A New Model System for the Study of Complex Dynamical Enzyme Reactions. I. A Nonlinear Enzyme Reaction in a Chemical Gradient

Gerold Baier, Peter Urban, and Klaus Wegmann

Institute for Chemical Plant Physiology, University of Tübingen, Tübingen, FRG

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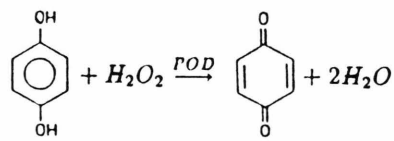
An experimental system for the study of biochemical reaction dynamics is introduced and described. A one-enzyme reaction is extended by an artificial feedback loop in an electrochemical device. Cyclic voltammetry is used to show that the reaction rate depends nonlinearly on the amount of cosubstrate offered. For some sets of fixed parameter values a damped oscillatory approach of the steady state was observed. The usefulness of the systems theoretical concepts is discussed.

Keywords: Reaction-diffusion Systems, Peroxidase, Feedback, Nonlinearity, Damped Oscillations.

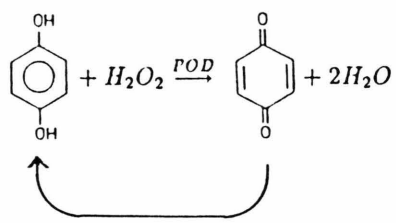
1. Introduction: The System

A well-known *in vitro* enzyme system containing horseradish peroxidase can exhibit oscillations and chaos in continuously stirred tank reactor (CSTR) experiments [1]. The search for instabilities in non-stirred solutions and for the minimal volume required for the formation of dissipative structures to apply synergetic concepts [2] to biochemical reaction networks on a subcellular level certainly stimulates the investigation of artificial enzyme systems. In the mentioned case, however, this search is handicapped by the involvement of molecular oxygen. If the model of reaction-diffusion equations applies, then a remarkably small number of kinetic units linearly coupled by diffusional terms should be sufficient to give rise to spontaneous symmetry breaking [3], as well as oscillatory, chaotic [4], and even hyperchaotic (cf. [5]) flows in the state space.

At first we introduce a variant of the published peroxidase system, wich has not been shown to oscillate yet. It is the reduction of hydrogen peroxide to water. This reaction has the asset that it does not contain a gaseous substrate, it can be investigated in aqueous solution, with no mass transfer occurring between liquid phase and gas phase. For the reduction of hydrogen peroxide, while NADH is not effective, numerous cofactors can be used; for reasons of our new approach we choose hydroquinone as electron donor. Thus the kinetic part of our system reads



Next we started runs in a continuous flow stirred tank reactor with arbitrary concentrations and found steady state behaviour only. We then considered conclusions from several theoretical investigations [6, 7], which argued that instabilities and complex behaviour in a dynamical system might be the consequence of a properly inserted feedback loop, where the output of a complex reaction sequence is fed back into substrate information, thus serving as a system immanent control parameter. A possibility for our system would be



Various ways to realize this seem possible, for example a second enzyme could be used to recover the reduced form of cosubstrate. However, we choose a different method. The most convenient reduction of

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benzoquinone can be achieved electrochemically on the surface of an inert electrode. But this means that the feedback loop is not homogeneous in the solution, it rather works locally and serves to introduce chemical gradients. This is a most interesting extension of reaction-diffusion systems, which has only recently been introduced for the widely studied Belousov-Zhabotinsky reaction [8] in an unstirred medium.

Four advantages are in favor of this technique:

- Using glassy carbon as electrode material and at low benzoquinone concentrations, passivation of the electrode as well as side reactions are negligible.
- The recovering reaction takes place on a well-defined surface only. The feedback rate is precisely controlable by means of the applied electrode potential, which is easier to handle than a second enzyme system.
- Starting with a solution of homogeneously distributed benzoquinone, the enzyme reaction will take place only in a very limited volume in front of the electrode due to diffusion of hydroquinone into the solution. Speaking in terms of a reaction-diffusion equation, we realize the couling of reaction cells with continuously decreasing substrate input, until for irreversible reactions like the present one, a cell is reached where there is no reaction at all.
- Under potentiostatic as well as amperostatic conditions the electrochemical set-up simultaneously is used as a continuous measuring device, most important in the study of dynamical systems. Here the variable to be traced is the instantaneous benzoquinone gradient at the surface of the electrode.

