Pattern formation in an immobilized bienzyme system

A morphogenetic model

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Experimental and theoretical studies of a reaction–diffusion model of two immobilized enzymes participating in the cellular acid–base metabolism, namely glutaminase and urease, are presented. The system shows an unstable steady state at pH 6.0, where any perturbation will drive the system towards a more alkaline or more acidic pH, owing to the autocatalytic behaviour with respect to pH exhibited by both enzymes. When diffusion is coupled to reaction by means of immobilization, different patterns of the internal pH profile appear across the membrane. If the bienzymic membrane is subjected to a perturbation at its boundaries, of the same amplitude but in opposite directions, the internal pH evolves through an asymmetric pattern to attain a nearly symmetric distribution of pH. The pH value at the final steady state is more acidic or more alkaline than the initial state according to the initial and boundary conditions. The final nearly symmetric state is attained more rapidly when less enzyme is immobilized ($1.8 \times 10^{-4} \text{ M} \cdot \text{s}^{-1}$ as against $3.3 \times 10^{-4} \text{ M} \cdot \text{s}^{-1}$ of total enzyme activity in the membrane volume). The experimental results agree rather well qualitatively with numerical predictions of the model equations.

INTRODUCTION

One of the major challenges in biochemistry and biology today is the problem of the manner in which the activities of billions of molecules are co-ordinated in space and time to produce cellular functions. In spite of our everyday deeper knowledge of the structure and function of isolated biological molecules, understanding of the functional organization of the cell is rather less developed [1,2]. Many hypotheses have proposed rates of chemical change (reactions) coupled to transport of matter (diffusion) as a plausible mechanism to explain how biological organization arises. These reaction–diffusion models have been extensively elaborated, although their experimental use lags far behind their theoretical development [3]. About 37 years ago, Turing [4] demonstrated mathematically that reaction–diffusion models are able to generate form, i.e. morphogenesis. The concept of a morphogen was introduced in the pioneering paper of Turing [4] as a name for a chemical substance able to generate form through a reaction–diffusion mechanism. Autocatalysis, or reaction activation by its product, or inhibition by its substrate, under far-from-equilibrium conditions, is a necessary requirement in Turing's [4] model for pattern formation and in all models derived from it [5–7].

Many examples of pattern formation have been reported in chemical and physical systems, such as chemical waves in the Belousov–Zhabotinsky reaction, and photochemical structures for a large number of photochromic and chromogenic compounds [8–10]. However, most examples of photochemical pattern formation or stable patterns in Belousov–Zhabotinsky reactions do not arise via a Turing instability, i.e. a diffusion-driven instability, but contain convective or surface effects [11,12]. In biochemical systems only a few experimental studies support the ability of reaction–diffusion mechanisms to produce patterns. The recent work of Aon *et al.* [13] shows a phenomenon of

pattern formation in a photobiochemical system. Those authors suggested that the non-linear reduction of an electron acceptor by photosynthetic activity, when coupled to diffusion, can trigger a symmetry change that redistributes a monotonic gradient of the electron acceptor in a banding pattern. The coupling of autocatalysis with diffusion has also been postulated to account for periodic precipitation processes, namely Liesegang rings, where the threshold for crystal growth would be the non-linear mechanism participating in the phenomenon [14].

The immobilization of enzyme systems gives rise to diffusional limitations (mass-transfer constraints) on the supply of substrates or removal of products in the immediate environment of the enzymes. In consequence, local metabolite-concentration profiles may be established across the membrane width, which will, in turn, determine the kinetic behaviour of the enzymes. In addition, immobilized enzymes constitute a well-defined system from the point of view of composition, thereby allowing knowledge and control of all variables. Thus modelling of these systems with reaction–diffusion equations is straightforward [15]. Although concentration profiles can be calculated from reaction–diffusion equations, little attention has been paid to their experimental visualization [16].

In the present work, a theoretical and experimental approach to a reaction–diffusion model of two immobilized enzymes participating in acid–base metabolism is developed. The enzymes involved are glutaminase and urease, which show autocatalytic behaviour with respect to pH. When diffusion is coupled to the autocatalytic reaction by means of immobilization, different patterns of the internal pH profile of the membrane appear. Theory predicts, and experiments confirm, that the evolution of the internal pH of the membrane towards a nearly symmetric steady state, through a transient asymmetric pattern of pH, is triggered by slight perturbations of the pH in an asymmetric fashion at the boundaries.

