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Biological modelling / Biomodélisation

Biological processes in organised media

Processus biologiques en milieux structurés

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Abstract

Embedding a simple Michaelis–Menten enzyme in a gel slice may allow the catalysis of not only scalar processes but also vectorial ones, including uphill transport of a substrate between two compartments, and may make it seem as if two enzymes or transporters are present or as if an allosterically controlled enzyme/transporter is operating. The values of kinetic parameters of an enzyme in a partially hydrophobic environment are usually different from those actually measured in a homogeneous aqueous solution. This implies that fitting kinetic data (expressed in reciprocal co-ordinates) from in vivo studies of enzymes or transporters to two straight lines or a sigmoidal curve does not prove the existence of two different membrane mechanisms or allosteric control. In the artificial transport systems described here, a functional asymmetry was sufficient to induce uphill transport, therefore, although the active transport systems characterised so far correspond to proteins asymmetrically anchored in a membrane, the past or present existence of structurally symmetrical systems of transport in vivo cannot be excluded. The fact that oscillations can be induced in studies of the maintenance of the electrical potential of frog skin by addition of lithium allowed evaluation of several parameters fundamental to the functioning of the system in vivo (e.g., relative volumes of internal compartments, characteristic times of ionic exchanges between compartments). Hence, under conditions that approach real biological complexity, increasing the complexity of the behaviour of the system may provide information that cannot be obtained by a conventional, reductionist approach. ***To cite this article: M. Thellier et al., C. R. Biologies 326 (2003).***

Résumé

La fixation d'enzymes michaéliennes dans une lame de gel peut suffire à les rendre aptes à catalyser des processus, non seulement scalaires, mais également vectoriels, y compris le transport actif d'un substrat entre deux compartiments, et à les faire se comporter comme le feraient un double mécanisme enzymatique ou de transport ou un processus allostérique. Dans un environnement partiellement hydrophobe, les paramètres cinétiques apparents n'ont en général rien à voir avec les véritables

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paramètres caractéristiques du comportement de la même protéine en solution aqueuse. Réciproquement, lorsque l'on observe (en coordonnées inverses) que des systèmes enzymatiques ou de transport in vivo s'ajustent mieux à deux approximations linéaires ou à une courbe sigmoïde qu'à une seule droite, ceci ne prouve pas qu'interviennent deux systèmes enzymatiques ou de transport différents ou un processus allostérique. Avec les systèmes de transport étudiés ici, il est apparu qu'une asymétrie fonctionnelle pouvait suffire à induire un transport à contre-gradient en l'absence de toute asymétrie structurale ; aussi, bien que tous les systèmes de transport actif isolés jusqu'ici correspondent à des protéines à structure asymétrique par rapport à la membrane où elles sont insérées, on ne peut pas exclure que des systèmes de transport à structure symétrique existent ou aient existé au cours de l'évolution. Avec des systèmes réels, tel que celui qui maintient le potentiel électrique de la peau de grenouille, augmenter la complexité du comportement du système par l'induction d'oscillations électriques par addition de lithium a permis d'atteindre des données sur ce système qu'il n'aurait pas été possible d'obtenir par l'approche réductionniste traditionnelle. ***Pour citer cet article : M. Thellier et al., C. R. Biologies 326 (2003).***

Keywords: immobilised enzymes; enzyme kinetics; aqueous/hydrophobic reaction media; artificial transport systems; electric oscillations

Mots-clés : enzymes immobilisées ; cinétique enzymatique ; réaction en milieu partiellement aqueux/partiellement hydrophobe ; systèmes artificiels de transport ; oscillations électriques

1. Introduction

In the last century, considerable progress has been made in separating, identifying and purifying the molecules that constitute living cells. In particular, purification of information-bearing molecules such as nucleic acids, proteins and polysaccharides has elucidated the relationship between structure and function under rigorously defined, controlled in vitro conditions. Despite the continuing importance of this reductionist approach to both fundamental biology and biotechnology, it is clear that macromolecules are in completely different conditions in vitro from in vivo. In the latter, they are located in compartments or at compartment boundaries and are surrounded by a variety of small mobile solutes that may be ionised; this heterogeneity means that the important parameters are not only concentrations of reactants and products but also electric fields, gradients of solute activities (including pH gradients), and transport and reaction rates.

In the present paper, we approach the problem of biological complexity by studying the behaviour of two different types of systems: (i) simple, in vitro enzymatic systems in which the media is either of a controllable structural complexity or of an aqueous-hydrophobic nature and (ii) a complex, *in vivo* system subjected to an extra constraint that in the case presented here is the frog skin after a specific alteration of its ionic environment.

2. Simple, in vitro enzymatic systems

This section concerns simple, in vitro enzymatic systems in which the media is either of a controllable structural complexity or of an aqueous-hydrophobic nature.

A number of studies have dealt with the in vitro behaviour of immobilised enzymes [1–3]. Studies in our group, as explained in the examples given below, illustrate the emergence of a complex behaviour in cases in which simple, 'monosteric' (as opposed to 'allosteric') enzymes were inserted in various types of artificial structures.