2. Experimental

The electrochemical device consisted of a glassy carbon disk electrode (diameter 2 mm), a platinum wire counter electrode (in a frit), and a saturated calomel electrode (SCE) as a reference system. All potentials are given versus the SCE. Before each experiment the working electrode was polished with moistured and then dry Al_2O_3 (particle size 0.3 μm) until a bright surface was obtained. The absence of particles after washing was controlled under a microscope. The solution was coated with a cooling (and heating, respectively) device to maintain a temperature of 19.0 ± 0.1 °C. Reaction volume was 25 ml. Concentrations were 4.0 or 2.0 mM freshly sublimated 1,4-benzoquinone and 4.8 mM of hydrogen peroxide ($\varepsilon_{240} = 43.6$ cm^2 Mol^{-1}) taken from a 30% stock solution, both in 0.1 M acetate buffer of pH 5.0. Horseradish peroxidase (EC 1.11.1.7) was obtained from Sigma (Type II, saltfree powder) and its concentration was 0.4 mg/25 ml, yielding a 0.21 μM solution ($\varepsilon_{403} = 9.1 \cdot 10^7$ cm^2 Mol^{-1}). Neither dichlorophenol nor methylene blue were added [1]. The whole solution was bubbled with nitrogen for approximately 15 minutes. Cyclic voltammetry was carried out using a Wenking Laboratory Potentiostat, model LB 81 and a Wenking Voltage Scan Generator, model VSG 72. In every experiment a cyclic voltammogram of the buffer was recorded as a reference line. The potential range for cyclic voltammograms was $E_s = +600$ mV and $E_\lambda = -900$ mV.

3. Results and Discussion

A series of ordinary cyclic voltammograms is shown in Figure 1. The solution contains buffer, 1,4-benzoquinone, and hydrogen peroxide, but no changes occur in the absence of H_2O_2. 1,4-Benzoquinone is reduced at potentials $E < 70$ mV with a peak current at $E = -50$ mV at a scan rate of $v_1 = 5$ mV/sec (Figure 1a). At 20, 80, and 320 mV/sec the peak continuously shifts towards negative potentials ($E_p = -80$, -115, and -165 mV, respectively) and the peak current i_p increases with the scan rate. Successive values for $i_p \cdot \sqrt{v^{-1}}$ (scan rates as in Fig. 1 a–d) are 0.49, 0.48, 0.45, and 0.41, respectively, small deviations from the

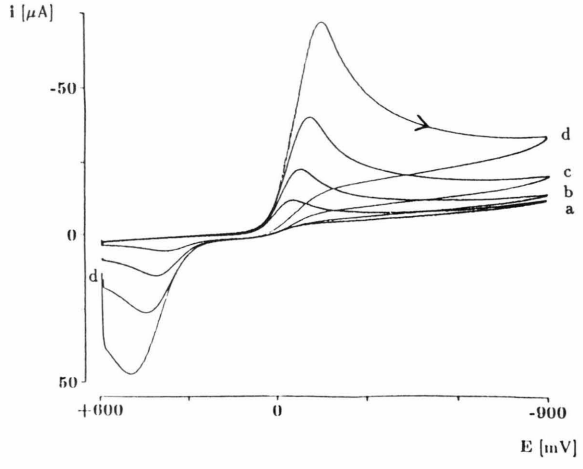

Fig. 1. Cyclic voltammograms of a solution containing 4.0 mM p-benzoquinone and 4.8 mN hydrogen peroxide in 0.1 M acetate buffer, pH 5.0. Scan rates are a) 5 mV/sec, b) 20 mV/sec, c) 80 mV/sec, and d) 320 mV/sec.

