calculated by using eqns. (15) and (16) with the previous given parameters.

Fig. 3. Product concentration pattern obtained versus time when using a hexokinase-symmetrical Pall Biotryne Immuno-Affinity membrane

Compartment I stirring rate = $450 \text{ rev} \cdot \text{min}^{-1}$; Compartment II stirring rate = $70 \text{ rev} \cdot \text{min}^{-1}$. The experiment is initiated by adding to Compartment II a solution of glucose/ATP/MgCl₂ in order to obtain an initial concentration of 2 mM. The open (○) and closed (●) circles are the data for Compartment I and Compartment II respectively. For symbols, see Table 1.

Thus, for the cases considered and described in Figs. 1 and 2, our experimental results, obtained with two different porous membranes, two different enzymes and for different hydrodynamic conditions, demonstrate the validity of our mathematical analysis. In all cases, a product hyperconcentration effect appears in the smaller compartment of our diffusion cell.

Immobilized hexokinase

Experimental data were also obtained using a Pall Biotryne ImmunoAffinity membrane of $0.2 \mu\text{m}$ size cut-off bearing hexokinase grafted on both sides of the membrane. The experimental conditions were: a constant stirring rate of $450 \text{ rev} \cdot \text{min}^{-1}$ in Compartment I, a constant stirring rate of $70 \text{ rev} \cdot \text{min}^{-1}$ in Compartment II and a 2 mM-glucose/ATP/MgCl₂ initial concentration in Compartment II.

As hexokinase catalyses a two-substrate enzymic reaction involving charged compounds, this case appears far more complex than the previous one and could not be modelled using the equations given above. Only the experimental plots of P_I and P_{II} versus time are represented in Fig. 3. Nevertheless, the goal of this last experiment was merely to show that glucose 6-phosphate can also be translocated into Compartment I of our diffusion cell as shown in Fig. 3.

DISCUSSION

If glucose oxidase cannot be considered as representative of translocation processes *in vivo*, invertase, by generating glucose, may simulate the behaviour of the intestine brush border β -glucosidase. As a matter of fact, several years ago, it was reported in a study dealing with intestinal absorption that the glucose derived from a glycoside reaches a high concentration in the aqueous compartment adjacent to the microvilli intestine membrane (Crane, 1975).

More recently (Hanke *et al.*, 1980; Warden *et al.*,

1980) summarized their findings concerning the transport into hamster intestine of glucose generated from phlorizin by brush border β -glucosidase as follows: '...it enjoys a kinetic advantage for tissue entry over free glucose in the medium by virtue of the position of the site where it is formed, i.e. inside the unstirred layer and near normal entry portals. No special hydrolase-related transport system like the one proposed for dissaccharides needs to be considered to account for our findings'. In our opinion, our mathematical analysis emphasizing the role of the diffusion layer adjacent to a bioactive surface may give an explanation of this transport into intestine of glucose generated by phlorizin.

We have also been able to simulate a well-studied product translocation process, i.e. that of glucose 6-phosphate. One can argue that a product hyperconcentration effect would obviously be obtained in Compartment I of our diffusion cell when using a symmetrical enzyme membrane. But, as demonstrated experimentally and corroborated by our mathematical analysis, the product hyperconcentration effect occurs even if there is no enzyme grafted on the membrane side facing Compartment I, i.e. when $k_1 = 0$, whatever the enzyme localization on the membrane side.

In such a system, a major role is played by the diffusion layer existing at the membrane level in Compartment II, which acts as a third compartment in which the reaction product accumulates and is distributed into Compartments I and II. Furthermore, the high local product concentration at the membrane level prevents the retrodiffusion to Compartment II of the product accumulated in Compartment I.

The key role of this third compartment on product distribution in a compartmentalized enzyme membrane system recently reported in our group (Maïsterrena *et al.*, 1986, 1987) was also stressed by other authors (Antonenko & Yaguzhinsky, 1986) using a bilayer lipid membrane.

As demonstrated by our results, a product hyperconcentration appears in the small compartment of our diffusion cell in all cases. The theoretical analysis, proposed to explain such a phenomenon, is in good agreement with our experimental data and may contribute to a better understanding of certain kinds of translocation processes occurring *in vivo*. Even if we are still far from situations *in vivo*, such simulations *in vitro* appear to be the most promising approach, as sensed by Mitchell many years ago (Mitchell, 1961).

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