SOME DIFFERENT WAYS TO USE ADSORPTION OF MOLECULES ON ELECTRODES TO MEASURE ENZYMATIC ACTIVITY

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1. Introduction

We have described an electric method to measure enzymatic activity [1–3]. The method is based on a synthetic substrate, which has the ability to adsorb on metal electrodes inserted into a buffer containing the substrate molecules. The molecules adsorb in such a way that the electrodes are effectively screened from the ions in the buffer. The reason for this, we believe, is the presence of benzene rings in the molecule which, when the molecule sits on the metal, causes this screening effect. The adsorption gives rise to a large change in electrode capacitance which is easily measured with a capacitance bridge. The method has been applied so far to reactions where the substrate is hydrolysed by an enzyme. Schematically we have the following reaction:

$$\text{Substrate} + H_2O \rightarrow \text{Part 1} + \text{Part 2}$$

A requirement is here that the two parts of the cleaved substrate do not adsorb on the electrode, or do adsorb in such a way that the electrode capacitance is not altered significantly. Another important observation is that although proteins (e.g., the enzymes) may adsorb on electrodes, they do not show up as a large capacitance change. The reason for this is that proteins in general do not screen the electrodes effectively [4].

There are a number of questions regarding the way the substrate molecules adsorb on the electrodes, how the enzyme works at and in the neighbourhood of the electrode, the pH and potential dependence of the adsorption process. Some of these questions will be dealt with in a future communication. The present paper is concerned with some different ways in which electrode adsorption can be used to quantitatively determine enzymatic activity.

2. Methods and results

The measurements were carried out at 37°C in Tris–HCl buffer (pH 8.2) in 0.15 M NaCl. The capacitance between two platinum electrodes in a test tube containing buffer solution was measured with a capacitance meter [3]. The substrates we use are tripeptides originally synthesized to be used in a chromogenic method for serine proteases (thrombin, trypsin) [5–7]. We have mainly studied the hydrolysis by bovine thrombin (SAF, Hoffmann-La Roche) of a substrate called S-2160, which is shown schematically in fig.1. This substrate has the properties described above, and although it adsorbs reversibly

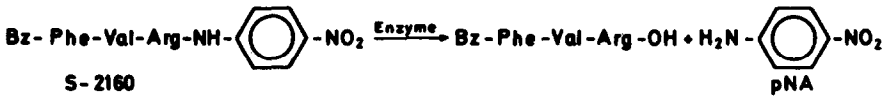

Fig.1. Chemical formula of the substrate *N*-benzoyl–L-phenylalanyl–L-valyl–L-arginine–*p*-nitroanilide (S-2160) and the hydrolytic cleavage of S-2160 by proteases.

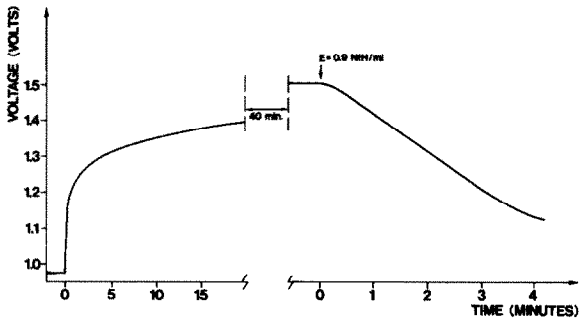

Fig.2. Adsorption of S-2160 (S_o = 50 μM) on platinum electrodes recorded by an automatic capacitance meter. The voltage is proportional to $1/C$, where C is the electrode capacitance. At the point indicated by the arrow thrombin is added. Note the change in time scale at this point.

it has a large affinity for metal surfaces. It can be seen in fig.2 that the introduction of S-2160 into a test tube containing buffer and two electrodes gives rise to a large change in capacitance between the electrodes. The adsorption properties of S-2160 can be utilized in some different ways as described below.