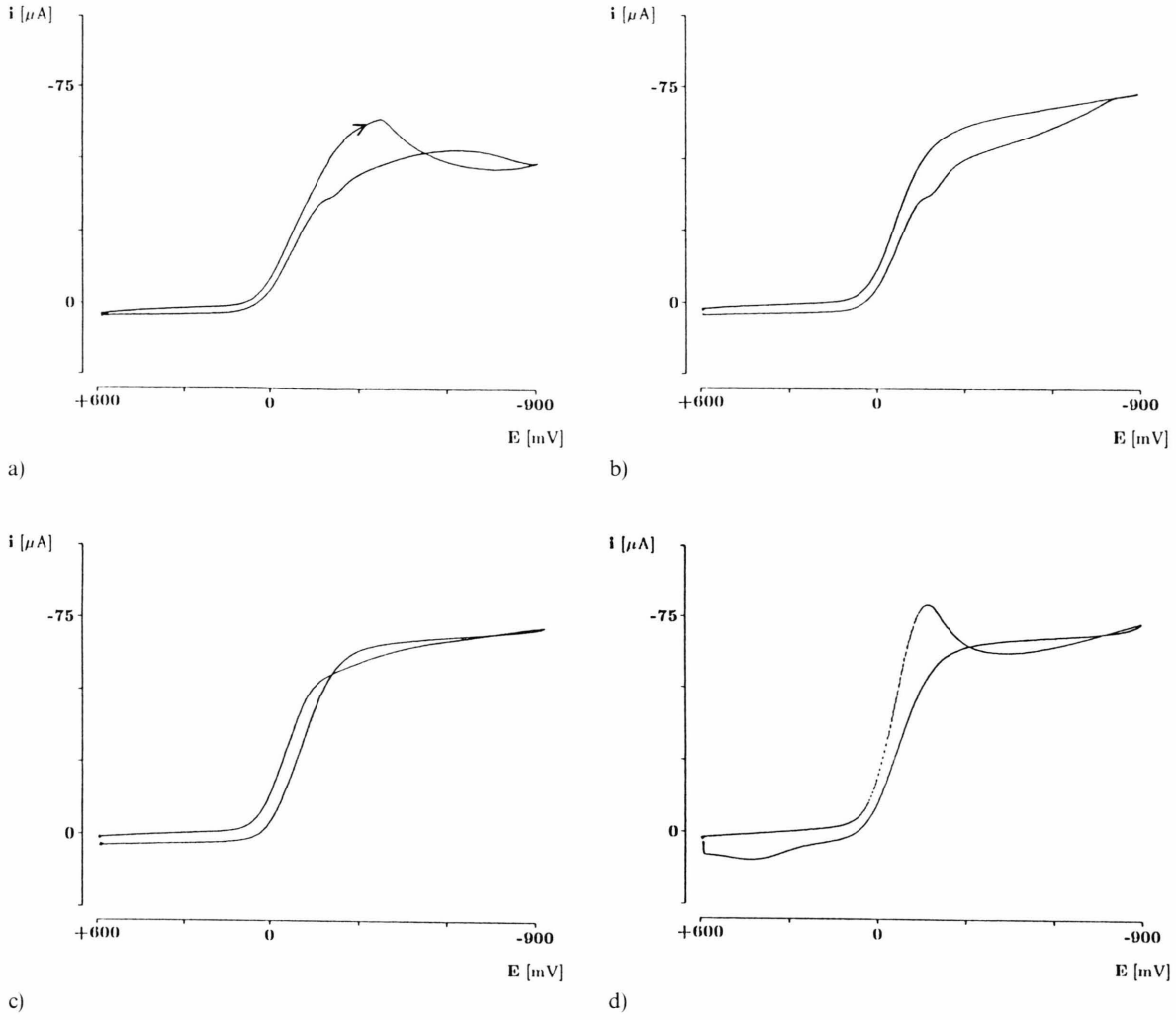

Fig. 2. Cyclic voltammograms of a solution containing 4.0 mM p-benzoquinone, 4.8 mM hydrogen peroxide and 0.21 μM horseradish peroxidase. Scan rates are a) 5 mV/sec, b) 20 mV/sec, c) 80 mV/sec, and d) 320 mV/sec.

expected constant value for a reversible single electron transfer. In the present case we deal with a two electron redox agent with two protonations, therefore the process is not reversible, the peak distance $\Delta E = -E_{red} - E_{ox}$ is found to be 410 mV at v_1 and is increasing with scan rate. In spite of the complicated mechanism this subsystem's response is simple with respect to parameter changes at the chosen conditions.

Now compare $E - i$-plots of the completed system including peroxidase (Figure 2). The cyclic voltammograms are displayed separately to avoid confusion. Starting with a seemingly ordinary result (Fig. 2 b) on the first scan, from E_s to E_λ the cyclic voltammogram looks similar to what would be expected in the case of electron transfer followed by a chemical reaction. Qualitatively similar results can be obtained in computer simulations of the most simple catalytic mechanism (EC_{cat}), assuming a reaction of pseudo-first order with a large excess of oxidizing substrate:

$$A \overset{e^-}{\rightleftharpoons} B \xrightarrow{k_1} A\,.$$

On the way back, at -800 mV the current does not follow this pathway with an expected slight decrease caused by the capacity of the electrode, which in our case could explain a deviation of approximately 5 μA.