2.1. The problem

The simplest possible behaviour for a monosteric enzyme, E, catalysing the reaction between substrate, S, and product, P, according to:

$$\mathrm{S} \overset{\mathrm{E}}{\longleftrightarrow} \mathrm{P} \tag{1}$$

in a homogeneous, aqueous medium, is that described (when $P = 0$) by Michaelis and Menten's kinetic equation [4]

$$V = V_{\mathrm{m}} S/(K_{\mathrm{m}} + S) \tag{2}$$

in which the italic symbol (e.g., S) characterises the concentration of the corresponding substance (e.g., S), and V_{m} and K_{m} are the maximum rate and the Michaelis constant of the reaction. The corresponding

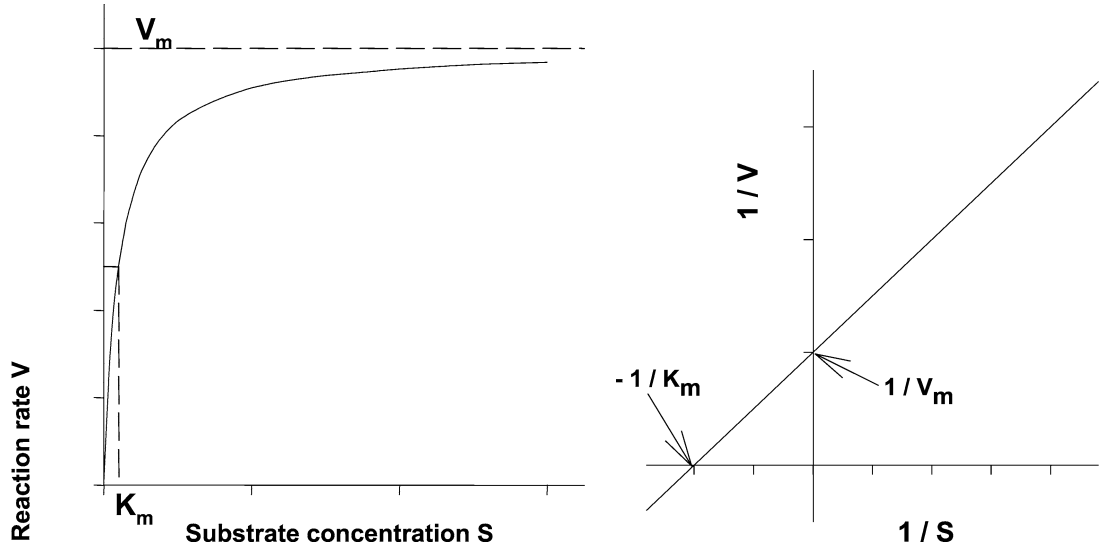

Fig. 1. Representative curve of the kinetics of a Michaelis and Menten enzyme reaction using either (a) the direct or (b) the reciprocal system of coordinates.

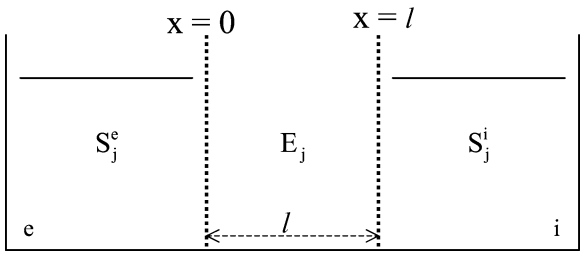

Fig. 2. General scheme of the in vitro systems studied. A gel slice, l in thickness and containing one or several enzymes, E_j, distributed at random, is used to separate two aqueous media, **e** and **i**, containing one or several solutes S_j.

curve in the direct system of co-ordinates is a hyperbola (Fig. 1a), but it is customary to use the reciprocal system of co-ordinates $\{1/S, 1/V\}$ (equation (3)):

$$\frac{1}{V} = \frac{K_m}{V_m}\frac{1}{S} + \frac{1}{V_m} \tag{3}$$

which gives a straight line, whose intercepts with the co-ordinate axes are $-1/K_m$ and $1/V_m$ (Fig. 1b) [5]. In such a case, the reaction kinetics are said to be 'monophasic'.

Now, let us consider model systems in which monosteric enzymes, E_j, are inserted at random in a gel slice, with thickness l, separating two aqueous solutions, **e** and **i** (Fig. 2) [6]. Locally, the rate, V_j, of the catalysed reaction(s) of substrate(s) S_j and product(s) P_j (4):

$$S_j \overset{E_j}{\longleftrightarrow} P_j \tag{4}$$

is again a hyperbolic function of S_j (for $P_j = 0$), which may be written as:

$$V_j = V_{mj}\gamma_j\lambda_j \tag{5}$$

$$\lambda_j = S_j/(K_{mj} + S_j) \tag{6}$$

In these equations V_{mj}, γ_j, λ_j and K_{mj} (assumed to be constant with respect to pH) are the maximum rate, the possible pH dependence, the substrate dependence and the Michaelis constant of enzyme E_j in solution, respectively. Within the gel slice, concentration S_j obeys the differential equation:

$$\partial S_j/\partial t = D_j(\partial^2 S_j/\partial x^2) - V_j(x,t) \tag{7}$$

where t is time, D_j the diffusion coefficient of S_j and x the distance along l with $x = 0$ and $x = l$ at the boundaries of **e** and **i**. The flux, J_j of S_j at time t and at point x in the gel slice is written as:

