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Structured Bienzymatical Models Formed by Sequential Enzymes Bound into Artificial Supports: Active Glucose Transport Effect

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Summary. Two different artificial membrane systems bearing two built-in sequential enzymes are studied and compared in this communication.

The first is a nonstructured membrane bearing two mixed enzymes: β-galactosidase and glucose-oxidase. Its use enables a mathematical model to be formulated describing the heterogeneous phase kinetics of a bienzymatic system. The second is a multi-layer membrane system in which the structural dissymmetry involves a spatial orientation of the reacting metabolites, resulting in active glucose transport.

The latter system consists of two active leaflets, the first phosphorylating glucose (hexokinase + ATP), the second dephosphorylating glucose-6 phosphate (phosphatase). On either side of this system, a perm-selective proteic layer allows the passage of glucose but not of glucose-6 phosphate. When positioned between two compartments containing glucose, such a membrane accumulates glucose on its phosphatase side, while degrading ATP.

The accumulation of glucose as a function of the initial concentration shows the classical saturation of the transport system. Fructose competes with glucose transport.

The chemical balance of these two reactions has the appearance of hydrolysis of ATP. Vectorial catalysis is a result of the dissymmetry in distribution of active sites and can be explained by an oscillatory concentration profile of glucose inside the membrane.

The bienzymatic mechanism, a model of which is given here, is valid for any thickness of active layers and applicable to a system where both active sides are part of the same molecule as soon as it forms a uniformly oriented monolayer throughout the membrane structure.

In his book "The Movement of Molecules Across Cell Membranes," Stein (1967) states the acceptability criteria of transmembrane transport models. The criteria selected require a molecular model which can take into account all the various types of molecules which participate, and notably require a specific correspondence between the material transported and the nature of the transporting agent. According to the same author, such a

model must be acceptable from both a physico-chemical and an energetic point of view; it must also be possible to carry out experimental measurements on it. The transport models which employ enzymic catalysts can satisfy these criteria, especially the most difficult one of specificity.

The models presented here are of the experimental type containing enzymes. The use of such experimental models does not necessarily imply that a classical enzymic mechanism is attributed to all biological transmembrane transports. When chemically well-defined materials are used, these experimental models offer the advantage of exhibiting, with the help of catalysts, mechanisms of accumulation and transport which have easily measurable characteristics of affinity and specificity, and which are of a proteic nature, as are the binding proteins of the membrane. Not only are the enzymes able to imitate the behavior of molecules which play a part in active transport, but they also constitute measurable markers which make it possible to establish the relationship between transport activity, number of sites, concentration of molecules to be transferred, and even concentration of the cofactor, etc.

The kinetic treatment of such systems is now sufficiently developed to allow computer simulation of all possible cases, resulting from previous equations (Thomas, 1971).

The experimental models presented here are produced by using as a support an inert film bearing enzymic molecules covalently bonded to it; this film is placed between two liquid compartments containing substrate solutions. An advantage of this type of film is that it simplifies the mathematical description of transport and other phenomena as it enables all the gradients in the system to be hypothetically restricted to a flow in one direction perpendicular to the film.

Whatever the form or dimensions of the system, the effect of the bonded biological catalyst differs in two ways from that of a free catalyst in solution: first, by the limitations connected with approach and diffusion constraints of the substrate and product of the reactions taking place in these supports; second, by the vectorial character which can be introduced by the geometrical structure of these supports, and by the arrangement of the catalysts within them.

The creation of metabolic sequences or cycles within cellular structures cannot be explained simply by the addition of various catalysts and necessary cofactors: a simple mixture of molecules in solution will eliminate the specific diffusion constraints of a given molecule. Neither does it take into account the mean distances to be covered, nor, consequently, the probability of each product formed meeting the specific catalyst necessary for its trans-

formation. This probability is greatly increased in juxtaposed sequential enzymic systems (such as on the walls of mitochondrial cristae).

The binding of enzymes into insoluble supports permits the creation of representative models which introduce the same diffusion constraints and which may cause the molecules to move in a given direction.

The growing interest in enzymes immobilized within structured supports (Goldman, Silman, Caplan, Kedem & Katchalsky, 1965; Money, Hornby & Crook, 1965; Goldman, Kedem, Silman, Caplan & Katchalsky, 1968) is partly justified by the necessity of a new approach to this important aspect of subcellular metabolism.

Production of such structure necessitates the improvement of methods enabling artificial structures to be realized within which enzymes will remain active; moreover, knowledge of the statistical distribution of the active sites is indispensable if theoretical reasoning on such systems is to prove valid.

An even more important step in the reproduction of cellular structures by simplified models is the well-defined spatial arrangement of enzymes on a given support. The coupling of enzymic reactions and diffusion can explain such processes, to which the name of "vectorial catalysis" has been given.

For these enzymes to initiate "vectorial catalysis," the catalytic properties of enzymes immobilized in an insoluble phase should not differ from those in solution. This has been proved by a study of the specificity, affinity and kinetics of several enzymic reactions (Thomas, Broun & Selegny, 1972).

Although the properties of the enzymes investigated do not appear to be modified when they are incorporated into insoluble supports (at least within the limits of our operating conditions), their effects and kinetics are substantially modified by certain physico-chemical parameters of the supports in which they are immobilized.