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MODEL DESCRIPTION

Nomenclature used

Parameter	Description	Units
E_i	Enzyme of the reaction i	
S_i and P_i	Substrate and Product of the reaction i	M
$V_{M(pH)}^i$	Maximal rate of enzyme i at a given pH	$M \cdot s^{-1}$
$V_{M(opt)}^i$	Maximal rate of enzyme i at the optimal pH	$M \cdot s^{-1}$
α_i	Ratio of maximal rate at a given pH to maximal rate at the optimal pH of the enzyme i	Non-dimensional
K_{es1}^i, K_{es2}^i	Ionization constants of the enzyme i	M
pH*	pH of the unstable steady state	Non-dimensional
β	Normalizing constant	M^{-1}
e	Membrane width in the direction parallel with diffusion	cm
D_{H^+}	Diffusion coefficient for H^+	cm^2/s
$\sigma_i = V_{M(opt)}^i e^2 / D_{H^+}$	Kinetic parameter for enzyme i	M
$\theta = e^2 / D_{H^+}$	Characteristic time constant of the system	s
σ	Normalized factor of the system	Non-dimensional

The model considers an artificial protein membrane in which two enzymes, E_1 and E_2 , are homogeneously immobilized. The product of the reaction catalysed by E_1 is an acid, whereas that produced by E_2 is a base. For the present experimental system, E_1 and E_2 are glutaminase and urease respectively. The reactions involved may be schematically represented as follows:

The model assumes that both enzymes obey Michaelis–Menten kinetics and that the substrates are present in excess to assure zero-order kinetics for both reactions. Moreover, the conditions of the reaction were assumed to allow the substrate concentrations to remain constant inside the membrane; that is, substrate consumption was assumed to be negligible compared with initial concentrations.

The pH-dependence of the enzymic activity may be treated using the classical assumption that the enzymes behave as dibasic acids, exhibiting two ionization equilibria. Of the three forms in which each enzyme may exist, only the monoprotonated species shows activity. The pH-dependence may be expressed by the following equation [17]:

$$\alpha_i = \frac{V_{M(pH)}^i}{V_{M(opt)}^i} = \frac{K_{es1}^i 10^{-pH}}{10^{-2pH} + K_{es1}^i 10^{-pH} + K_{es1}^i K_{es2}^i} \quad i = 1, 2 \quad (1)$$

where K_{es1}^i and K_{es2}^i are the ionization constants of enzyme i , and $V_{M(opt)}^i$ and $V_{M(pH)}^i$ are the maximal rates at the optimum pH or at a given pH respectively. This expression is valid only if the relation between the ionization constants of the enzyme $K_{es1}^i / K_{es2}^i > 10^2$ holds [17].

The optimum pH for E_i activity will occur at:

$$pH_{(opt)}^i = \frac{pK_{es1}^i + pK_{es2}^i}{2} \quad i = 1, 2 \quad (2)$$

The pH optima for the activities of E_1 and E_2 should be different, with that of E_1 lower than that of E_2 (Fig. 1) in order to obtain an asymmetric pattern (see below). Another condition required by the model is that the immobilized enzymes are homogeneously distributed and that only substrates and products can diffuse in the membrane.

The instantaneous pH is assumed to be described by the following phenomenological expression:

$$pH = pH^* + \beta ([P_2]/[P_1]) \quad (3)$$

with pH^* , the pH of the unstable steady state, given by $\frac{1}{2}(pK_{es2}^1 + pK_{es1}^2)$.

This expression is strictly valid only around pH^* , where $[P_1]/[P_2] \ll 1$, and may be verified experimentally close to this pH. Although the expression may look rather *ad hoc*, it may be obtained by expanding the function $pH = pH^* + \log[(\beta [P_2] + 1)/(\beta [P_1] + 1)]$ as a Taylor series and neglecting the quadratic and higher-order terms. This function was derived from the following reasoning: since P_1 is an acid, an increase in its concentration will lead to a decrease in the pH with respect to pH^* ; the converse is true for an increase in the concentration of the alkaline product, P_2 . Unity is added to the $[P_i]$ values in order to account for their positive contributions to the change of pH (since for an argument of the logarithm lower than unity the logarithmic function will be negative); then only the non-dimensional ratio $(\beta [P_2] + 1)/(\beta [P_1] + 1)$ is allowed to reach values over or under unity. From the experiments with reaction products of glutaminase and urease the order of magnitude of β was estimated as $1 M^{-1}$.