Instead a drop occurs leading to an early decrease in current. At -200 mV there is a shoulder in the drop, and from $E = -160$ mV on the system is back to regular behavior. There is no peak at all the oxidation range of hydroquinone. Comparing currents at $E = -E_\lambda$ we found an enhanced catalytic current with more than six times the value of the uncatalyzed case. As no hydroquinone can be detected in the cyclic voltammogram, the kinetic part can be assumed to be irreversible.

In Fig. 2c the scan rate is four times faster than in Fig. 2b, the scan forward is almost identical. What would be expected from a simple first order model is that independent of the applied scan rate the current would reach the same steady state value in the limit of a long cathodic branch, but that, as long as this point is not reached, the corresponding current at faster scan rates would always be larger than the current at slower scan rates. Referring to Figs. 2a–d we can write: $i_\lambda(a) \leqq i_\lambda(b) \leqq i_\lambda(c) \leqq i_\lambda(d)$. This is not the case in Figure 2c. Careful and repeated measurements show that here i_λ is slightly below i_λ at 20 mV/sec. This time it is only about four times the value for the uncatalyzed electron transfer.

Note that the reaction is assumed to be irreversible and that it takes some time to be completed. Therefore at higher scan rates the ratio of the current caused by products of the reaction and the current caused by simple diffusion in the absence of any kinetic will decrease; the cyclic voltammogram will become more similar to the uncatalyzed case. Line crossing cannot occur in the simple EC_{cat} model, but has been found in simulations of an EEC_{cat} mechanism (*two* electron transfer steps followed by first order catalysis) [9], because catalysis lags behind and reaches maximum productivity in benzoquinone synthesis while the potential is already on its way back.

On the way back, differing from Fig. 2b, the current does not stay below the first scan or even drops; instead the line cross at -720 mV. This situation is kept up for about 500 mV until again at $E = -160$ mV the system is back to normal behavior; still there is no peak in the oxidation branch.

The next plausible step to keep up with the model is to assume that the enzyme is in the range where it is saturated and the turnover rate is independent of substrate and dependent of enzyme concentration only. Still, this cannot explain why the current at $E = E_\lambda$ is below the value at 20 mV/sec (73.5 μA compared to 74.5 μA). Although the benzoquinone gradient at the electrode is steeper (because the time to reach maximum turnover at the electrode is smaller), the catalytic enhancement is slightly smaller in Fig. 2c, meaning the overall enzyme reaction rate is smaller.

Figure 2d now confirms that we do not observe a simple zero order saturation effect plus a negligible deviation caused by some experimental error. Rapid scans (at 320 mV/sec) show an increase in current followed by a decrease, i.e. a peak at $E = -170$ mV instead of a plateau. The current at $E = E_\lambda$ again is below the value for slower scan rates (69.5 μA).

Gradients are extremely steep in this case and hydroquinone will hardly be able to diffuse far from the electrode's surface. The reaction is not fast enough, so the peak on the first scan is similar to the one in the uncatalyzed case. The effect of the enzyme's action can be traced in the following as a deviation from the uncatalyzed case, because the decrease is stopped and there is an increase in current due to the catalytic activity. On the way back lines cross again at $E = -320$ mV, but this time the current behaves as expected, there is a plateau of enhanced current until the peak vanishes. As there was not enough time for the enzyme to consume all of the reduced cosubstrate, a small peak can be detected in the oxidation region, and there is nothing like a shoulder as in Figure 2b. This record is a superposition of the uncatalyzed and the catalyzed case, with passive diffusion dominating at short times and steep gradients and kinetics coming into play significantly about one second after the very onset of reduction. Reversely, this means that the effect of the enzyme can be traced at the electrode only with some finite time delay. This might be important for the mechanism of the feedback control.

Given the results of Figs. 2c and 2d, one would expect for slow scan rates that the enzyme displays its full catalytic capacity, and the current should be enhanced during the complete reduction cycle. The enhancement, however, would not be proportional to the concentration of hydroquinone due to the fixed amount and therefore the limited capacity of the enzyme leading to a saturation function in the substrate-velocity plot.