$$J_j(x,t) = -D_j(\partial S_j/\partial x)_{x,t} \tag{8}$$

while the rate of disappearance, $\overline{V_j}$, of S_j from one of the aqueous solutions (e.g., **e**) is written

$$\overline{V_j^e} = -J_j(0,t)A/v^e \tag{9}$$

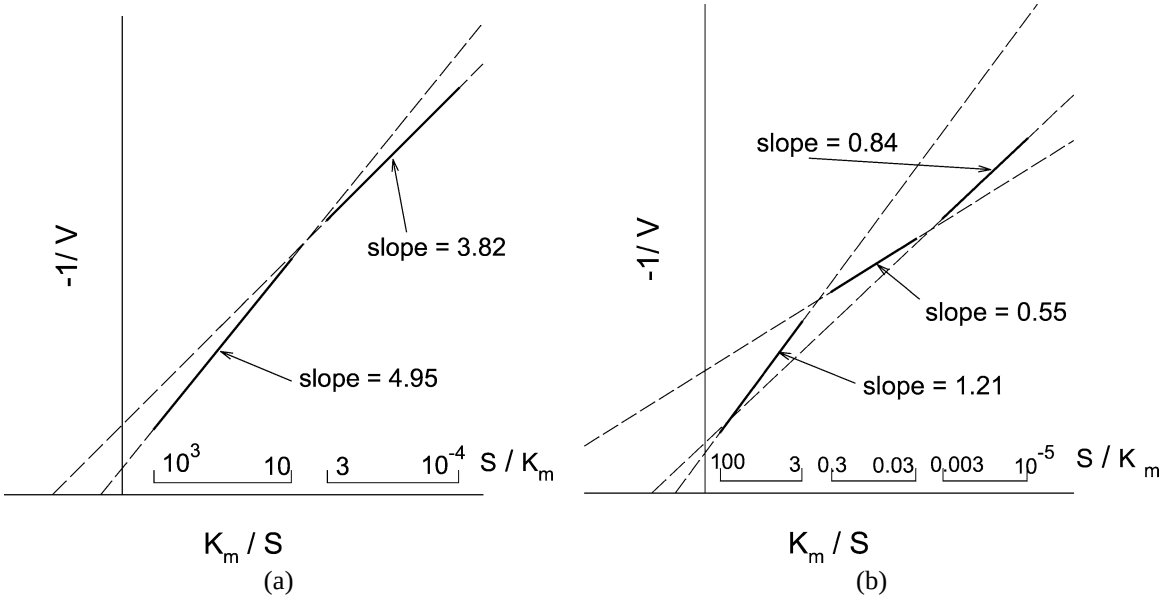

Fig. 3. Kinetic behaviour (reciprocal system of coordinates) of a monosteric enzyme system, similar to that described in Fig. 2, for increasing values of αl: (a) dual-phasic behaviour and (b) sigmoidal behaviour.

where A is the surface area of the gel slice and v^{e} the volume of compartment **e**.

2.2. Kinetic behaviour of a monosteric enzyme inserted in a gel slice separating aqueous compartments

In the simplest possible case, i.e. when the system is monoenzymatic (index j thus may be deleted), the concentration of S is the same in **e** and **i** ($S^{\mathrm{e}} = S^{\mathrm{i}}$), the system is without any pH effect ($\gamma =$ constant) and the stationary state has been established everywhere in the gel slice ($\partial S/\partial t = 0$), one may consider the dimensionless, diffusion–reaction parameter:

$$\alpha l = \sqrt{V_{\mathrm{m}}\gamma l^2/K_{\mathrm{m}}D} = \sqrt{\tau_{\mathrm{diff}}/\tau_{\mathrm{react}}} \qquad (10)$$

In equation (10), τ_{diff} and τ_{react} are the characteristic times for the diffusion of S and for the reaction, respectively, with:

$$\tau_{\mathrm{diff}} = l^2/D \quad \text{and} \quad \tau_{\mathrm{react}} = K_{\mathrm{m}}/V_{\mathrm{m}}\gamma \qquad (11)$$

The larger the contribution of the reaction and the smaller that of diffusion, the larger is the value of αl and vice versa. Computing the dependence of $-1/\overline{V^{\mathrm{e}}}$ as a function of $K_{\mathrm{m}}/S^{\mathrm{e}}$ (reciprocal system of co-ordinates), the case when $\alpha l \ll 1$ gives a linear plot (i.e. there are Michaelis–Menten kinetics in the gel slice at all values of substrate concentration and the system behaviour thus remains monophasic) with the apparent K_{m} being equal to the actual K_{m} of the enzyme in solution and $\overline{V_{\mathrm{m}}}$ being proportional to the V_{m} of the enzyme in solution. However, as αl increases, the plot of $\{K_{\mathrm{m}}/S^{\mathrm{e}}, -1/\overline{V^{\mathrm{e}}}\}$ ceases to be linear. The curve has a monotonous negative curvature and its extremities (low and high S values) can be approximated by two different straight lines (dual-phasic behaviour). In this case, although there is a single monosteric enzyme present in the system, one could be tempted to wrongly interpret the kinetic data as corresponding to the presence of two different monosteric enzymes (one detectable at low and the other at high substrate concentrations). With pH-dependent systems (i.e. when the reaction produces or consumes protons or hydroxyl groups), situations are encountered in which the plot in the reciprocal system of co-ordinates $\{K_{\mathrm{m}}/S^{\mathrm{e}}, -1/\overline{V^{\mathrm{e}}}\}$ ceases to be linear and possibly becomes sigmoidal. Therefore, even with an enzyme of the Michaelis–Menten type, it is possible to obtain in the reciprocal system of co-ordinates (Fig. 3) a complex, non-linear (e.g., dual-phasic or sigmoidal) kinetic behaviour by imposing

appropriate (and possibly rather simple) structural conditions.