Under these conditions, the temporal change of pH at any point in the membrane width (the spatial co-ordinate actually taken into account) ($\partial pH / \partial t$) is accounted for by a diffusional term ($\partial^2 pH / \partial x^2$) and a reaction term $[\sigma F(pH)]$. Thus the kinetic equation describing the pH dynamics is a reaction–diffusion equation:

$$\left. \begin{aligned} \frac{\partial pH}{\partial t} - \frac{\partial^2 pH}{\partial x^2} - \sigma F(pH) &= 0 \\ \text{Boundary conditions: } pH(0, t) &= pH_0 \text{ and } pH(1, t) = pH_1 \\ \text{Initial conditions: } pH(x, 0) &= g(x) \end{aligned} \right\} \quad (4)$$

where

$$\begin{aligned} F(pH) &= \alpha_2 - \alpha_1 \\ \sigma_1 &= \frac{V_{M(opt)}^1 e^2}{D_{H^+}}; \quad \sigma_2 = \frac{V_{M(opt)}^2 e^2}{D_{H^+}} \\ \sigma_1 &= \sigma_2; \quad \sigma = \beta \sigma_1 = \beta \sigma_2 \end{aligned}$$

where $pH(0, t)$ and $pH(1, t)$ indicate the pH values at any time, t , at the boundaries of the membrane width (spatial co-ordinates 0 and 1). The initial condition is accounted for by the function $g(x)$ describing the spatial distribution of pH at time 0. In this case $g(x)$ is the equation of a straight line with slope $(pH_0 - pH_1)/e$ and y-intercept pH_1 .

In the model we have chosen $\sigma_1 = \sigma_2$ in order to reduce the number of parameters to be investigated. The experimental conditions were selected so that $V_{M(opt)}^1 = V_{M(opt)}^2$ to fulfil $\sigma_1 = \sigma_2$.

Eqn. (4) has been made non-dimensional by introduction of the factors e^2 / D_{H^+} and e , the membrane width, as characteristic time and space units respectively. This characteristic time is proportional to the time constant of the system, equal to $\theta = e^2 / D_{H^+}$. Thus the parameter σ contains kinetic and spatial information about the system. Mathematically, the existence and uniqueness of a solution to the system of eqns. (4) can be proved by the Leray–Schauder–Tychonov theorem [18]. The numerical

simulation is done with an explicit-finite-difference method. pH_i^n is an approximation of $\text{pH}(i\Delta x, n\Delta t)$, where Δx and Δt are the space and the time steps respectively, and i and n the number of the spatial and temporal steps being considered. The approximation is defined by the following scheme:

$$\left. \begin{aligned} \frac{\text{pH}_i^{n+1} - \text{pH}_i^n}{\Delta t} - \frac{\text{pH}_{i+1}^n + \text{pH}_{i-1}^n - 2\text{pH}_i^n}{\Delta x^2} + \sigma F(\text{pH}_i^n) &= 0; \\ 1 < i < N-1; \quad n > 0 \\ \text{pH}_0^n = \text{pH}_0, \quad \text{pH}_N^n = \text{pH}_1, \quad n > 0 \\ \text{pH}_i^0 = g(i\Delta x) \quad 1 < i < N-1 \end{aligned} \right\} \quad (5)$$

This scheme is eqns. (4) written in a discrete manner with finite increments instead of infinitesimals. N is the number of spatial intervals, so that $N\Delta x$ will account for the total length of the space considered (e). By analysis of the stability of the explicit scheme (5), the approximate function pH_i^n is bounded (i.e. is stable) for $1 < i < N-1$ and for $n > 0$, if $F(\text{pH})$ is bounded and for the absolute value of $\Delta t/(\Delta x)^2 < \frac{1}{2}$.

If the pH-dependence curves of the enzymes were found to exactly overlap each other, then one homogeneous steady state might be expected to occur at $\text{pH} = \frac{1}{2}(\text{p}K_{\text{es}2}^1 + \text{p}K_{\text{es}1}^2)$. This value corresponds to the point where the two curves of pH-dependence cross each other (Fig. 1). However, thermodynamic analysis for the excess entropy production shows that this solution is always unstable (D. Thomas, A. Goldbeter & R. Lefever, unpublished work). The behaviour of the system, the internal pH profile across the membrane, and its evolution until achievement of the final state will depend on the initial and boundary conditions.

EXPERIMENTAL

Materials

Urease (urea amidohydrolase EC. 3.5.1.5.) from jack beans (*Canavalia ensiformis*), type IV (63 units/mg dry wt.), and glutaminase (L-glutamic aminohydrolase, EC 3.5.1.2) from *Escherichia coli*, grade II (5.4 units/mg dry wt.), were from Sigma Chemical Co.