This communication deals with sequential, bienzymic systems, and gives both theoretical and experimental results for two particular cases: in the first, two products of successive transformations are formed from the substrate by enzymes distributed homogeneously in a membrane support; in the second, two enzymes that catalyze two inverse reactions are incorporated into two different active layers.

The first is a model of a cellular reaction compartment comprising a short metabolic chain, while the second is a model of active transport coupled with two metabolic reactions using the energy provided by a cofactor immobilized within the membrane. The latter has already been described among the naturally occurring mechanisms of active transport. The example given is the phosphorylation of glucose through the action of hexokinase and ATP, followed by its dephosphorylation by a phosphatase.

Only the asymmetry of the system and its permeability to glucose and glucose-phosphate, respectively, can explain the selective transport of glucose from one compartment to the other. Glucose is therefore accumulated against the chemical potential gradient owing to the energy provided by ATP degradation; a characteristic primary active transport is thus realized.

Since the work of Sols (1956), as well as that of Crane and Krane (1956), it is known that the reactive transport of glucose in the intestine cannot be linked to this type of mechanism. Yet, even if this double enzymic reaction cannot be envisaged in this particular case, some mechanisms of phosphorylation followed by dephosphorylation during transport have been shown to exist (Morgan, Post & Park, 1960) and have been analyzed for *Saccharomyces cereviciae* (Van Steveninck, 1970). Nordlie and Soodsma (1969) have shown that glucose-6 phosphatase plays a part in the reabsorption of glucose by the kidneys.

Finally, the contribution of Kropp and Wilson (1970) must be mentioned. They revealed the presence of fixation sites for hexokinase on the external mitochondrial membrane. This would suggest that this enzyme plays a part in the phosphorylation of sugars prior to their entry.

Therefore, it is not impossible that the mechanism proposed here for a model exists as a natural mechanism in the cellular accumulation of glucose, the criteria of Stein (1967) being taken into account. Even if it constitutes an oversimplified version of mechanisms observed in certain bacteria cells, as well as in the tissues of higher organisms, it does provide a fairly faithful illustration of these natural processes.

Previous attempts in this direction were described earlier (Nims, 1968; Pasynski, Moisseyeva & Zvyagil'skaya, 1964), but our system is the first artificial one to allow true active transport of biological material across a membrane.

Theoretical Analysis

Let us take two sequential enzymes E_A and E_B, each of which catalyzes one practically irreversible reaction such as:

$$S \xrightarrow{E_A} p \xrightarrow{E_B} P.$$

Under stationary state conditions, the equations are established in the same way whether the enzymes E_A and E_B are regulary distributed throughout the insoluble phase, or whether they are localized in two different layers. The symbols used in both cases are summarized in Fig. 1 *a* and *b*.

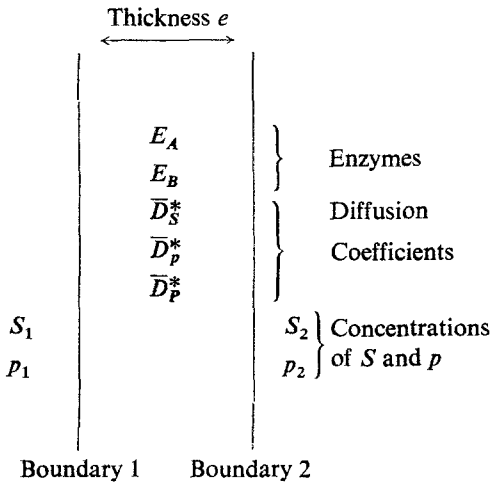

Fig. 1a

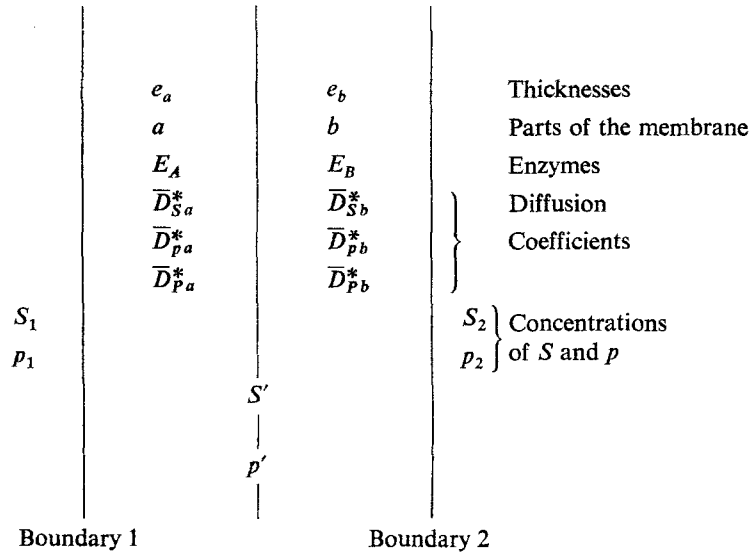

Fig. 1b

Fig. 1. (a) Diagram of the homogeneous type bienzymatical artificial membrane experimentally studied. (b) Diagram of a double-layer artificial membrane with two sequential enzymes experimentally studied

In each case the product of E_A constitutes also the substrate of E_B, but a particular case must be distinguished where E_B catalyzes the formation of the initial substrate, thus creating the two reactions:

$$S \xrightarrow{E_A} p \xrightarrow{E_B} S.$$

First Case: Homogeneous System

The two enzymes are distributed throughout the system as is shown in Fig. 1*a*. The membrane being studied separates two compartments in which the concentrations of the substrate are given as S_1 and S_2, those of the intermediary product as p_1 and p_2, and those of the final product as P_1 and P_2.