2.1. *Pre-adsorbed substrate molecules*

In this method substrate molecules are added to the test tube and are allowed to adsorb on the electrodes. When equilibrium is reached the test cell is ready to use. As shown in fig.2 the introduction of thrombin into the test cell gives rise to an increase in the electrode capacitance (a decrease of $1/C$). This is explained as follows: The hydrolysis of the substrate decreases the concentration of whole substrate molecules which causes desorption from the electrodes. The enzyme may also hydrolyse molecules directly on the electrode surfaces. This method, detailed in [1–3], is time consuming, or requires 'pre-adsorbed' substrate molecules, i.e., electrodes inserted into a solution with substrate molecules long before they will be used. The method is still practical, however, and from the initial rate of change of the capacitance after the addition of enzyme, the enzymatic activity can be determined. It was found that after a calibration curve had been obtained by the addition of known amounts of thrombin, a measured unknown thrombin activity fitted well with that determined by a spectrophotometric method [2]. The practical sensitivity of the method is comparable to the spectrophotometric method in which the light adsorption of formed paranitroaniline groups is used to determine the number of hydrolysed molecules.

2.2. *Enzyme and electrodes added simultaneously*

There are, however, other more practical ways in which the special properties of the substrate can be used. We recall that:

(i) The substrate molecule has a high affinity for metal surfaces;
(ii) The substrate adsorbs on electrodes in such a way that it gives rise to a large decrease in capacitance;
(iii) The two parts of the cleaved substrate do not have this property;
(iv) The adsorption of large molecules (proteins) do not, in general, change the capacitance of an electrode significantly.

These facts make it possible to use the substrate as illustrated in the insert to fig.3, where the electrodes are inserted into the buffer containing the substrate. The experimentally observed change in capacitance versus time is easy to understand. When electrodes and enzyme are introduced into the buffer, the substrate molecules adsorb immediately. The substrate

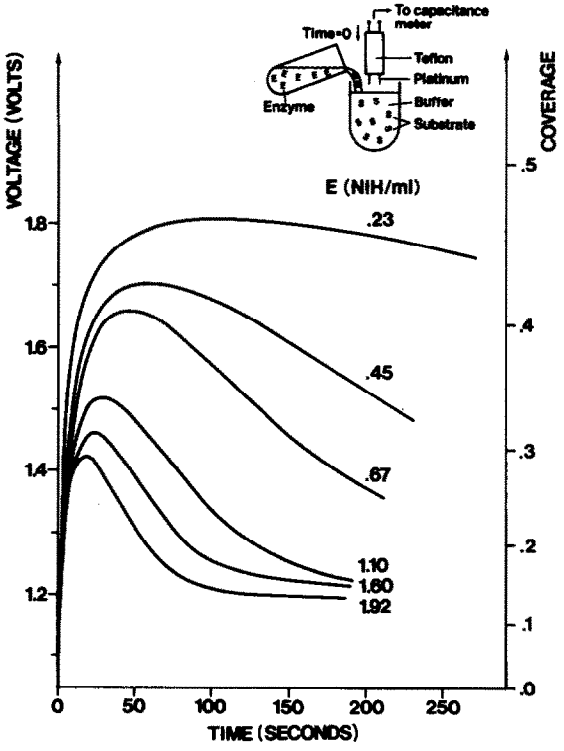

Fig.3. Adsorption of S-2160 (S_o = 50 μM) on platinum electrodes in presence of thrombin. The enzyme concentration is shown at each curve. The scale to the right gives the partial coverage of S-2160 on the electrode surfaces. The insert shows the test cell and how an experiment is performed.

molecules are small so the diffusion time to the electrodes will be small on the experimental time scale. Due to the presence of enzyme the concentration of substrate molecules decreases with time, and a point is reached when the concentration in solution becomes so small that a net desorption sets in. We have now passed the maximum of the curves in fig.3. Ideally, if we waited long enough the capacitance would go back to its initial value. Experimentally it is found that it levels off at some value indicating that some part of the substrate molecule is left on the electrodes, or that thrombin is adsorbed, giving a (small) capacitance change. Information about the enzymatic activity is found in the position and the height of the maximum of the curves in fig.3. Concerning these peaks we make the following simple calculations. In our experiments the enzymatic reaction is monitored for a long time, and the substrate concentration decreases significantly. Thus the integrated form of the Michaelis-Menten equation is applicable for mathematical interpretation. As the Michaelis-Menten constant, K_m, is 80 μM [7] and the substrate concentration is ~50 μM (initially), the disappearance of the substrate molecules may be approximated by an exponential decay. Thus the variation with time in substrate concentration, $S(t)$, may be written as:

$$S(t) = S_o \exp(-\alpha E t) \quad (1)$$

where S_o is the initial substrate concentration, E is the enzyme concentration and α is a constant. In fig.3 the measured voltage is proportional to $1/C$, where C is the electrode capacitance. We now introduce the coverage, θ, of the substrate molecules on the electrodes. Coverage here means the fraction of the electrode surface which electrically is screened from ions in the solution. This is not necessarily the same as the physical coverage. The relation between the coverage and the measured voltage (see θ-scale in fig.3) is determined from adsorption experiments like those in fig.2 [8]. Furthermore a first order kinetic equation is assumed for the adsorption of the substrate molecules, or:

$$\frac{d\theta}{dt} = c_1 S(t)(1-\theta) - c_2\theta \quad (2)$$

where c_1 and c_2 are rate constants. In reality a more complex kinetic equation is followed [8], but for low coverages (<0.5) this model is a good approximation and is sufficient for our calculations. At the maximum of the curves in fig.3 eq. (1) and (2) yield $d\theta/dt = 0$ at $t = t_m$ (with $\theta = \theta_m$) and we obtain:

$$E = \frac{1}{\alpha t_m} \ln\left(S_o \frac{c_1}{c_2} \frac{1-\theta_m}{\theta_m}\right) \quad (3)$$

If the constants α, c_1 and c_2 are measured this equation can be used to determine an unknown enzymatic activity from t_m and θ_m in fig.3. A more practical way is to make a calibration curve (E versus $1/t_m$) for known activities.

It is obvious, however, that the evaluation of experiments like these in fig.3 is rather cumbersome and will only give E in an indirect and uncertain way. A rapid, direct and more practical method is described below.

2.3. *Clean electrodes inserted in a cell containing enzyme and substrate*

A very neat way to use the adsorption properties of the substrate is as follows. Enzyme is added to a test cell containing a given amount of substrate, S_o. Clean electrodes are inserted into the cell when the enzymatic reaction has run for a certain time, t_e (say 60 s). The non-cleaved substrate molecules adsorb on the electrodes and give rise to a capacitance change. The initial rate of change is related to the amount of non-cleaved substrate molecules (S_e), which for $S_o << K_m$ is obtained from eq. (1) as $S_e = S_o \exp(-\alpha E t_e)$.

From eq. (2) we now obtain:

$$\left(\frac{d\theta}{dt}\right)_{t=0} = c_1 S_e = c_1 S_o e^{-\alpha E t_e} \quad (4)$$

The initial rate of change is therefore a measure of enzyme activity. Examples of capacitance changes obtained in this way are shown in fig.4a. On a larger time scale these curves will be similar to those in fig.3 since the enzymatic reaction continues all the time. It may, of course, be possible to stop the enzymatic reaction before the electrodes are inserted. This was not necessary in the present case due to the high affinity of the substrate molecules to the electrodes. Figure 4b is a calibration curve, which on a logarithmic scale shows the initial rate of change in capacitance versus known amount of added thrombin. The slope of this curve is $-\alpha t_e$ and with t_e = 60 s, α is calculated to

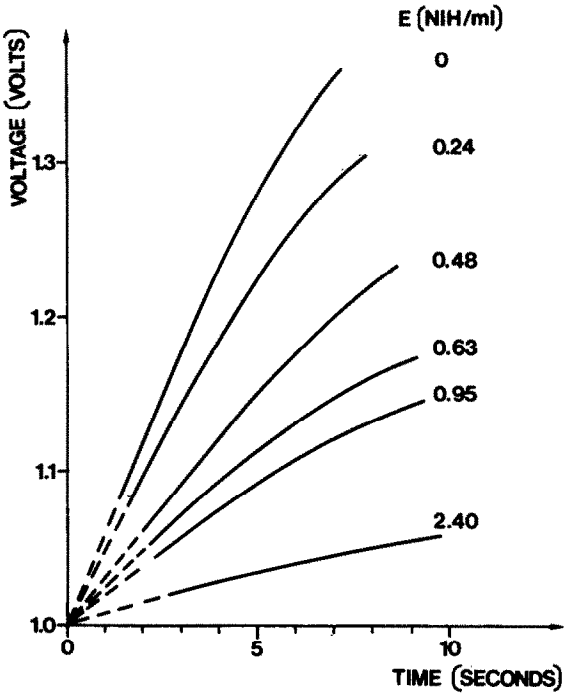

Fig.4a. Initial rate of adsorption of S-2160 (S_o = 50 μM) on platinum electrodes after incubation with thrombin for 60 s. The total enzyme concentration is shown at each curve. The first 2 s of each curve (dashed lines) are not available due to transients. The transients can be avoided with a modified experimental setup.