Methods

Membrane preparation. Artificial protein membranes were prepared by co-cross-linking methods [19]. A solution of 0.4% (v/v) glutaraldehyde, enzymes (variable concentrations) and 4% (w/v) BSA in 20 mM-phosphate buffer, pH 7.0, was spread on a plane glass surface. This solution was dried under a cold airstream, producing a protein film of 80 μm thickness when 1 ml of the above solution was spread on an 8 cm^2 surface. The film thus obtained was washed with 20 mM-phosphate buffer, pH 7.0, and immersed for several hours in glycine solution (10 g/l) in order to block free glutaraldehyde groups. The activity of the immobilized enzymes was stable for at least 2 weeks.

Enzyme-activity measurements. A pH-stat assay was employed to determine enzyme activity. The urease assay was performed at 25 °C in a reactor containing a total volume of 4 ml. The reaction mixture contained 0.15 M-urea and 0.1 M-KCl in 20 mM-potassium phosphate (for pH 8.0–6.5) or 20 mM-potassium phosphate/acetate (for pH 6.5–5.0) and the enzyme immobilized in a membrane volume of $3 \times 10^{-2} \text{ cm}^3$. The rate of 1 mM-HCl addition was continuously recorded (pH-meter E 300; Impulsomat E 473 titrator; Dosimat E 535 electronic burette; electrodes A 9100; Metrohm A. G., Herisau, Switzerland).

The glutaminase assay was performed at 25 °C in a reactor containing a final volume of 4 ml. The reaction mixture contained

0.32 M- γ -methylglutamic acid and 0.1 M-KCl in 20 mM-potassium phosphate/acetate buffer and the enzyme immobilized in a membrane volume of $3 \times 10^{-2} \text{ cm}^3$. The titration curve with 1 mM-NaOH solution was continuously recorded.

The reactions may be written as follows:

For both enzymes, the rates of activity measured as mol of acid (urease) or base (glutaminase) consumed/min per mg of protein did not necessarily show the same optimal pH when these activities were expressed as mol of NH_3 (urease) or glutaric acid monomethyl ester (glutaminase) production. This effect is attributable to the weak basic (urease) or acid (glutaminase) properties of the reaction products and consequently the variations in the degree of dissociation of the product as a function of pH. The two types of measurements may be related through a factor derived by the method of Bücher *et al.* [20].

Since enzyme activities were determined through the H^+ flow from the membrane or from the soluble phase, membranes containing very low concentrations of immobilized enzymes were used (activity less than 2 $\mu\text{mol}/\text{min}$ per cm^3 of membrane volume) in order to avoid diffusion becoming the rate-limiting step. This effect will in turn provoke product accumulation, changing also the pH inside the membrane with respect to that of the surrounding solution [21]. The magnitude of this effect may be estimated by considering the Thiele modulus [22], which relates the characteristic diffusion time, e^2/D_{H^+} , to the characteristic time for reaction, C_p/R_p , where C_p is the product concentration and R_p is the rate of product formation. The concentration of product inside the membrane is assumed to be at steady state, thus the characteristic time for reaction should be lower than the characteristic time for diffusion (in our case, the Thiele modulus $< 10^4$).

Diffusion-coefficient measurement. The H^+ diffusion coefficient in the protein membrane was measured in a diffusion cell consisting of two compartments of equal volume separated by a membrane 100 μm thick with 12 cm^2 of interfacial surface. After addition of a known quantity of a strong acid (HCl), the pH evolution in both compartments was monitored until equilibration, which was attained after 5 h. The slope of the curve $\log\{([\text{H}^+]_I - [\text{H}^+]_{II})/([\text{H}^+]_I + [\text{H}^+]_{II})\}$ versus time is equal to $2D_{\text{H}^+} \cdot S/d \cdot V$, where $[\text{H}^+]_I$ and $[\text{H}^+]_{II}$ are the H^+ concentrations in compartments I and II respectively, S is the interfacial surface area, V is the volume in each compartment, d is the thickness of the membrane and D_{H^+} is the diffusion coefficient of H^+ [16].