In this system, the presence of enzyme E_B does not modify the flux of the substrate S. Our investigation is carried out on the intermediary product. For each elementary volume of the membrane, the variation of the concentration as a function of time is caused by three different phenomena: the formation, degradation and diffusion of the intermediate product. This may be expressed:

$$\frac{\partial p}{\partial t}=\left\{\frac{\partial p}{\partial t}\right\}(\text{diffusion})+\left\{\frac{\partial p}{\partial t}\right\}(\text{formation})+\left\{\frac{\partial p}{\partial t}\right\}(\text{degradation}). \tag{1}$$

The Michaelis formula gives the rate of formation and transformation of p. The diffusion kinetics of p are expressed by Fick's second law.

In the stationary state, the following differential equation is obtained, in the case where S is in excess:

$$\bar{D}_p^* \frac{d^2 p}{dx^2}=V_{MA}-V_{MB}\frac{p}{K_{MB}+p} \tag{2}$$

where V_{MA} = maximal activity of E_A and V_{MB} = maximal activity of E_B.

This equation is solved by a development in series already published for monoenzymatical systems (Selegny, Broun & Thomas, 1969*a*, *b*; Thomas, Broun & Selegny, 1972). These solutions give the ingoing and outgoing fluxes of p, using the following symbols:

$$\alpha_{p_1}=\frac{p_1}{K_{MB}}; \qquad \sigma_{pB_1}=\frac{e^2}{\bar{D}^*}\frac{V_{MB}}{K_{MpB}};$$

$$\beta_{pB_1}=\frac{1}{12}\sigma_{pB_1}\frac{1}{(1+\alpha_{p_1})^2}; \qquad \rho_{pB_1}=\frac{1}{1+2\beta_{pB_1}};$$

$$\lambda_{pB_1}=\frac{\alpha_{p_1}}{1+\alpha_{p_1}}.$$

They give Eqs. (3a) and (3b) of the fluxes.

$$J_{p_1}\cong\bar{D}_p^*\frac{p_1-p_2}{e}\rho_{pB_1}+\frac{e}{2}(V_{MB}\lambda_{pB_1}-V_{MA})\rho_{pB_1}(1+\beta_{pB_1}), \tag{3a}$$

$$J_{p_2}\cong\bar{D}_p^*\frac{p_1-p_2}{e}\rho_{pB_2}-\frac{e}{2}(V_{MB}\lambda_{pB_2}-V_{MA})\rho_{pB_2}(1+\beta_{pB_2}). \tag{3b}$$

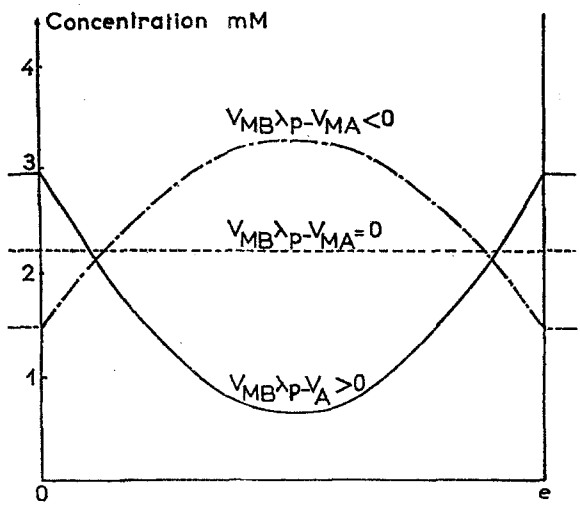

Fig. 2. Substrate concentration profiles in a homogeneous bienzymic membrane. If the value $V_{MB}\lambda_{pB} - V_{MA}$ is negative, the system produces p. If this quantity is positive, p is degraded by the system. If it is zero, the concentration profile in the membrane is horizontal and the concentration of p remains unmodified. These theoretical profiles coincide with the experimental results

Eq. (2) also enables the *concentration profiles* of p in the membrane to be calculated.

We have previously shown that substrate concentration profiles are at a minimum at one point within a membrane bearing one enzyme activity. It follows that the global transformation of the substrate is modulated within the membrane as a function of its concentration in the outside medium. In the system described here, p is transformed within the membrane where its concentration may be maintained at a given value by a stationary state established between production and degradation.

For a symmetrical case ($p_1 = p_2$), if the value $V_{MB}\lambda_{pB} - V_{MA}$ is negative, the system will tend to produce p; if this quantity is positive, p will be degraded by the system. If it is zero, the concentration of p remains unmodified, and the concentration profile is horizontal. These profiles are shown in Fig. 2.

A permanent equilibrium thus exists between the production and degradation of p, resulting in a regulation of the concentration of this intermediary product within the membrane; this concentration remains constant in spite of variations in the external concentration of S.