2.3. Two-enzyme artificial first-order transport systems

Let us consider again [6] a model system made of a gel slice separating two aqueous compartments, **e** and **i**, but now let us insert two monosteric enzymes, E_1 and E_2, differing in their optimum pH value and catalysing the associated reactions

$$S + XY \overset{E_1}{\longleftrightarrow} PX + Y \tag{12}$$

$$PX \overset{E_2}{\longleftrightarrow} S + X \tag{13}$$

at random in the gel slice. The overall reaction

$$XY \longleftrightarrow X + Y \tag{14}$$

is assumed to be exergonic in the direction of the splitting of XY into X and Y. Different pH values, pH^e and pH^i, are imposed on compartments **e** and **i** to create a pH gradient in the gel slice, and the pH^e and pH^i values are chosen so as to make E_1 active only in a layer, l_1, of the gel slice close to compartment **e** and E_2 active only in a layer, l_2, close to compartment **i** (Fig. 4). Under such conditions, substrate S diffusing from compartment **e** is transformed into PX by enzyme E_1 in layer l_1; the part of PX which diffuses towards l_2 is transformed back into S by enzyme E_2; this newly-formed S diffuses partially towards compartment **i** and, depending on the concentration profiles in the gel slice, the overall process can produce an uphill transport of S at the expense of the energy provided by the exergonic splitting of XY. The system

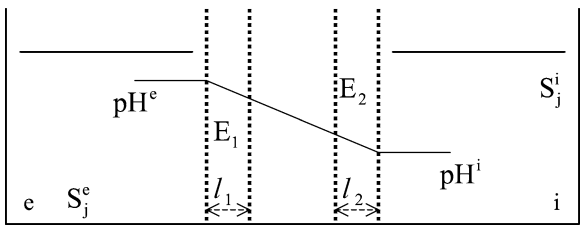

Fig. 4. Two-enzyme systems. In a system structurally similar to that described in Fig. 2, two enzymes, E_1 and E_2, are distributed at random in the gel slice. Two different pH values, pH^e and pH^i, are imposed on compartments **e** and **i**, in order that only enzyme E_1 is active in layer l_1 facing **e** and only E_2 is active in layer l_2 facing **i**.

under consideration thus is a model of a first-order [7] process of transport.

As an example, let us consider the simple case when the two enzymes have the same V_m and the same K_m in solution ($V_{m1} = V_{m2} = V_m$ and $K_{m1} = K_{m2} = K_m$) and the two layers, l_1 and l_2, have the same thickness ($l_1 = l_2 = \bar{l}$). Within each layer, the enzyme activity is treated as constant with x, while the intermediate layer (thickness $n\bar{l}$) is considered as purely diffusive, without any significant enzyme activity. The experiment starts with identical concentrations of S in compartments **e** and **i** (i.e. at time 0, $S^e = S^i = S$). Under these conditions, a dimensionless, diffusion–reaction parameter:

$$\alpha\bar{l} = \sqrt{V_m \gamma \bar{l}^2 / K_m D} \tag{15}$$

can again be defined and the initial rate of transport, $\overline{V'}$, can be computed as in section 2.1. When plotting these data in reciprocal co-ordinates $(1/S, 1/\overline{V'})$, three cases may be considered.

- When $\alpha\bar{l} \ll 1$, whether or not there is a pH feedback, the plot is linear; the transport process thus behaves as a single Michaelis–Menten type process; moreover, in this case, the apparent K_m of the transport process is found to be equal to the actual K_m of the enzymes in solution.
- When $\alpha\bar{l} \sim 1$, again whether or not there is a pH feedback, the plot in reciprocal co-ordinates appears to be made of two asymptotic straight lines, corresponding to $S \ll K_m$ and $S \gg K_m$ (dual-phasic plot), connected by a monotonous curve. Although there are only two types of enzymes with identical values for K_m and for V_m, it might seem that there are two transport processes, one with low apparent K_m and V_m and the other with high apparent K_m and V_m playing the major role at low and high substrate concentrations, respectively. Moreover the apparent K_m and V_m of transport no longer bear a simple relationship to the actual K_m and V_m of the enzymes in solution.
- When $\alpha\bar{l} \gg 1$ and when a pH feedback exists (e.g., consumption or production of protons in reactions (12) and (13)), the curve connecting the two asymptotic straight lines may become sigmoidal.