Experimental observation of patterns. In order to observe the spatial patterns of pH, the membrane was saturated with an acid-base indicator, Bromothymol Blue, which undergoes a transition from yellow to blue between pH 5.7 and 7.7 ($\text{p}K = 6.7$). The membrane was equilibrated at pH 6.0 and then placed in the reactor shown in Scheme 1, thermostatically controlled at 25 °C. The reactor consisted of two compartments containing the substrate solutions in which the pH was controlled and maintained at a constant value with a pH-stat. Each compartment contained, in a final volume of 10 ml, 0.12 M-urea, 0.28 M- γ -methylglutamic acid and 0.1 M-KCl in 20 mM-potassium phosphate/acetate buffer at a pH specified for each experimental situation. The compartments were separated by a bridge on which the membrane was placed in such a way that both membrane borders were immersed. According to the model, the

Scheme 1. Device used to visualize the pattern in the enzyme membrane

The upper semicircumference indicates the distance between the two reservoirs, i.e. the membrane width e , corresponding to the direction parallel with diffusion, across which the pattern could be visualized (see the text).

patterns are expected to appear across the distance between the two reservoirs, the membrane width (e), in the experimental device used in this work (Scheme 1). The time course of pH evolution inside the membrane was followed through the colour shift of the pH indicator. Photographs and densitometric analysis (at 610 nm) of the membrane were recorded at regular time intervals. Densitometric profiles were obtained with a Cliniscan densitometer (Helena Laboratories, Beaumont, TX, U.S.A.).

RESULTS

Study of the pH-dependence of immobilized glutaminase and urease activities

The activities of immobilized glutaminase and urease were studied as a function of pH in order to investigate if model conditions were fulfilled. Since the point where the two curves of pH-dependence cross each other is highly unstable (Fig. 1), it is important to know its value in order to reproduce the conditions in which the spatial asymmetric pattern can appear (see below). Fig. 2(a) shows the activity of urease with an optimum at pH 7.0. Owing to the basic properties of the reaction product, the branch corresponding to acid pH is autocatalytic, i.e. product formation alkalizes the medium, driving the pH closer to the optimum and therefore increasing the reaction rate. From this curve, the values of $K_{es1}^2 = 1 \times 10^{-4}$ M and $K_{es2}^2 = 7.08 \times 10^{-7}$ M were calculated and used to integrate eqns. (4) numerically.

The rate of the reaction catalysed by glutaminase exhibits a maximum at pH 5.1 (Fig. 2b). In this case the alkaline branch is autocatalytic, owing to the balance of acid-base properties between substrate and products. Namely, acidification of a

Fig. 1. Hypothetical bienzyme E_1 and E_2 system activities as a function of pH

The point at which the curves cross each other corresponds to an unstable steady state. Arrows indicate the autocatalytic branches of the two enzymic reactions.

Fig. 2. pH-dependence of immobilized urease and glutaminase activities

(a) Specific activity of immobilized urease expressed as $\mu\text{mol of acid consumed} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$. (b) Specific activity of immobilized glutaminase expressed as $\mu\text{mol of alkali consumed} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$. Enzyme-activity measurements were performed as indicated in the Materials and methods section. (c) Difference between urease and glutaminase activities ($V_{ur} - V_{glu}$) for a membrane containing a ratio of 25 mg of glutaminase per mg of urease. Activities are expressed as $\mu\text{mol of acid consumed} \cdot \text{min}^{-1}$, and negative values indicate that the system consumed alkali rather than acid owing to a predominance of glutaminase activity. Arrows indicate the direction of evolution of the system when it crosses through the unstable steady state. In all cases each point is the average of four independent measurements, and S.E.M. being less than 5% of the value.

reaction mixture initially, at pH > 5.1 occurs as the reaction evolves, driving the pH closer to its optimum and therefore increasing the reaction rate. The values of $K_{es1}^1 = 1.58 \times 10^{-6}$ M and $K_{es2}^1 = 1 \times 10^{-8}$ M obtained from this curve were used in the numerical simulations.

The specific-activity ratio of immobilized urease/glutaminase is approx. 25. Therefore a ratio of 0.04 mg of urease to 1 mg

glutaminase was used to prepare the proteic membranes, in order to obtain the same V_M at the respective $\text{pH}_{(\text{opt})}$ and thus to fulfil the imposed condition of $\sigma_1 = \sigma_2$.

Fig. 2(c) shows the difference between the reaction rates of urease and glutaminase for the immobilized bienzyme system corresponding to an activity ratio of 1, i.e. 25 mg of glutaminase/mg of urease. When the difference between enzyme activities is zero, at $\text{pH} = 6.0$, the bienzyme system changes from a mode in which glutaminase activity prevails over urease activity to a mode in which urease activity predominates. This pH value is important, because at this value the sense of the amplification, driven by autocatalysis, towards acidification or alkalization, will abruptly change. Thus the reaction system behaves like a marble at the apex of a convex surface: the slightest disturbance will be amplified and the marble will roll downhill towards the left or the right depending on the direction of the disturbance. In consequence, the behaviour of the system will depend strongly on the initial and boundary conditions of the assay.