Second Case: Double-Layer System

In this case, two membrane layers are juxtaposed: layer 1 containing only the homogeneously distributed enzyme E_A and layer 2 only enzyme E_B, also homogeneously distributed. The reactions are of the form:

$$S \xrightarrow{E_A} p \xrightarrow{E_B} P.$$

The same reasoning is carried out with respect to the intermediary product p whose concentration is studied for each of the membrane layers.

The first method provides the following expressions for ingoing and outgoing fluxes of the intermediary product p (*see* Fig. 1*b*):

$$J_{p_1} = \frac{\bar{D}_p^*}{e_a}(p_1 - p') + \frac{\bar{D}_S^*}{e_a}(S_1 - S')\rho_{SA_1}\beta_{SA_1} - \frac{e_a}{2} V_{MA_1}\rho_{SA_1}\lambda_{SA_1}(1+\beta_{SA_2}), \tag{4a}$$

$$J_{p_2} = \frac{\bar{D}_p^*}{e_b}(p' - p_2) - \frac{e_b}{2} V_{MB}\rho_{pB_2}\lambda_{pB_2}(1+\beta_{pB_2}). \tag{4b}$$

In the particular case where E_B transforms p back to S, the substrate of E_A, we have:

$$S \xrightarrow{E_A} p \xrightarrow{E_B} S.$$

This system will result in a banal solution if the mean diffusion of p at each boundary of the system is of the same order of magnitude as that of the substrate S. Conversely, if the diffusion of p is restricted to the space formed by the two membranes externally limited by the selective surfaces impermeable to p, then S will tend to decrease in the compartment situated on the same side as E_A, and to increase in that on the other side of E_B. The substrate concentrations are designed as follows: S_1' and S_2' on the external sides of the system; S_1 and S_2 on the active layer boundaries; and S_0 in the middle of the system.

The fluxes and profiles of S can be calculated by a process similar to the one previously described. The flux entering the first active layer is:

$$J_{S_1} = \bar{D}_S^* \frac{S_1 - S_0}{e}\rho_{S_1} + \frac{e}{2} V_M \lambda_{S_1}\rho_{S_1}(1+\beta_{S_1}). \tag{5}$$

The flux leaving the first active layer is

$$J_{S_0} = \bar{D}_S^* \frac{S_1 - S_0}{e}\rho_{S_1} - \frac{e}{2} V_M \lambda_{S_0}\rho_{S_0}(1+\beta_{S_0}), \tag{6}$$

and that entering in the second active layer is

$$J_{S_0} = \bar{D}_S^* \frac{S_0 - S_2}{e} - \frac{e}{2} V_M \tag{7}$$

while that leaving the second active layer is

$$J_{S_2} = \bar{D}_S^* \frac{S_0 - S_2}{e} + \frac{e}{2} V_M. \tag{8}$$

The different parameters employed are:

$$e = \text{thickness}; \qquad \alpha_{S_1} = \frac{S_1}{K_M}; \qquad \sigma_{S_1} = \frac{e^2}{D_S} \frac{V_M}{K_M};$$

$$\beta_{S_1} = \frac{1}{12} \sigma \frac{1}{(1+\alpha_{S_1})^2}; \qquad \rho_{S_1} = \frac{1}{1+2\beta_{S_1}}; \qquad \lambda_{S_1} = \frac{\alpha_1}{1+\alpha_1}.$$

The simple passive diffusion of the initial substrate through the two selective films may be represented by two diffusion equations linking S_1 and S_2 to the external solution concentrations S_1' and S_2'. Values for the fluxes $J(S_1)$ and $J(S_2)$ at the external limits of the system may thus be obtained using the external concentrations S_1 and S_2. The profiles of the concentration of S within the membrane may also be calculated (Fig. 3).

The analytical expression of the fluxes and profiles of p may easily be obtained if a zero value is attributed to the fluxes at the limits of the active system (the selective films being impermeable to p).

Fig. 3 shows a group of profiles calculated for a membrane separating two compartments whose respective substrate concentrations are S_1 and S_2. These profiles show the evolution of the system if, after initial conditions where S_1 and S_2 are identical, S_1 is subsequently maintained constant. Compared to S_1, S_2 will increase as a function of time.

A regular spatial distribution of two different enzymes theoretically appears to allow the transformation of diffusion reaction phenomena into a transfer of substances from one compartment to another in a direction opposite to that normally occurring under the effect of the concentration gradient. It is quite clear that if these two inverse and successive transformations of the substrate into the product and then of the product back into the initial substrate are to be irreversible, at least one, if not both, of the reactions must be accompanied by a supply of energy. If this is not the case, then an equilibrium state would soon be attained, thus rendering

