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# An experimental enzyme–membrane oscillator

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SUMMARY

Periodic behavior has been observed in a synthetic membrane bearing papain activity. A membrane-coated glass pH electrode is exposed to a solution containing benzoyl-L-arginine ethyl ester at high pH. If the pH and substrate levels in the outer solution lie within a well-defined range, and if the rate of stirring is appropriate, spontaneous oscillations of the inner pH occur with a period of about 20 s. The oscillation depends on both the autocatalytic character of the enzymatic reaction and the feedback effect due to structural diffusion limitations. Certain relevant parameters of the cross-linked papain–albumin membrane used are reported.

Regulation and control in cellular metabolism are frequently attributed to conformational changes governing the kinetic behaviour of key enzymes, but so far little attention has been paid to the role of diffusion (and the spatial distribution of reactants) in the feedback process. This is surprising since the majority of enzymes *in vivo* are either attached to membrane structures or contained within cell organelles, and are thus strongly influenced by membrane properties. Recent studies have shown that membrane-bound arrays of enzymes can differ markedly in their kinetic behaviour from homogeneous solutions of the same enzymes[1,2]. The advent of well-characterized synthetic enzyme membranes makes it possible to investigate the mutual effects of reaction and diffusion in a variety of simple model systems.

Immobilization of enzymes has been widely studied[3,4], and methods exist for the

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production of well-defined distributions of bound enzymes within a membrane[5,6]. It is now recognized that in such systems the internal microenvironment, as determined both by the membrane structure and by the local concentrations of reactants and products, exerts a profound effect on the mode of action of the enzymes[6,7]. Consequently it seems feasible to design enzyme–membrane systems which exhibit instabilities and periodic behaviour by (a) providing the proper local conditions for autocatalysis, and (b) introducing the appropriate negative feedback in the form of a diffusional process.

The experimental evidence for instabilities in biochemical systems is limited to a small number of studies of sustained oscillations in homogeneous solution, involving principally the glycolytic system[8] and the peroxidase system[9]. Both temporal and spatial oscillations characterize the multi-step catalytic oxidation of malonic acid by bromate, not only in homogeneous solution[10,11] but also, as recently shown, within a membrane which eliminates possible convection effects[12]. Instabilities and oscillatory phenomena in membranes due entirely to interaction between flows, without chemical reaction, have been known for a number of years and are well understood[13,14]. Remarkably, no synthetic enzyme–membrane has been constructed up to now in which chemodiffusional coupling gives rise to periodic phenomena. Such systems are not difficult to devise; one example based on a single well-defined reaction has been shown to oscillate in principle by means of computer simulations[15]. Its experimental realisation is described below.

A protein membrane bearing papain activity is produced on the surface of a glass pH electrode by a previously described co-cross-linking method[6]. A solution containing 40 mg/ml albumin (Sigma Chemical Co., St. Louis, Mo.; Fraction V bovine serum albumin), 10 mg/ml non-activated papain (Miles–Yeda Ltd, Rehovot; specific activity 19 I.U. benzoyl-L-arginine ethyl ester), and 5 mg/ml glutaraldehyde in phosphate buffer (0.02 M, pH 6.8) is poured over the surface of the bulb of a "combined" pH electrode (Radiometer, Copenhagen; No. GK 2311 C). During this procedure the electrode is rotated horizontally about its axis of revolution at 100 rev./min; after 15 min complete insolubilisation occurs and the bulb is found to be covered with a thin protein coating. The coating is rinsed in distilled water or phosphate buffer, and the immobilized enzyme activated by immersion in a solution of EDTA (1 mg/ml) and cysteine (1 mg/ml). The active membrane-coated electrode is then introduced into a solution of KCl (0.1 M) prepared from doubly-distilled water, and the pH at the membrane–glass interface determined. This is equivalent to determining the pH at the center of a membrane mounted between identical solutions.

Experiments with the substrate benzoyl-L-arginine ethyl ester (benzoyl-L-arginine ethyl ester·HCl, Miles–Yeda AR) were conducted at room temperature (24 °C) using 500 ml of the bulk KCl solution stirred by a magnetic stirrer at a maximum speed of 350 rev./min. On introduction of substrate into the solution the pH within the membrane decreases immediately, while the external pH (adjusted to a selected value with NaOH) remains constant. If the pH and substrate levels in the bulk solution lie within a well-defined range, a spontaneous time oscillation of the inner pH occurs, as shown in Figs 1 and 2.