Visualization of the pH profiles inside the membrane

According to the result of numerical simulations, when the system is placed at the unstable steady state, analysis of the pH profile evolution across the membrane reveals three possible situations. If a small disturbance takes place at the boundaries, it will be translated into a local pH perturbation inside the membrane. The numerical resolution of the system predicts that it will be amplified and propagated across the membrane width. Figs. 3(a)–3(c) show the internal profile of pH in the membrane obtained by numerical integration of eqn. (4) for three different boundary conditions and/or σ . The temporal evolution of the pH inside the membrane passes through a transient pH profile to which we will refer as the 'asymmetric pattern'. The final state is a nearly symmetric distribution of pH.

The asymmetric pattern implies a difference between acid and alkaline regions of 4 or even 6 pH units, depending on the value of σ : the larger the value of σ , the greater the difference between extreme pH values and the steeper the gradient. The asymmetric pattern is, according to simulations, numerically stable for σ values higher than 10^3 (not shown). When experiments were performed with $\sigma = 10^3$, however, slight variations of the pH at the membrane borders create gradients in the direction perpendicular to the membrane width, causing the asymmetric pattern to become unstable and distorting it.

Figs. 4–6 show the experimental results obtained with parameter values corresponding to Figs. 3(a)–3(c) respectively. The phenomenon is triggered when the membrane equilibrated at $\text{pH} 6.0$ is placed in the reactor. Fig. 4 corresponds to an experimental situation in which a perturbation of 0.3 unit of pH is applied to each compartment, but in opposite directions, i.e. one compartment at $\text{pH} 5.7$ ($\text{pH} = 6.0 - 0.3$) and the other compartment at $\text{pH} 6.3$ ($\text{pH} = 6.0 + 0.3$).

Fig. 5 corresponds to the same boundary conditions, but for different concentrations of the immobilized enzymes. The membrane contained the same activity ratio between both enzymes but with concentrations 2.4-fold lower.

The propagation of the alkaline front is observed through the change in Bromothymol Blue colour (Fig. 4a). Densitometric analysis of the membrane reveals that the pH gradient between the extreme acidic and alkaline regions is rather steep (Fig. 4a). It should be pointed out that the quantification of the difference between extreme pH values is limited by the resolution given by the colour change undergone by the indicator. By simultaneous scanning of membranes equilibrated at pH values between 6 and 8 and membranes where the pattern formation is taking place, we were able to conclude that the difference between acid and alkaline regions is at least 2 pH units.

Fig. 3. Numerical simulation of the pH profile in the membrane

The space co-ordinate represents the width of the membrane, e . Initial conditions comprise a gradient between the pH values indicated as boundary conditions. In (a) the temporal co-ordinate corresponds to a value of 0.35θ . Thus each curve represents the pH distribution across the membrane each 0.035θ . Boundary conditions are 6.3 and 5.7 and $\sigma = 450$. (b) The temporal co-ordinate corresponds to a value of 0.2θ , which implies that each curve represents the pH distribution across the membrane at 0.02θ . The boundary conditions are the same as in (a) and $\sigma = 250$. In (c) the temporal co-ordinate corresponds to a value of 0.2θ ; thus each curve represents the pH distribution across the membrane at 0.02θ ; 6.4 and 5.9 are the pH values at the boundaries and $\sigma = 250$.

Fig. 4. Photographs (a) and densitometric profiles (b) of the membrane at the indicated time intervals at high enzyme concentration

The experiment was started by placing the membrane in the reactor and perturbing the pH in each compartment: 6.3 and 5.7 (boundary conditions). The equivalent immobilized activity for each enzyme was $3.3 \times 10^{-4} \text{ M} \cdot \text{s}^{-1}$ in the membrane volume, and the distance between the two compartments was 1 cm, which gives a σ value of 430. The densitometric profiles of the membrane at the time intervals indicated in a were obtained at 610 nm (see the Materials and methods section). Arrows indicate the absorbance of membrane pieces equilibrated at the indicated pH values and subjected to densitometric analysis simultaneously with the membrane.