With these considerations in mind the experimental finding shown in Fig. 2a is a big surprise. In this cyclic voltammogram at 5 mV/sec catalysis starts at the onset of the reduction peak. The current rises to a volume nine times the corresponding value during the benzoquinone reduction at -355 mV, and it appears as if it would reach a plateau. At -355 mV, however,

there is a sudden drop of he current until, at −750 mV, it starts to rise, keeps rising on the back scan, reaches a smooth maximum and starts to decrease again. At −160 mV, once more we find a shoulder at the potential where the system is back to its "regular" behavior. The oxidation peak has completely vanished, of course, as is the case for 20 mV/sec and 80 mV/sec.

Our first sep in interpreting this cyclic voltammogram is that the enzyme does not reach a steady rate of catalysis at the given conditions, but that at −355 mV during the first scan a constellation is built up where the activity of horseradish peroxidase decreases, although cosubstrate input does not. Decrease in activity then leads to a counter effect resulting in a second increase, until the applied potential is too positive for the process to be continued. At slow rates in a completely linear domain of benzoquinone reduction we witness the onset of nonlinear changes in enzyme activity. Cautiously speaking we have found a set of parameters for which the reaction rate deviates from a normal saturation function. Another point worth to be noted is that the current drop at −700 mV during the back scan in Fig. 2b is obviously the same phenomenon as the one at −355 mV in Figure 2a. Moreover, in repeated cyclic voltammograms at 5, 10, amd 20 mV/sec we find that this edge always appears some 40 seconds after the potential has passed −160 mV, the point we repeatedly referred to as the return to a regular behavior of the back scan. We end this description of Fig. 2 by concluding that a specific regulatory mechanism in the kinetic part of our system causes the enzyme – with a certain time delay – to switch its activity nonlinearly back and forth.

The reaction of hydroquinone occurs within a few seconds, it also is irreversible. During passive diffusion into the solution, therefore, all of the reduced cosubstrate is consumed by the enzyme and, as long as there is enough hydrogen peroxide for the reaction to continue, a gradient of hydroquinone can be upheld by a given potential at the electrode: a fixed Nernst concentration at the surface of the electrode and zero concentration inside the solution. The reverse happens to benzoquinone, because it is synthesized wherever hydroquinone is consumed. Whenever a potential in the limit current region of its reduction peak is applied, the benzoquinone concentration is zero at the electrode and has a fixed value (defined by the initial conditions) inside the solution. By means of the catalytic recovering, the gradients of both benzoquinone and hydroquinone are not allowed to slowly expand into the solution as a function of time, and thus the reaction is limited to a narrow diffusion layer.

It is the dynamics of the reaction in this diffusion layer, where there is a gradient of one reacting species and the reverse gradient of the product of the reaction, that we are going to investigate (see Figure 3).

Of course, cyclic voltammetry is not a technique to study the dynamics of a chemical reaction continuously. The changes incurrent are a function not only of the system but of a continuously sweeping potential as well, the reaction is never allowed to settle into an attracting subset of state space, it is constantly turn away from it. On the other hand, cyclic voltammetry is an easy to perform and tell-taling technique, which in our case has been used to detect the domain of the interesting nonlinear response. The fact that the current rises on the reverse scan, while the potential grows more and more positive, led us to the assumption that there is at least on peak to be found in an otherwise monotonously decreasing current-time curve in this potential range.

We now turn to the application of single-step chronoamperometry for our purpose. This means we choose a potential in the range where benzoquinone is reduced, starting with conditions, where there is no reaction at all. We force the system to suddenly jump from zero potential to the chosen value and record the current response in the potentiostatic mode as a function of time. In order to be able to observe chemical oscillations, which are expected to show up in the order of 0.1–0.001 sec^{-1}, we extend the range of observation up to several hours. In the following article we specifically deal with the problem arising during such long-time observations from the participation of convection in the solution [10].

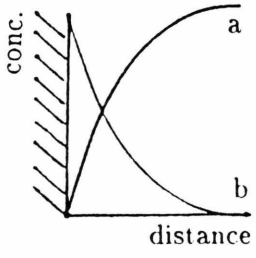

Fig. 3. Steady state concentration profiles in the chronoamperometric experiment for oxidized (a) and reduced (b) form of the electroactive species.