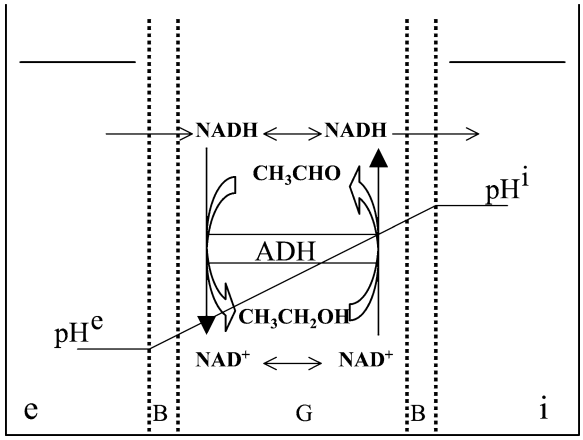

Fig. 5. A gel slice, G, with both sides covered with a barrier, B, is used to separate two aqueous compartments, **e** and **i**. The gel slice contains a random distribution of the enzyme ADH which catalyses the oxido-reduction reaction CH_3CHO/CH_3CH_2OH with the cofactor NAD. Two different pH values, pH^e and pH^i, are imposed on **e** and **i** in order that the reaction is in favour of the alcohol on the **e** side of the gel and in favour of the aldehyde on the **i** side. NADH, but not NAD^+, can diffuse freely through barrier B.

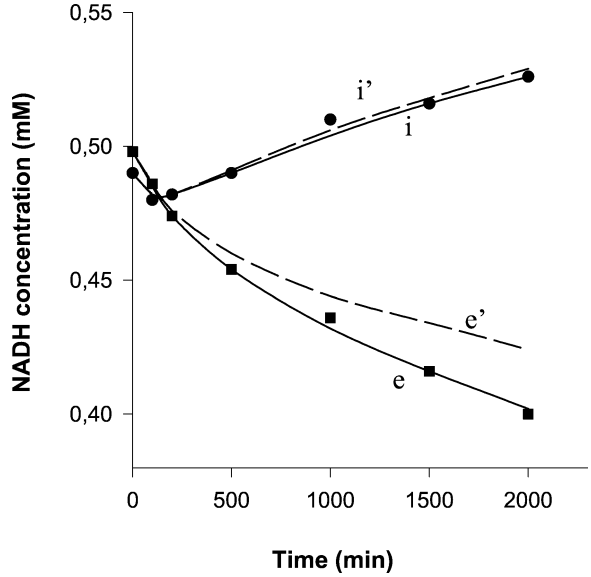

Fig. 6. Computed time-courses of the NADH concentrations in compartments **e** (curve e) and **i** (curve i). Curves e' and i' correspond to the concentration changes due only to the transport (i.e. after subtracting the spontaneous, non-enzymatic NADH degradation). Experimental points in compartments **e** (■) and **i** (●).

2.4. Monoenzymatic artificial second-order transport systems

In the preceding sections 2.2 and 2.3, we discussed purely theoretical systems. Let us now consider from both the theoretical and experimental points of view a soluble enzyme constrained to work as a transporter by particular conditions of structure and concentrations [8,9].

Yeast alcohol deshydrogenase (ADH) catalyses the reversible reaction:

$$CH_3CHO + NADH + H^+ \overset{ADH}{\longleftrightarrow} CH_3CH_2OH + NAD^+ \quad (16)$$

A gel slice, G, containing a homogeneous distribution of ADH was inserted between two barriers, B, that prevented enzyme leakage and were practically impermeable to NAD^+ while being permeable to NADH and OH^-. Gel slice and barriers were then used to separate two aqueous compartments, **e** and **i**, containing identical mixtures of alcohol, acetaldehyde, NADH and phosphate buffer, but no NAD^+ (Fig. 5). Constant pH values, more alkaline in **i** than in **e**, were imposed in the aqueous compartments, thus creating a pH gradient in the gel slice. After an initial decrease of the NADH concentration in both compartments, corresponding to NADH equilibration of the gel slice with the bathing solutions, NADH concentration began to increase in **i**, while it continued to decrease in **e**. After a few days, the NADH concentration in **i** was higher than the initial NADH concentrations in the aqueous compartments and almost twice as high as the final concentration in **e** (Fig. 6). The qualitative interpretation is as follows. At the outset of the experiment, NADH diffuses from **e** and **i** into the gel slice. In the part of the gel close to **e**, where the concentration of H^+ is the highest, NADH is transformed into NAD^+ as a consequence of the pH-dependence of the enzyme, then NAD^+ diffuses into the gel but cannot diffuse into **e** and **i** because of the barriers B. In the part of the gel close to **i**, where the concentration of H^+ is lowest, NAD^+ tends to be transformed back into NADH, thus increasing the NADH concentration in this part of the gel. As a consequence, NADH tends to diffuse toward compartment **i**. This corresponds to an uphill transport of NADH driven by the pH gradient (a transport of OH^- ions from **i** to **e** rather than a transport of H^+ ions from **e** to **i**), while electric neutrality in compartments **e** and **i** is ensured via exchanges of saline ions

provided by the buffer. Such a system is therefore a model of second-order active transport [7].

The analysis and the numerical calculations [9] were performed essentially as above (sections 2.1 and 2.2) using diffusion–reaction equations of the type:

$$\partial c_j/\partial t = D_j(\partial^2 c_j/\partial x^2) + V_j(c_j, c_k) \quad (17)$$

where c_j and c_k represent the concentrations of species j and k, t the time, D_j the diffusion coefficient of j, x the space co-ordinate in the gel and V_j the enzymatic rate of the reaction involving j. Initially, the experimental data did not seem to be in accord with the time-courses of NADH concentrations predicted from the numerical simulations. However, when a non-enzymatic, pH-dependent degradation of NADH (as determined in a complementary experiment) was taken into account, the fit between the experimental data and the predictions of the model became extremely good (Fig. 6).