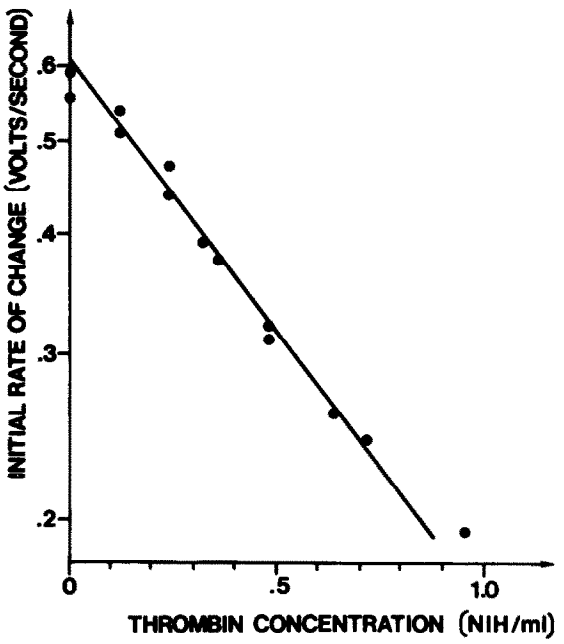

Fig.4b. Initial rate of change in voltage on a logarithmic scale versus thrombin concentration. The solid line corresponds to α = 0.022 ml/s NIH.

0.022 ml/s · NIH. According to the data sheet [7] the ratio $V_{max}/K_m \cdot E$, which is $\simeq\alpha$, is 0.014 ml/s · NIH. This discrepancy may be explained by uncertainty in the thrombin activity, which is specified to 51 NIH/mg enzyme as determined by clotting. However, the activity is expected to be higher on small substrates like S-2160. Another contribution to the deviation is that eq. (1) is not valid initially and for low enzyme concentrations, which means that α is not identical with $V_{max}/K_m \cdot E$. It is also seen that for low and high enzyme concentrations there is a deviation from a straight line in fig.4b. For low enzyme concentrations this may be caused by loss of enzyme molecules due to adsorption on the glass walls of the test tubes.

It is concluded that the above method can be conveniently used to determine thrombin activity.

3. Discussion

The substrate we use is very convenient to use for the measurement of enzymatic activity. Only a small number of steps are required, the measurements are rapid and the interpretation is simple. This is especially the case for the last method discussed above, where the initial adsorption rate is determined during the first 3 or 4 s. As well as the spectrophotometric method the electric methods described are well suited for automation. Compared to the optical use of the substrate the electric methods have some additional advantages. Measurements may be performed in light scattering and in light absorbing solutions, e.g., in whole blood and in tissue extracts. Depending on the application it is also possible to choose different ways of measurement. Since the size of the electrodes does not matter, the electric methods can be developed for very small sample volumes. Measurements on blood from finger tips may be possible.

The methods are mainly limited by the electrode which must be cleaned in a controlled way between each measurement. In some cases poisoning phenomena have been seen. They are probably caused by irreversible adsorption of some organic compounds. With the use of throw away electrodes this problem is solved. It should be mentioned that other metals than platinum can be used as electrode material.

The use of adsorption of molecules on electrodes should make it possible to make simple measurements of enzymatic activities in whole blood and in crude extracts. By the use of different substrates, other reactions may be studied also.

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References

[1] Arwin, H. and Lundström, I. (1976) Rev. Sci. Instrum. 47, 1394.
[2] Arwin, H., Lundström, I., Claeson, G., Ekenstam, B. and Aurell, L. (1976) FEBS Lett. 64, 95.
[3] Arwin, H. and Lundström, I. (1976) Physics in Industry, Pergamon, New York.
[4] Stenberg, M., Arwin, H. and Nilsson, A. (1980) J. Colloid Interface Sci. in press.
[5] Svendsen, L., Blombäck, B., Blombäck, M. and Olsson, P. (1972) Thromb. Res. 1, 267.
[6] Blombäck, M., Blombäck, B., Olsson, P. and Svendsen, L. (1974) Thromb. Res. 5, 621.
[7] Synthetic chromogenic substrates, Data Sheet, Kabi Diagnostica, S-122 87 Stockholm, Sweden.
[8] Arwin, H. and Lundström, I. (1980) in preparation.