An alkaline, nearly symmetric pattern is attained comparatively more rapidly in the case where lower enzyme concentrations were immobilized for the same small perturbation applied at the boundaries. For the experiments shown in Figs. 4 and 5, whereas the membrane with $1.8 \times 10^{-4} \text{ M} \cdot \text{s}^{-1}$ of total enzyme activity has practically arrived at the final, nearly symmetric steady state by 10 h, the membrane containing $3.3 \times 10^{-4} \text{ M} \cdot \text{s}^{-1}$ of activity exhibits almost the same pattern as at 8 h (Fig. 4). However, the first spatial asymmetric pattern takes more time to appear, and the pH gradient is less steep (Fig. 5). The latter agrees rather well with theoretical predictions, since for $\sigma = 250$ the steady state is attained after 0.14θ (Fig. 3b), whereas for $\sigma = 450$ an equivalent of 0.28θ should pass before achievement of the final pattern (Fig. 3a).

The transient asymmetric pattern is not obtained either

Fig. 5. Photographs (a) and densitometric profiles (b) of the membrane at the indicated time intervals at low enzyme concentration

The experiment was performed as described in Fig. 4. The equivalent immobilized activity for each enzyme was $1.8 \times 10^{-4} \text{ M} \cdot \text{s}^{-1}$ in the membrane volume, and the distance between the two compartments was 1 cm, which corresponds to a σ value of 230. The densitometric profiles of the membrane were obtained as in Fig. 4.

theoretically or experimentally when the same kinetic conditions used in Fig. 3(b) are assayed, i.e. $1.8 \times 10^{-4} \text{ M} \cdot \text{s}^{-1}$ of total enzyme activities, with pH 6.0 and 6.3 at the boundaries. In contrast, a steady uniform alkalinization across the membrane was observed (Fig. 6).

DISCUSSION

The aim of the present study was to develop experimental studies and a mathematical model of an immobilized bienzyme system that is able to exhibit a phenomenon of pattern formation through a reaction-diffusion mechanism. Qualitative agreement between theoretical predictions and experimental results was observed. The principal results of the work show that the pH profile in the enzymic membrane after small perturbations at the boundaries evolves, under well-defined conditions, through an asymmetric pattern towards a final, nearly symmetric, distribution of pH (Figs. 4–6). It should be stressed that for the same parameter values used in the experiments, such as σ and the pH at the boundaries, the qualitative behaviour of the pH-profile

Fig. 6. Photographs (a) and densitometric profiles (b) of the membrane at the indicated time intervals upon an asymmetric pH perturbation

The reaction was started by perturbing asymmetrically the pH (6.3 and 6.0) in both compartments. Enzymes were immobilized with an activity equivalent to $1.8 \times 10^{-4} \text{ M} \cdot \text{s}^{-1}$ in the membrane volume. The distance between the two compartments was 1 cm, which corresponds to a σ value of 230. Densitometric profiles of the membrane were obtained as in Fig. 4.

evolution inside the membrane is described by resolution of the model equations. Moreover, the experimental values of the constants K_{es1} , K_{es2} for both enzymes were used in the equations.

Although experimental results corroborate the model predictions, there is not an exact quantitative agreement. Possible explanations of this disagreement include the following points.

(i) The model assumes a linear relationship between the pH and the difference of reaction product concentrations (eqn. 3), an assumption which is valid only at low concentrations of the products, i.e. at the beginning of the reaction. This approximation leads to an overestimation of the difference: $\text{pH} - \text{pH}^*$ for high values of $([P_2] - [P_1])$. This overestimation is equivalent, mathematically, to an increase in σ , which will lead to an increase in the steepness of the pH gradient ($\text{pH} - \text{pH}^*$) and thus to an increase in the time necessary to attain the steady state. Therefore the difference between predicted and experimental times for the evolution of the pattern may be at least partially accounted for by this approximation (see below).

(ii) The model does not take into account the buffer capacity of the membrane or of the solutes diffusing through it. In addition, diffusion is taken into account only in one dimension, the membrane width. In the experimental device used here

spontaneous gradients may also occur in the direction perpendicular to diffusion, thus giving rise to inhomogeneities. The latter may distort the gradients or the asymmetric patterns once generated, thereby always leading to the nearly symmetric final state.