2.5. Enzyme-catalysed reactions in an aqueous/hydrophobic reaction medium

The kinetics of the hydrolysis and synthesis reactions of the peptide bond of the dipeptide, *N*-Cbz-L-tryptophanyl-glycineamide, catalysed by α-chymotrypsin, have been studied in mixtures of water and 1,4-butanediol [10]. Although the polarity of 1,4-butanediol is not very high, it is miscible with water in all proportions. The initial reaction rates decreased exponentially with decreasing water content in the solvent mixture. The study of the substrate dependencies have revealed that both the apparent and the actual kinetic parameters were dependent on the water content, and thus on the polarity, of the solvent mixture. However, the exponential decrease in the initial rate of hydrolysis was due mainly to the K_m increase and only slightly to the modification of the V_m.

A reduction of the water content from 100 to 20% (v/v) did not alter V_m by more than a factor of 4. Such variation in V_m is due to changes in the conformation of the enzyme. One of the main causes of such changes in mixtures of water and organic solvent is the substitution of essential water molecules in the vicinity of the protein surface by organic solvent molecules. However, with organic solvents like 1,4-butanediol, the interactions between organic solvent molecules and the enzyme are similar to those between water molecules and enzyme; these organic solvents thus do not alter the enzyme conformation very much. Another cause is the possible modification of the ionisation/neutralisation constant of ionisable groups of the protein, in particular in the active site, as a consequence of the variation of the polarity of the reaction medium.

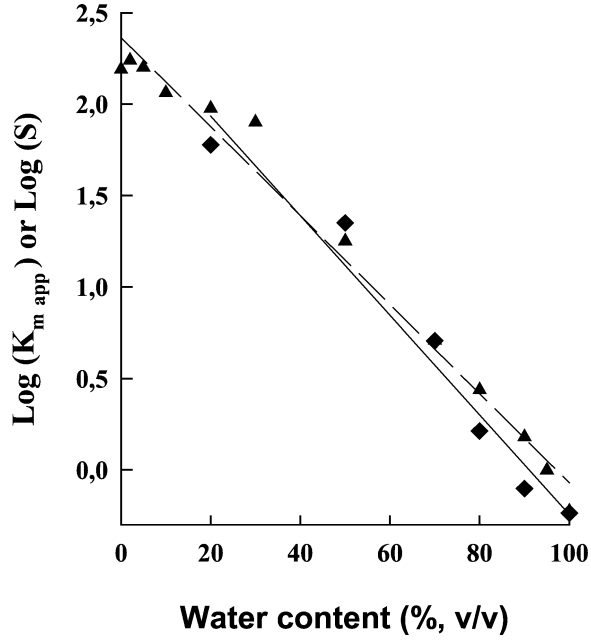

Fig. 7. Logarithms of the apparent K_m, $K_{m\,app}$, for the hydrolysis of the dipeptide (◆) and for the solubility limit, S, of this dipeptide (▲) as a function of the water content of the solvent mixture.

Decreasing the polarity of the reaction medium by decreasing its water content led to an exponential increase in the apparent K_m for the hydrolysis of the dipeptide (see above) and in the solubility of this dipeptide (Fig. 7). Since the interactions between substrate and the active site of α-chymotrypsine are mainly hydrophobic, this means that decreasing the water content of the reaction medium tends to favour the interactions of the dipeptide with the solvent mixture and thus to weaken the interactions of the dipeptide with the active site of the enzyme. Moreover, it has been established that the ratio of the actual rate constants for the formation/dissociation of the enzyme–substrate complex in the solvent mixture is given by the corresponding ratio in aqueous medium divided by the equilibrium constant for the transfer of the dipeptide from water to the solvent mixture (which

is equal to the ratio of the dipeptide solubility in the solvent mixture to that in water).

3. Effect of lithium on the electrical potential of frog skin

Frogs maintain the saline concentration of their internal medium, even when immersed in freshwater ponds, due to the activity of a Mg-dependent Na-K-ATPase that creates a steady transepithelial potential difference in the range of a few tens to more than a hundred millivolts (positive inside). This active ion-pumping system has been studied in great detail, especially by Ussing's group [11,12], using the ventral skin of frogs mounted between two aqueous compartments filled with appropriate saline solutions. Maintaining the electrical potential of frog skin in these conditions requires sodium in the external medium. Lithium is the only ion that can be used instead [13]. However, when all or part of the external sodium is replaced by lithium, the electric potential frequently oscillates [14–16], which it never does in the absence of lithium (Fig. 8).