The average thickness of membrane coatings prepared as above ranges from 10 to 20 $\mu$m (by weight and area measurements), and hence it is to be expected that unstirred

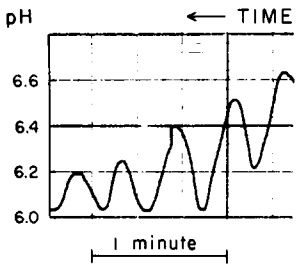


Fig.1. Time dependence of pH at the enzyme membrane–glass electrode interface. Nominal membrane thickness 10 μm, gentle stirring. Substrate concentration and pH in the bulk solution were initially 4.5 mM and 9.3, respectively.

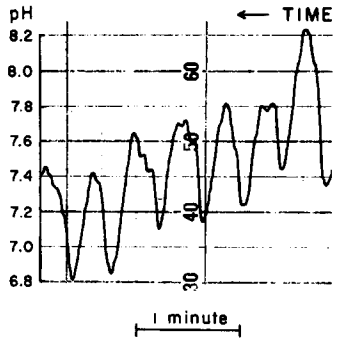


Fig.2. Time dependence of pH at the enzyme membrane–glass electrode interface. Nominal membrane thickness 20 μm, rapid stirring. Substrate concentration and pH in the bulk solution were initially 4.5 mM and 9.5, respectively.

layers at the outer surfaces may play an important role in the phenomenon. This was found to be the case: with a 10-μm membrane oscillations are only obtained in the absence of stirring or with gentle stirring (Fig. 1), while with a 20-μm membrane under comparable conditions rapid stirring is necessary to produce oscillations (Fig. 2). Membranes prepared with albumin alone do not give oscillations or trans-membrane pH differences at any stirring speed in otherwise identical experiments. The addition of $10^{-4}$ M $HgCl_2$, a papain inhibitor, to the bulk solution in the presence of an active membrane completely abolishes both oscillations and steady pH differences. To facilitate comparison with simulations the basic parameters of the papain–albumin membranes were measured independently and are reported in Table I.

The oscillatory mechanism is readily understood in conceptual terms. Papain exhibits maximal activity at neutral pH, hence ester hydrolysis is autocatalytic at alkaline pH values. In the papain membrane this behaviour is modified by a feedback effect due to diffusion of $OH^-$ into the membrane and to local substrate consumption, and thus at appropriate pH and substrate levels oscillations can result. There is qualitative agreement between the experimental results and the results of computer simulations[15]; differences may be attributed primarily to boundary layer effects and inhomogeneities in the membranes, especially in membrane thickness. Such inhomogeneities will tend, *inter alia*, to decrease the amplitude of the oscillations. An additional factor is that time-independent conditions are not strictly maintained in the bulk solution in these simple experiments, and consequently the oscillations "drift".

It is important to note that papain, an enzyme which has been exhaustively studied, never gives rise to periodic behaviour in homogeneous solution. Thus the membrane described here demonstrates rather clearly the limitations of classical enzymology

TABLE I

DIFFUSION COEFFICIENTS AND ENZYMATIC ACTIVITY OF THE PAPAIN–ALBUMIN MEMBRANE (WATER CONTENT 90–93% BY WEIGHT)

BAEE, benzoyl-L-arginine ethyl ester.

| $D_{H^+}$ * ($cm^2 \cdot s^{-1}$) | $D_{OH^-}$ * ($cm^2 \cdot s^{-1}$) | $D_{BAEE}$ ** ($cm^2 \cdot s^{-1}$) | *Rate of BAEE hydrolysis per unit volume of membrane* *** ($mole \cdot cm^{-3} \cdot s^{-1}$) |
|---|---|---|---|
| $5.5 \cdot 10^{-4}$ | $3.2 \cdot 10^{-6}$ | $1.2 \cdot 10^{-6}$ | $4.0 \cdot 10^{-5}$ |

* Measured in a diffusion cell using membranes cast on a glass plate. Gradients of HCl or NaOH at pH values less than 6 or greater than 8, respectively, were established in 0.1 M NaCl. Precision within ± 30%.

** Gradient of benzoyl-L-arginine ethyl ester·HCl established in KCl at neutral pH, using an albumin membrane. Precision within ± 10%.

*** Measured by means of a pH-stat. Precision within ± 5%.

with respect to complex biological systems, where structural factors may introduce totally new phenomena. It has been suggested that enzymatic reactions could, under suitable conditions, produce spatially ordered states[16,17]. Enzyme membranes or other systems involving immobilized enzymes may well be important in this regard, since they are governed by diffusion and reaction alone, without the occurrence of convection or random motion of active sites, and boundary conditions can readily be specified.

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