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Determination of Butyrylcholinesterase Inhibition Using Ion Transfer across the Interface between Two Immiscible Liquids

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An investigation was made into the inhibition of the enzyme butyrylcholinesterase by paraoxon (diethyl *p*-nitrophenyl phosphate), using butyrylcholine chloride as the substrate. Experimental measurement was based on the transfer of the butyrylcholine cation across the interface between water and 1,2-dichloroethane using cyclic voltammetry. By this method it was possible to determine the rate constants for both the inhibition of the enzyme and the hydrolysis of butyrylcholine.

The aim of this work is to show that the enzyme butyrylcholinesterase (BCHE) can be studied using the transfer of the butyrylcholine cation (ButCh) across the water/1,2-dichloroethane interface, by means of cyclic voltammetry, and that the inhibition of the enzyme by the organophosphate compound paraoxon can also be demonstrated.

Organophosphate compounds, and related substances, are the most widely used insecticides in the world, having replaced the older, and more persistent, organochlorine compounds. The target of these substances is the central nervous system (CNS) of insects but because the CNS of both vertebrates and invertebrates operates by cholinergic transmission, accidential poisonings due to crop spraying or other uses may severely harm humans.[1]

Nerve transmission ends when the enzyme acetylcholinesterase (ACHE) hydrolyses the neurotransmitter acetylcholine to choline and acetic acid in order to reestablish the initial state of the postsynaptic membrane.[1] Organophosphate compounds imitate the overall molecular shape of acetylcholine and can bind irreversibly to the esteratic active site of the enzyme, rendering it nonfunctional.[2]

Due to the health threat posed by the buildup of pesticide residues in the environment, legislation has made the detection of these compounds at the parts per billion (ppb) and sub–ppb domains a necessity. At the moment, the best techniques are gas chromatography (GC), high-performance liquid chromatography (HPLC), and GC coupled with mass spectrometry (GC/MS).[3] Although such techniques are the best available, they are expensive and they require skilled personnel to operate them efficiently. In the search for less costly alternatives, a lot of effort has been devoted to the development of relatively inexpensive electrochemical techniques, such as enzyme or antibody biosensors, to try and effect the same performance as the conventional methods. Some examples of enzymatic methods developed specifically for the detection of organophosphate compounds are described below. The methods developed generally fall into two broad categories—amperometric and potentiometric.

Potentiometric Methods. A commercial, field-based instrument utilizing replaceable pads with gel-entrapped BCHE as the sensor was developed by Goodson et al.[4] in the 1970s. Pesticide detection was based on the production of electroactive thiocholine from the hydrolysis of butyrylthiocholine. The inhibition of the enzyme produced a change in the measured voltage of the cell.

Durand et al.[5] and Kumaran and Tran-Minh[6] used pH electrodes modified with an enzymic layer to investigate ACHE[5] and BCHE[6] inhibition, using acetylcholine (ACh) and butyrylcholine (ButCh) as the respective substrates. The method of detection was based on the fact that one proton is produced per hydrolyzed substrate molecule.

Amperometric Methods. Razumas et al.[7] studied BCHE inhibition in solution using indoxyl acetate as the substrate, producing indoxyl, which could be measured amperometrically.

Campanella et al.[8] investigated ACHE and BCHE inhibition using a method based on an oxygen electrode covered with a membrane, on which choline oxidase was immobilized. ACHE or BCHE was either free in solution or immobilized with the choline oxidase. The substrates used were ACh and ButCh. Any inhibition of the enzyme led to an increase in the oxygen concentration being measured. Their results showed that BCHE was more sensitive to organophosphate inhibition than ACHE for a given inhibitor.