A body of evidence obtained by others, cited in Lassalles et al. [17], demonstrates that all the ionic processes are controlled at the level of the skin

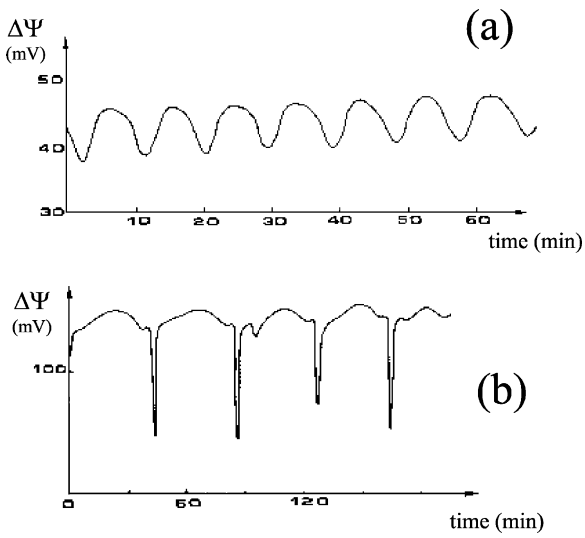

Fig. 8. Two cases of oscillations of the electric potential difference, $\Delta\Psi$ (mV), of the frog skin, (a) almost sinusoidal and (b) much more complex.

epithelium, which comprises a few layers of cells separating the external medium **e** from the internal medium **i**. The epithelium may be modelled as follows (Fig. 9): it is made of two main compartments, C_1 and C_2; the membrane, **a**, at the external face of the epithelium is relatively permeable to Na^+ and Li^+ whilst it is almost impermeable to K^+; the membrane, **b**, between compartments C_1 and C_2 is permeable to K^+ but not to Na^+ and contains the Na-K-ATPase pumping sodium and lithium from C_1 to C_2 and potassium from C_2 to C_1; the face, c, between epithelium and internal medium is not an actual membrane, but may be considered rather as a non-selective diffusive barrier.

In studies of the oscillatory process, our group [17–20] has shown experimentally that (i) when no transepithelial potential is imposed, sustained oscillations with a period of about 10 min are maintained for several hours, (ii) an oscillation of the Na^+ influx accompanies the electric oscillation (the two oscillations have approximately the same period but are not

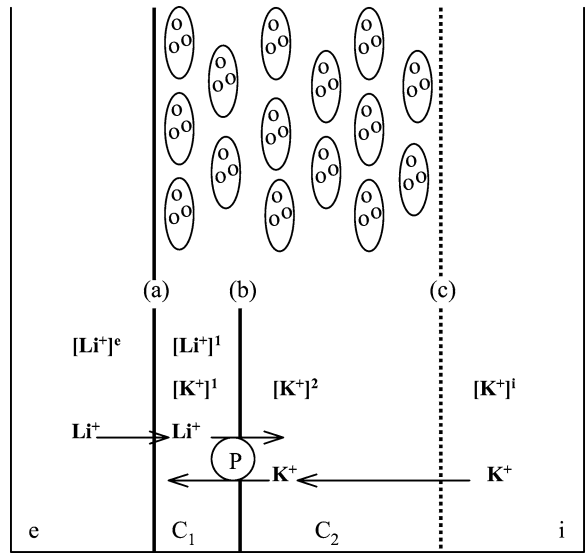

Fig. 9. Modelling frog skin epithelium. Top: schematic representation of the cellular structure of frog skin epithelium, mounted between two aqueous, saline solutions, **e** and **i**. Bottom: the epithelium model for ionic exchange. The frog epithelium is modelled as corresponding to two compartments C_1 and C_2 with three barriers, **a** between **e** and C_1, **b** between C_1 and C_2 and **c** between C_2 and **i**. Symbol $[x]^j$ stands for concentration of cation x in compartment j. The Na-K-ATPase, P, pumps actively Li^+ from C_1 to C_2 and K^+ from C_2 to C_1.

in phase), (iii) under conditions of imposed potential the transepithelial electric current has damped oscillations, (iv) the shape of the oscillations in potential is quite variable (from almost sinusoidal to very complex), (v) theophyllin (which induces an accumulation of cyclic AMP within the cells) promotes a significant decrease in the mean electric potential of the skin but does not affect the characteristics of the oscillation very much, (vi) important factors influencing the oscillations include temperature, the permeability of the external membrane to lithium and the potassium concentration in the internal medium, (vii) no evident correlation exists between skin area and the characteristics of the oscillations, which may therefore have a local origin (possibly in local oscillators at the level of the cell) and the coupling of these oscillators would generate the macroscopic oscillations and (viii) synchronisation of local oscillators can be controlled by varying the coupling resistance in the absence of diffusion, which is consistent with electrical coupling rather than diffusion being responsible for the synchronisation.

After extensive numerical simulation of the oscillatory process, it proved impossible to obtain all the above characteristics unless the model had a few well-defined properties, the following ones in particular.