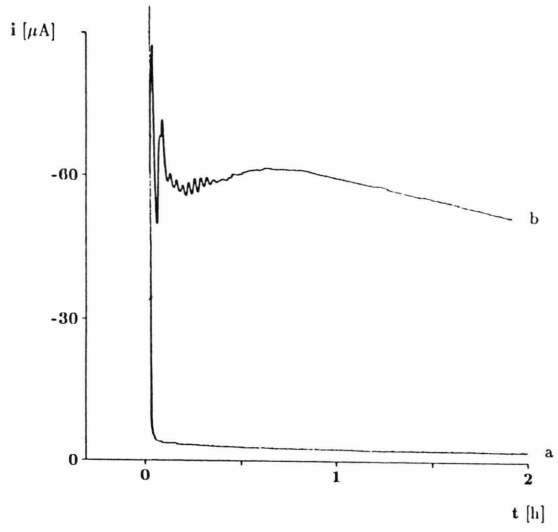

Fig. 4. Chronoamperometric experiments at $E = -300\,\text{mV}$. Concentrations are a) 2.0 mM p-benzoquinone and 4.8 mM H_2O_2, and b) 2.0 mM p-benzoquinone, 4.8 mM H_2O_2, and 0.21 mM peroxidase.

The next figure (Fig. 4) shows the result of two chronoamperometric experiments at a constant potential of -300 mV with parameters as in Figs. 1 and 2, except that a lower benzoquinone concentration has been used to minimize the extend of side reactions like polymerization of quinone induced by small amounts of semiquinone radicals or the formation of a quinhydrone complex. Cyclic voltammograms at this concentration are less pronounced but exhibit the same basic features as discussed for an initial concentration of 2.0 M. Again in a) the system behavior is as expected: drop of the current, proportional to $\sqrt{t^{-1}}$ as expressed in the Cottrell equation, down to a steady state value close to zero. The final value deviates from zero because of convection, for instance as a result of local density gradients during the course of the reaction. Comparing Fig. 4b, the current is catalytically enhanced, the second part is that of a slowly shifting quasi-steady state. The shift is probably due to the slow side reaction. During the first twenty minutes, however, we witness an interesting small amplitude, damped oscillation. It is introduced by one large amplitude single peak and a medium size double peak. The current increase of the first peak is related to the current increase in the back scan of Fig. 2a at 5 mV/sec. Cyclic voltammetry test thus proves valid as a tool for the search of nontrivial nonlinear aspects of the reaction under investigation.

Two conclusions can be drawn from the chronoamperometric experiment: (i) The idealized solution of the corresponding reaction-diffusion equation of our system is that of a stable focus which – as an attracting set – is approached in a damped oscillatory manner. (ii) The surrounding of the fixed point attractor instate space is nontrivial. There is a two peak oscillation, which might indicate bending of a stable manifold, and there is an increase in amplitude of the small peaks before the final damping terminating the oscillation. This leads us to the assumption that not only do we stand in the vicinity of a possible instability (of the focus) in parameter space, giving rise to sustained oscillatory behavior, but also that these oscillations could be of a higher complexity than single peak. While the behavior reported so far was reproducible, we also observed sustained oscillations in some of the experiments, but we could keep them under control only after introducing an extension of the set-up for the control of convection motion, which will be described and discussed in our subsequent paper in detail.

4. Theory

The system under investigation is likely to be described by a set of partial differential equations of the type:

$$\frac{\partial b}{\partial t} = F + D_b \cdot \nabla^2 b\,,$$

where b denotes the concentration of species B, with the kinetic term F and spatial diffusion.

This set consists of three building blocks: the reaction kinetic cell, respective coupling of cells via diffusion, and boundary conditions as provided by the electrochemical experiment.

The homogeneous reaction unit is oxidation of the reduced form of cosubstrate B. B is oxidized by the enzyme to yield A, the reaction follows a function $F(b, c)$, where C is the other reaction partner (hydrogen peroxide in our case). Assuming c constant for simplicity F is a function of b only. A natural approach is a steady state approximation for enzyme-substrate complexes, yielding a Michaelis-Menten-type term:

$$F(b) = \frac{\mathrm{d}b}{\mathrm{d}t} = -\frac{k_1 b}{K + b} = -\frac{\mathrm{d}a}{\mathrm{d}t}\,,$$

where the concentration of enzyme and substrate C are implicit in the constants.