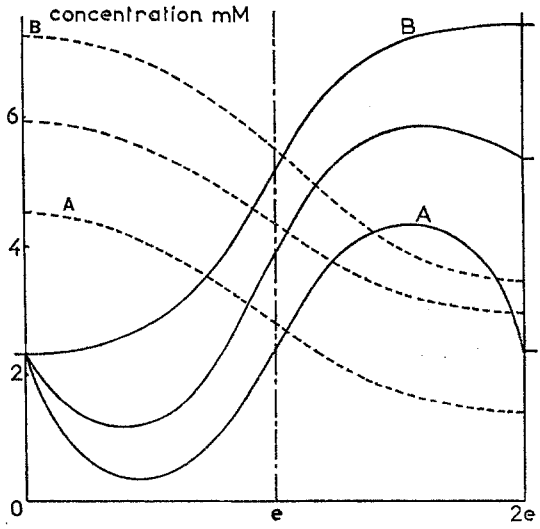

Fig. 3. Evolution of substrate concentration profiles in a bienzymic membrane with a hexokinase-phosphatase double-layer system. Glucose concentration profiles are given in ———; glucose-6 phosphate concentration profiles in -------. The membrane separates two compartments: the concentration (S_1) within the first compartment can be considered as constant, as its volume is far larger than that of the second. In the initial conditions $S_1 = S_2$, the glucose profile is given by the curve *A*. At the stationary state, S_1 is smaller than S_2 and $J_1 = J_2 = 0$. The glucose profile is shown by *B*. In the third glucose profile, S_1 is smaller than S_2 and the fluxes (J_1, J_2) are positive. Active transport appears in this case. These calculated profiles explain its mechanism. They coincide with the experimental results

translocation impossible. Now that these implicit hypotheses have been set forth, it should also be noted that the preceding treatments have a broad application and do not restrict the choice of the reaction to be studied.

One example of each of these couplings of two different enzymic reactions has been tested experimentally. The systems were chosen either because of the facility of analysis they offered (monolayer system) or because of their immediate biological interest (double-layer system). Experiments have confirmed the validity of the hypotheses and calculations exposed in this paper, as will now be shown by an analysis of the experimental procedures and results.

Materials and Methods

Principle

Two sequential bienzymatic systems were produced. The first consists of a homogeneous mixture of β-galactosidase and glucose-oxidase in an insoluble phase; this system, which forms a chain in which the initial substrate does reappear, is obtained by reticulation

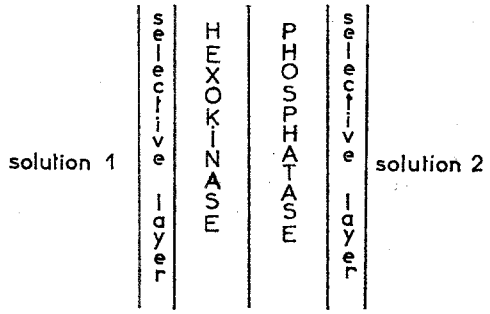

Fig. 4. Diagram of a double-layer membrane covered on its external side by two selective films

of these enzymes with an inactive protein (albumin). The substrate is lactose; it is hydrolyzed into galactose and glucose, and the latter is subsequently oxidized into gluconic acid in the presence of molecular oxygen.

The second system provides an example of glucose transport against its concentration gradient. It is composed of two *active protein layers* prepared separately and then bound to *selective films* to form a single membrane (Fig. 4).

The selective films are obtained by the reticulation of a nonenzymic protein (albumin) in a cellulose matrix. There films are permeable to glucose, but impermeable to glucose-6 phosphate. This selectivity is due on the one hand to a "molecular sieve" effect produced by the films, and on the other to the electrical charges of the reticulated protein. Both glucose-6 phosphate (with pK_a values 0.94 and 6.11) and albumin are charged negatively at the pH values at which the experiments are carried out.

The "active enzymic films" respectively bear hexokinase and phosphatase reticulated on an inert protein (albumin); both are impregnated with ATP and covered on their external side by two "selective films".

In this asymmetric membrane, glucose is temporarily phosphorylated:

(1) glucose + ATP $\xrightarrow{\text{hexokinase}}$ glucose-6 phosphate + ADP;

(2) glucose-6 phosphate $\xrightarrow{\text{phosphatase}}$ glucose + inorganic phosphate.

Since the sum of the two reactions is: ATP → ADP + inorganic phosphate, *the system behaves chemically as a simple ATPase.*

Formation of the Membrane

In this previously published method (Avrameas, Broun, Selegny & Thomas, 1968) a mixture containing 0.4% glutaraldehyde and 6% bulk protein (e.g., serum albumin) in a 0.02 M, pH 6.8 phosphate buffer is used. A planar glass surface is coated with this solution for reticulation, to realize the active membranes. To realize selective films, a cellulose matrix is impregnated with this reactive mixture (Thomas *et al.*, 1972).

Measurement of the Fluxes

Single-Layer Bienzymatic System. The experimental device used for measuring the fluxes has already been described (Selegny, Avrameas, Broun & Thomas, 1968). The

membrane to be studied is positioned between two compartments, each containing 100 ml of solution; both solutions are stirred and their temperature is controlled. The glucose fluxes are determined by enzymatical titration of samples (Keilin & Hartree, 1952).

Double-Layer System. The membrane separates two compartments: one large enough for the concentration within it to be considered as constant (900 ml); the other holding a volume of 6 ml. The fluxes are obtained by titrating samples using an enzymatical method. A portion of 5 cm^2 of the membrane is in contact with the compartments. Both compartments are thermostated and stirred with a magnetic stirrer.

Measurement of Maximal Activities

Each activity can be measured separately under the operating conditions of the bi-enzymic system.