- If $\pounds_1$ and $\pounds_2$ represent the volumes of compartments C_1 and C_2 per unit surface of epithelium, they obey the relation $\pounds_1/\pounds_2 \ll 1$, i.e. compartment C_2 has to be much larger than compartment C_1. This makes it possible to choose one of the various possible modes of transport of Na^+ through the epithelium proposed in the literature and cited in Lassalles et al. [17]. Indeed, $\pounds_2 \gg \pounds_1$ does not support the idea that compartment C_1 merely corresponds to most of the cytoplasmic volume and C_2 is restricted to a few endoplasmic cisternae, whereas it strongly supports the idea that compartment C_1 corresponds to a few cytoplasmic vacuoles transporting Na^+ (and Li^+) and compartment C_2 is part or all of the intercellular spaces and possibly some endoplasmic cisternae (in this case, most of the cell cytoplasm would remain inactive in Na^+ (or Li^+) transport).
- The main variables are the concentration of Li^+ in compartment C_1 and that of K^+ in compartment C_2. When the characteristic times τ_1 and τ_2 are defined as corresponding to Li^+ modifications in C_1 and K^+ modifications in C_2, respectively, the ratio of the characteristic times obeys the relation $0 < \tau_1/\tau_2 < 1$ with $1\ \text{min} \leqslant \tau_1 \leqslant 5\ \text{min}$.
- Two parameters, ρ and u, represent the relative importance of the active to the passive fluxes of Li^+ and K^+, respectively, such that $\rho \gg 1$ and $u \gg 1$, with $\rho \leqslant u \leqslant 2\rho$ (i.e., ρ and u are of the same order of magnitude).
- P represents the permeability coefficients of interfaces **a**, **b** and **c** for ions K^+ and Li^+, such that $P_K^b/P_K^c \ll 1$ and $P_{Li}^a/P_K^c \ll 1$.

4. Discussion and conclusions

Embedding simple Michaelis–Menten enzymes in a gel slice allows them to catalyse not only scalar processes but also vectorial ones, including the uphill transport of a substrate between two compartments. Moreover, a system containing a single species of a simple enzyme can be made to behave as if it contained two different enzymes (or transporters) or an allosterically controlled enzyme (or transporter). These effects are more pronounced the larger the term characteristic of the reaction ($V_m\gamma/K_m$) compared with that of substrate diffusion (D/l^2). The existence of a pH feedback also tends to increase these effects. In the presence of a partially hydrophobic environment (which is likely to be the case with membrane-bound enzymes or transport systems in vivo), the measured kinetic parameters are usually different, sometimes very different from those characteristic of the protein behaviour in homogeneous, aqueous solutions. Another group [21,22] has also shown that it is possible to induce oscillatory phenomena in immobilised enzyme systems or spatio-temporal pattern formation in immobilised bienzymatic systems.

These results are of general relevance to the interpretation of kinetic data on membrane-bound enzymes or transport systems. In reciprocal co-ordinates, such experimental data are often better fitted by two straight lines (one for the high and the other for the low concentrations) than by a single line. In line with the original proposal of Epstein and co-workers [23–25], most authors interpret such data as revealing the presence of two different membrane mechanisms of reaction or transport, with the apparent K_m and V_m of the membrane processes being equated to the actual K_m and

V_m of the catalytic proteins involved. Similarly, sigmoidal experimental curves have been taken as indicative of an allosteric character of the membrane-bound active proteins [26]. Our results clearly show that such interpretations can be wrong, since the complexity of the kinetic behaviour may as well correspond to the partially hydrophobic nature and other features of the cellular structures in which the enzyme or transport systems are inserted (for a more detailed discussion, see Thellier et al. [27]). Indeed, an alternative interpretation to allosteric interaction has been given for sigmoidal kinetic behaviour in an actual biological system in vivo [28].

A real cell membrane is obviously not the same as a thick homogenous gel slice or a water/hydrophobic mixture. Nevertheless, the values of the parameters used in the experiments reviewed here cover the range of biological possibilities [6,8,9]. Moreover, since real membranes are far more complex than our gel slices or aqueous/hydrophobic mixtures, it is likely that the effects of membrane structure and/or hydrophobicity in vivo are even more numerous and diverse than they are in vitro.

In the artificial transport systems discussed here (sections 2.3 and 2.4), a functional asymmetry such as a proton gradient sufficed to induce an active uphill transport even under symmetrical structural conditions. Therefore, although all the active-transport systems isolated until now correspond to proteins asymmetrically anchored in a membrane, one cannot exclude the possibility that structurally symmetrical systems of transport do exist in as yet unstudied organisms or did once exist earlier in evolution.

Situations exist in biology (see Vincent et al. [9]) where the same enzymatic activity is present on each side of a membrane and where the ionic conditions are different in the media on each side. Such a situation, which closely resembles the artificial ones studied here, is important in the homeostasis of inorganic ions in cellular compartments or in the operation of relay and amplification mechanisms (in which the direction of transfer depends on differences in ion concentrations in the compartments). Moreover, the frog epithelium in vivo resembles some of the in vitro set-ups described here (sections 2.3 and 2.4), since it can be modelled as a thick two-compartment slice with both passive ion diffusion and active ion transport by an enzyme.

It is remarkable that increasing the complexity of the behaviour of the living frog-skin system by inducing electric oscillations via lithium addition to the external solution provided information on this system (such as the relative size of compartments and the values of other parameters) that could not have been provided by a conventional, reductionist approach. This may be the basis for the introduction of 'complexification', as opposed to reductionism, as a method for studying those properties of a complex system that are destroyed when adopting the conventional, reductive, biochemical approaches.

Further study of the model systems discussed above would benefit from knowledge of the distribution of ions. This may be provided by secondary ion mass spectrometry (SIMS), a method largely used by physicists for materials studies (see, e.g., [29,30]), now available for application to biological specimens [31–33] after appropriate preparation of dehydrated tissue sections [34,35] or using frozen hydrated samples [36].

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