This is a saturation function which is close to first order for small concentrations of B, and then continuously changes into zero order for higher concentrations of B. The cyclic voltammetry experiments shown in Fig. 2 claim that such a term is not sufficiently yet. For high scan rates the resulting gradients are very steep and only a limited amount of enzyme action will take place, so it is straightforward to expect that cyclic voltammograms will tend to become more similar to the ones for the uncatalyzed case, which is what can be observed. At slow scan rates, however, an inhibitory effect can be observed, which leads to decrease of the current response. There is more time for hydroquinone to be synthesized, there is more time for the enzyme to turn it into benzoquinone, yet, the reaction rate is characteristically slowed down. An additional (nonlinear) term has therefore to be taken into account, but it is too early to investigate any realistic mechanism for the present reaction using kinetic data.

This reaction kinetic cell now has to be multiplied and the units have to be properly arranged and coupled. A model for the spatial extension of our experiment is a one-dimensional array of reaction cells, which are linearly coupled via their respective concentration differences. The equation for species B (with diffusion coefficient D_B) in the n-th cell would thus read

$$\frac{\mathrm{d}b^n}{\mathrm{d}t} = F(b^n) + D_b(b^{n+1} + b^{n-1} - 2b^n).$$

The correct partial differential equation has been truncated into a finite number of linearly coupled ordinary differential equations.

Boundary conditions are as given by the electrochemical set-up. At the electrode (cell number 0):

$$b^0 = a^0 \exp((E - E_0)\, F/RT),$$

$$b^0 + a^0 = \text{const},$$

where E_0 is the formal potential and E the actual potential applied. At the boundary inside the solution (largest cell number considered):

$$b^\infty = 0, \qquad a^\infty = a_0.$$

Initial concentrations for reacting species A and B are

$$b_0^n = 0, \qquad a_0^n = a_0.$$

There is a single formal potential for electron transfer at the electrode, and both diffusion coefficients are set equal.

Apart from the fact that mechanistic details are hitherto unknown, a severe quantitative treatment of the partial differential equation for such a complex system seems an enormous task. At present it would be desirable to obtain theoretical predictions from abstract model equations for further experimental testing. We therefore suggest a more basic procedure, namely, to simply start with the fundamental types of rate equations from enzyme kinetics and put together a catalogue of simulated cyclic voltammograms for a feedback system as introduced in this paper. Such simulations of the outlined system of ordinary equations using standard integration routines could be performed with any personal computer and would yield qualitative results in reasonably short times. We deem qualitative results to be of valuable assistance for the interpretation of experimental results. Particularly in the field of self-organized dissipative structures (for which the present system has been designed) with its non-analytical equations enormous progress has been achieved by means of simulations. A first set of results will be provided in a forthcoming paper.

A remarkable feature of catalytic types of reactions is this: in the uncatalyzed case the gradients of species A and B are complementary (their relation is given by the Nernst equation) and they smoothly change as a function of time. They extending into the solution. As soon as the reduced form is oxidized again by some kinetic mechanism, however, this tends to eliminate the steady growth of the diffusion layer, and for sufficiently high reaction rates, e.g. as in enzyme catalyzed reactions, the distribution of A and B will be stabilized in a shape as shown schematically in Figure 3.

Therefore we expect such systems to produce interesting results in their long-term dynamics (not only transistory behavior) of coupled reaction cells with a constant gradient of substrate and product, a tempting configuration that is hard to achieve otherwise.

5. Summary

To sum it up, we made an attempt to extend the number of experimental systems for the study of self-organization in enzyme reactions by coupling of the kinetic part to an artificial electrode reaction. This can be done in any system where one of the products is electrochemically active. A special feature here was the enzyme's cosubstrate being the product of the electrochemical reaction itself, so that an arbitrary but

easy to specify feedback loop could be introduced. Such loops do naturally occur in many dynamical systems and are one of the prerequisites for instabilities. The system differs from simply nonstirred set-ups in that the reaction takes place in an environment of a controlled gradient of one of the substrates and that the reaction does not spread over the entire solution, but is limited to a narrow diffusion layer at the surface of the electrode. In addition to an exact parameter control (of the applied potential in potentiostatic experiments) this set-up provides continuous recordings of time series. So far missing is a spatial resolvement of the diffusion layer to distinguish simultaneous oscillations of the gradients from spatial structures like moving chemical waves, which could give rise to the same kind of temporal behavior.

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