Single-*Layer System.* The coupled system is studied at 25 °C with a 0.15 M lactose solution in a 0.1 M phosphate buffer at pH = 7.2. Air is bubbled through the solution. The β-galactosidase activity (bovine liver, Sigma) is measured under air-bubbling by observing the formation of glucose by enzymic titration. The glucose-oxidase activity (aspergillus niger, Sigma) is measured using a "pH-stat" in the absence of lactose and in the presence of a 0.15 M glucose solution in a 0.005 M phosphate buffer at pH = 7.2. This pH value is maintained constant with 0.1 N sodium hydroxide.

Double-Layer System (Each enzyme is first studied alone on its support, that is to say in a monoenzymic system). The experiment is carried out in a 0.42 M Tris-HCl buffer (pH = 7) containing 1.3 g/liter of $MgCl_2$ (necessary for hexokinase activity). Under these conditions:

(1) The hexokinase activity (yeast, Sigma) is determined by the coupling of this enzyme with glucose-6 phosphate dehydrogenase which oxidizes the glucose-6 phosphate formed in the presence of NADPH. The resulting NADPH is determined at 340 nm in a 10^{-2} M glucose solution containing 3 g/liter ATP.

(2) Phosphatase activity (potato, Sigma) is measured in a 10^{-2} M glucose-6 phosphate solution by observing the formation of glucose by the method described for a single-layer system.

Measurements of the Diffusion Coefficients

The effective diffusion coefficients of glucose and glucose-6 phosphate in the membrane are obtained by applying Fick's first law in the absence of any enzymic activity: (1) with nitrogen bubbling in the single-layer system; (2) without any cofactor present in the double-layer system.

Measurement of the Michaelis Constants

Determination of the true Michaelis constants is made according to a method already published by Selegny *et al.* (1969*b*).

Results

Homogeneously Distributed Enzymes

In this section we present both theoretical predictions and experimental data relative to the fluxes of the intermediary compound, glucose, for two different stationary states in which the fluxes at the limits of the system are of opposite signs. It should be remembered that the concentrations on either side of the membrane are equal, and the fluxes also have the same absolute value on each surface of the membrane.

The Maximal Activity of Each of the Enzymes May be Measured in the Presence of the Other. The maximal activity of glucose-oxidase, measured by a "pH-stat" in a glucose solution where no lactose is present, is: $V_{MB}=4.3\pm0.1\times10^{-3}$ mole cm^{-3} hr^{-1}; that of β-galactosidase, measured under oxygen-free conditions and therefore in the absence of glucose-oxidase activity, is $V_{MA}=1.25\pm0.05\times10^{-3}$ mole cm^{-3} hr^{-1}.

The Other Parameters of the System. (1) Michaelis constant for glucose-oxidase (in air at pH = 7.2): $K_{MB}=8.5\pm0.5\times10^{-6}$ mole cm^{-3}; (2) Effective diffusion coefficient of glucose: $\bar{D}^*=0.14\pm0.01\times10^{-2}$ cm^2 hr^{-1}; (3) Thickness of the membrane: $e=3.5\pm0.1\times10^{-3}$ cm.

As lactose is present in great excess in the medium, its diffusion coefficient is not to be taken into account in the equations.

Two Stationary States which Illustrate the Possibilities of Such a System. It will be remembered that p_1 is the concentration of p in the first compartment and p_2, that in the second. In one case, the intermediary product p is created by the membrane: $p_1=p_2=1.42\times10^{-6}$ mole cm^{-3}; the resulting outgoing flux is:

$$J=8\times10^{-7} \text{ mole } cm^{-2} \text{ } hr^{-1}.$$

In the other case, p is transformed by the membrane: $p_1=p_2=1.4\times10^{-5}$ mole cm^{-3}; the incoming flux is:

$$J=10^{-6} \text{ mole } cm^{-2} \text{ } hr^{-1}.$$

The concentration profiles calculated under stationary state conditions for these two cases were shown in Fig. 2.

A Comparison of Theoretical Previsions and Experimental Results. The preceding data provide the dimensionless parameters defined in the theo-

retical part of this paper:

$$\alpha_{pB1}=\frac{p_1}{K_{MpB}}=0.16\pm0.01; \qquad \sigma_{pB1}=\frac{e^2}{\bar{D}^*}\frac{V_{MB}}{K_{MpB}}=4.4\pm0.2;$$

$$\beta_1=\frac{1}{12}\sigma_{pB1}\frac{1}{(1+\alpha_1)^2}=0.27\pm0.04; \quad \rho_{pB1}=\frac{1}{1+2\beta_1}=0.65\pm0.15;$$

$$\lambda_1=\frac{\alpha_1}{1+\alpha_1}=0.14\pm0.01.$$

These values are introduced into the following equation:

$$J=e(V_{MB}\lambda_{pB1}-V_{MA})\rho_{pB1}(1+\beta_{pB1}).$$

This system is symmetrical and the values of the parameters are equal on each surface of the membrane. The calculated overall flux for these two surfaces is:

$$7\times10^{-7}\text{ mole hr}^{-1}\text{ cm}^{-2}<J<10^{-6}\text{ mole hr}^{-1}\text{ cm}^{-2}.$$

Under the above-mentioned conditions, direct experimental measurement shows the flux of glucose to be:

$$7\times10^{-7}\text{ mole hr}^{-1}\text{ cm}^{-2}<J<9\times10^{-7}\text{ mole hr}^{-1}\text{ cm}^{-2}.$$

These results coincide within the limits of experimental error. The validity of the reasoning when applied to the succession of two sequential enzymes bound onto a single insoluble membrane layer is thus confirmed. The same calculations may also be applied to the prediction of the evolution of such systems in real biological media.