(iii) A diffusion constant for H^+ through the membrane of $0.8 \times 10^{-6} \text{ cm}^2/\text{s}$ was obtained. It must be pointed out that D_{H^+} has only an operational value, since the translocation of H^+ as an entity has no physical meaning. What is actually translocated is a change of acid-base equilibria of the electrolytes present in the medium, triggered by the addition of HCl to one of the compartments. The low value of the H^+ diffusion coefficient results in a rather large time constant for the system, $\theta = 3 \times 10^2 \text{ h}$, for a 1 cm membrane width. Experimentally, the patterns evolved on the order of 10–15 h, which is well below the values predicted by the numerical simulations for achievement of the final steady state. The experimentally observed rate of propagation of the asymmetric pH pattern is significantly larger than the rate of diffusion alone. The effect of change of the indicator colour by simple diffusion was tested by placing the membrane in the reactor in which one of the compartments was at pH 9.0 and the other at pH 6.0. The compartment solutions did not contain the enzyme substrates, but only the salts and buffers normally present in the reaction media. The front of alkaline pH that propagates by diffusion took approx. 50 h to reach the opposite border of the membrane. As this time interval is much longer than the time taken when reaction coupled to diffusion drives the propagation of the alkaline front, it may be stated that the reaction accelerates the propagation because it amplifies the gradients of products inside the membrane.

The model presented here cannot be considered a Turing model. In the latter it is assumed that the initial homogeneous state is stable and that the asymmetric pattern arises because this homogeneous state becomes unstable due to perturbations as some control parameter is varied. In our model the initial state is unstable in order to be able to attain transiently the asymmetric pattern. In addition, in Turing's model the characteristic length of the pattern depends only on the reaction and diffusion features of the system, whereas in our model it is the geometric characteristic of the system, namely the membrane width, that determines the size and landscape of the pattern. The reaction and diffusion features of the system will determine the amplitude and temporal evolution of the phenomenon. It is worth noting that the appearance of coherent behaviour in the evolution of the pH profile across the membrane, i.e. the co-ordinated change of the pH towards the steady-state nearly symmetric pattern, with or without passage through a transient asymmetric pattern, constitutes a transition from microscopic to macroscopic order. This latter transition emerges when the non-linear properties of the implicated reactions are coupled to diffusion under well-defined boundary conditions. It has been suggested that the coupling of the non-linear photosynthetic reduction of an electron acceptor [23] to diffusion in the presence of a gradient was the underlying mechanism of the symmetry change of the electron acceptor in a photobiochemical system [13].

It should be stressed that, in the work presented here, the patterns did not appear to arise because of hydrodynamic instability. The pattern appears in a solid matrix in which the enzymes are immobilized and in which hydrodynamic currents are not possible. Although this example corresponds to a rather artificial system, the fact that hydrodynamics can be eliminated as a factor in the appearance of the patterns strengthens its biological significance. Owing to the highly structured nature of biological media, convection and in general hydrodynamic phenomena are likely to be precluded. On the contrary, it is possible that reactions occur coupled to diffusion, thereby giving

rise to the possibility of Turing instabilities. We do not mean to imply that these results support the notion that protons constitute a morphogen, in Turing's sense of the word, in a living organism, but rather to suggest that any substance participating in autocatalytic reactions could eventually lead to transient asymmetrical patterns which could, in turn, condition the future of a cell in a morphogenetic field.

The ability of spatial patterns to give orientational cues in a morphogenetic field has been proposed, especially in the form of a gradient profile (for a review, see [24]), which could in turn provide the cells with information about their position in the tissue [25]. Crick [26] has calculated how a chemical substance diffusing through a developing tissue from a localized source towards a sink located at the other end of the tissue could produce a gradient. The measured diffusion coefficient of H^+ is well above $10^{-8} \text{ cm}^2/\text{s}$, the threshold considered necessary for establishment of a gradient across a distance of 1 mm in a time as short as a few hours [26]. These ideas are now beginning to be accepted by experimental embryologists [27,28], and several experimental demonstrations of the existence of gradients have been reported [28–30].

The phenomenon described here, a transient rupture of pH symmetry across a membrane, may be considered as a spatial anisotropy-generating mechanism. It will be self-generated, owing to the non-linear nature of the biological reactions occurring inside the membrane, and dynamic, since it evolves in time. Several dynamic consequences may be envisaged from a 'dynamically self-generated anisotropy'. It could well be a mechanism for conditioning the activities of biological processes co-existing in space and thus a functional distribution in that field [31]. Earlier work from our laboratory showed that two compartmentalized enzymes in a selective membrane may produce a vectorial behaviour, namely a phenomenon of active transport [19]. A self-generated anisotropy would have the same dynamic consequences [32], but in this case by compartmentalization of relevant biochemical activities. It is tempting to speculate that, if a dynamically self-generated anisotropy of pH exists in the cytoplasm of a polarized cell, it would delimit several regions, or 'compartments', in which specific enzymic activities could be expressed, according to their pH-dependent activity profiles.

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