Active Glucose Transport

Only the results obtained using an artificial structured membrane onto which both hexokinase and phosphatase have been bound will be described.

Optimum Activity of Each Enzyme. The system will function optimally if V_M has the same value in each layer. Some preliminary investigations are necessary to obtain this equality, as the effectiveness of the binding (and its variation) must be taken into account. Mean enzymic activity remaining after binding is 30% of the initial activity for hexokinase, and 57% for phosphatase.

Once these preliminary determinations have been made, the activity of the preparation available for use enables the two V_M values to be fixed at 10^{-3} moles per cm^3 per hour.

Numerical Values of the Parameters of the System. The hexokinase Michaelis constant for glucose is $K_M = 10^{-7}$ mole cm^{-3}. The phosphatase constant was not determined, as under our experimental conditions its kinetics were of zero order.

The effective glucose diffusion coefficient in the two active layers was: $\bar{D}^* = 0.14 \times 10^{-2}$ cm^2 hr^{-1}. That of glucose-6 phosphate was: $\bar{D}^* = 0.04 \times 10^{-2}$ cm^2 hr^{-1}.

In the two selective films, the glucose diffusion coefficient was: $\bar{D}^* = 0.5 \times 10^{-3}$ cm^2 hr^{-1}, while that of glucose-6 phosphate was less than 10^{-6} cm^2 hr^{-1}.

Therefore, the diffusing quantities were undetectable by the glucose-6 phosphate dehydrogenase-NADP system at 340 nm.

The thickness of both active layers was 6×10^{-3} cm; that of the selective film was 4×10^{-3} cm.

Active Transport. The glucose solutions in the external compartments on either side of the membrane were equal when $t = 0$; $S_1 = S_2$.

As the volume of the donor compartment was 150 times greater than that of the receptor, it may be assumed that the concentration in the first would remain constant throughout the experiment.

Samples were taken at intervals for measuring the concentration in the receptor compartment; this concentration is shown in Fig. 5 for the three following initial concentration conditions: 2.8×10^{-3} M; 5.6×10^{-3} M; 1.12×10^{-2} M.

The concentration in the receptor compartment increases regularly at first, and subsequently reaches an equilibrium which is shown by the concentration profile *B* in Fig. 3. The concentration subsequently decreases once the ATP enclosed within the membrane has been consumed. The system is no longer able to furnish the energy necessary to maintain the concentration gradient, and it returns progressively to its initial state.

Trials are now being carried out for ATP to be continuously present in the system. Up to the present, the membrane was impregnated at the beginning of the experiment with ATP, which was not renewed. In such a system, the action permitting glucose accumulation in one compartment requires additional energy to maintain the gradient and to compensate passive diffusion.

Previous studies of transmembrane transport distinguish between active transport currents and passive diffusion inverse currents, the latter flowing inversely to the former. In the present system, these two components of the overall molecular movement coexist.

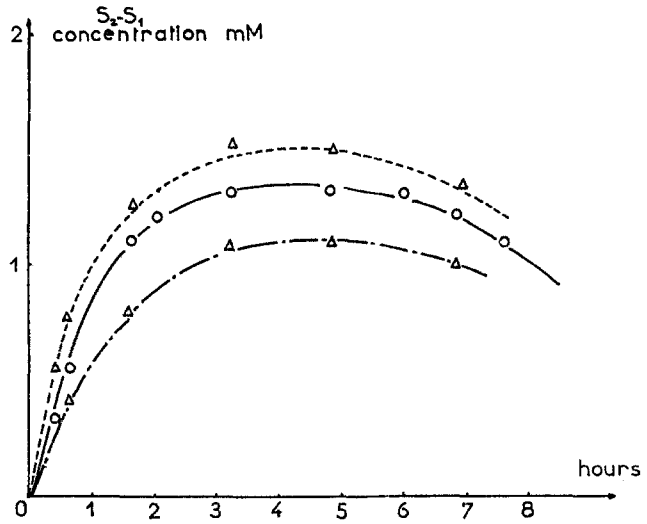

Fig. 5. A hexokinase-phosphatase double-layer membrane separates two compartments. This figure shows the evolution of the glucose concentration on the receptor side (S_2) in the case where the donor compartment concentration is constant (S_1). The results are obtained with three initial concentration values: (1) $S_1 = 2.8 \times 10^{-3}$ M ——— · ———; (2) $S_1 = 5.6 \times 10^{-3}$ M ———; (3) $S_1 = 1.12 \times 10^{-2}$ M --------

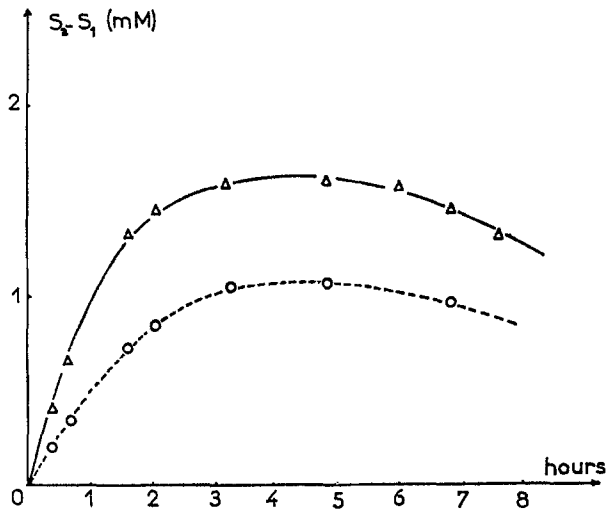

Fig. 6. For the same experimental conditions as in Fig. 5, this figure shows the evolution of the glucose concentration in the receptor compartment in the presence of fructose 2.8×10^{-3} M showing a competive phenomenon. (With fructose --------; without fructose ———)

At the present stage of our experiments, we cannot yet plot a complete curve $v=f(S)$ with respect to our transport system, but it already seems probable that *this system may be saturated.* In fact, from the values of S_1 equal to 10^{-2} M, the values of the initial rates of transport as well as those of the equilibrium level, remain constant.

As may be supposed, glucose competes with fructose for hexokinase, the latter substrate reducing the effect of active transport. Fig. 6 shows the results obtained with an initial fructose concentration of 2.8×10^{-3} M. More thorough experimentation on these competitive phenomena in such transport systems will be published at a later date.

If only the energy theoretically necessary for the concentration process is taken into consideration, the maximum net yield of our "glucose pump" is 5% of what could ideally be obtained at the initial stage.

Discussion

In this paper, two sequential bienzymatical insoluble phase systems are described. Although their characteristics and significance as models are essentially different, it would appear to be advantageous to compare them.

The first system is not, strictly speaking, structured. Yet solution and membrane constitute a heterogeneous system having some particular characteristics of intracellular microsurroundings where diffusion constraints are present. The homogeneous distribution of sites in this insoluble β-galactosidase glucose oxidase system results in a behaviour *qualitatively* similar to the behavior of these two enzymes mixed together in solution. Important *quantitative* differences of glucose concentration are, however, observed. This system is analogous to that described by Mattiasson and Mosbach (1971) in which insolubilized active sites are statistically homogeneously distributed. The accumulation of the intermediary product in the insoluble phase favors the second reaction. The mathematical model adapted to the membrane shape of the experimental system allows quantification of this accumulation using the calculated concentration profiles inside the insoluble structure. The rate glucose transformation within the insoluble structure, as well as its profile inside the membrane, render this concentration much more stable than in solution. The advantage of this regulative effect is that it maintains the concentration of a given intermediary metabolite near a constant value. Thus, within a specific compart-

ment, such a regulation can attenuate any abrupt discrepancies which may be caused by a discontinuity in metabolic processes. It may be preferable to consider the operation of this structure as a model of any insulated compartment where metabolite transformation takes place, rather than more specifically as a model of a biological membrane.

The second structured double-layer bienzymic membrane presented here is completely different. It demonstrates a specific effect of spatial transport which is closely related to an oriented structure, that is to say with the arrangement of functional elements in a logical sequence in space. If only the balance of the double chemical reaction involved is taken into account, the result is a simple hydrolysis of ATP due to the enzymic couple acting as an ATPase. Generally speaking, this hydrolysis of ATP gives no information about the real potentialities of this catalytic system. To realize the full importance of this system it is necessary to consider its structure in space and, more precisely, its dissymmetry. According to Curie's well-known principle, phenomena which do not present an asymmetrical structure cannot give rise to vectorial effects. Hence, systems which are completely isotropic or symmetrical, and which comprise reactions either in solution or in the insoluble phase cannot give result in "vectorial catalysis" or transport phenomena. Of course, the same situation occurs when an ion transporter is isolated from its supporting membrane and is dispersed in a solution. Under the experimental conditions described, transport is brought about by dissymmetric and geometrical anisotropy in the distribution of the active sites. This dissymmetric anisotropy increases the probability that a greater number of glucose molecules are present in one of the compartments of the system rather than in the other. The system thus gives an experimental physicochemical basis to the expression "vectorial catalysis".

It should be emphasized that the mechanisms proposed remain valid for a wide range of different thicknesses and dimensions of the active layer. They may be extended to the case where two different enzymic sites are situated on the same molecule, on the condition that such molecules are oriented within the structure. Such research seems well adapted to a tentative interpretation of the role of ATPases in active transport of cations in Na-K pumps; according to Post, Sen & Rosenthal, (1965), they have two different types of catalytical sites, kinase and phosphatase, and it now seems probable that they are directionally oriented inside the membrane. Indirectly, this raises the question of the possible role as various transports of ATPases which have been detected in cellular membranes. Such an interpretation may soon be proved or disproved owing to the numerous in-

vestigations of transmembrane transport that are at present being carried out by different research teams.

In any case, the molecular interpretation of observed natural phenomena will require the construction of more elaborate and more numerous models. Those described here seem quite promising. Hitherto they have only been subjected to an initial investigative study, but a more extensive analysis is already under way and the results will be published at a later date.

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