Bernabei et al.[9] studied ACHE inhibition using the same principles but replaced the oxygen electrode with a hydrogen

(1) Hassall, K. A. *The biochemistry and uses of pesticides: structure, metabolism, mode of action and uses in crop protection*, 2nd ed.; Macmillan: Basingstoke, U.K., 1990; Chapter 4.
(2) Matsumura, F. *Toxicology of insecticides*; Plenum Press: New York, 1975; Chapters 3 and 4.
(3) Seiber, J. N., Li, Q. X., Van Emon, J. M. In *Immunochemical methods for environmental analysis*; Van Emon, J. M., Mummar, R. O., Eds.; ACS Symposium Series 442; American Chemical Society: Washington, DC, 1990; Chapter 13.
(4) Goodson, L. H., Jacobs, W. B., Cage, B. R. *Proc. Natl. Conf. Control Hazard. Mater.* April 11–13, 1978.
(5) Durand, P.; Mallevialle, J.; Nicaud, J. M. *J. Anal. Toxicol.* **1984**, *8*, 112.
(6) Kumaran, S., Tranh-Minh, C. *Electroanalysis* **1992**, *4*, 949.
(7) Razumas, V. J., Kulys, J. J., Malinauskas, A. A. *Environ. Sci. Technol.* **1981**, *15*, 360.
(8) Campanella, L., Achilli, M., Sammartino, M. P., Tomassetti, M. *Bioelectrochem. Bioenerg.* **1991**, *26*, 237.
(9) Bernabei, M., Cremisini, C., Mascini, M., Palleschi, G. *Anal. Lett.* **1991**, *24*, 1317.

0003-2700/94/0366-0052$04.50/0

peroxide sensor and sandwiched the enzyme-loaded membrane between two membranes, one designed to protect the electrode from electrochemical interferences and the other to protect the enzyme from large molecules and bacteria. Their results showed that experiments with the ACHE free in solution were more sensitive than for immobilized case.

Kulys and D'Costa[10] and Skladal[11] studied BCHE inhibition using printed-strip amperometric sensors containing the immobilized enzyme and a redox mediator. The substrate used in each case was butyrylthiocholine.

From the various investigations cited above,[4-11] the general conclusions were that the inhibition of the enzyme is favored by incubation with the pesticide for a certain amount of time and that the longer the incubation time observed the greater the inhibition. It is clear, however, that amperometric methods tend to give much better sensitivity in general than the potentiometric methods.

Vanysek and Behrendt[12] investigated the hydrolysis of ACh by ACHE at the interface between two immiscible electrolyte solutions (ITIES), in their case water and nitrobenzene, in an attempt to demonstrate an alternative assay for enzyme activity. The problem with this method, however, is that the ACh and choline (Ch) peaks are poorly resolved, making it difficult to allow unambiguous determination of enzyme activity. The resolution of ButCh and Ch is much clearer, which makes the enzyme reaction easier to follow, as will be shown.

EXPERIMENTAL SECTION

BCHE (EC 3.1.1.8) from horse serum (500 units mg^{-1}), ButCh chloride, and paraoxon were supplied by Sigma. Acetone, methanol, tris(hydroxymethyl)aminomethane hydrochloride, tris(hydroxymethyl)aminomethane, anhydrous lithium chloride, and tetrabutylammonium chloride ((TBA)Cl) were supplied by Fluka. 1,2-Dichloroethane (1,2-DCE) was supplied by Merck. Tetrabutylammonium tetrakis(4-chlorophenyl) borate ((TBATPB)Cl) was prepared by metathesis of (TBA)Cl and K(TPB)Cl (Lancaster) and recrystallized twice from methanol. All reagents used were analytical grade or better.

The apparatus used was a four-electrode potentiostat, of a design similar to that given in ref 13. It consisted of an ordinary three-electrode potentiostat (Southampton University) and a "homemade" zerostat. The potential was supplied by a waveform generator PPRI (Hi-Tek Instruments), which was coupled to an X–Y recorder (Bryans Instruments). The cell used was constructed of glass and was of the design shown in Figure 1. Both the cell and the four-electrode potentiostat were housed in a Faraday cage. The cell was furthermore kept at a temperature of 37 ± 0.5 °C by means of a thermostated water bath (unless otherwise stated).

The paraoxon was dissolved in acetone before being diluted with water to make standard solutions, which also contained (ButCh)Cl. The enzyme and the lithium chloride were diluted with 0.1 M tris-HCl buffer to give a concentration of x units L^{-1}/10 mM LiCl (x is a value specified in the text) and a pH

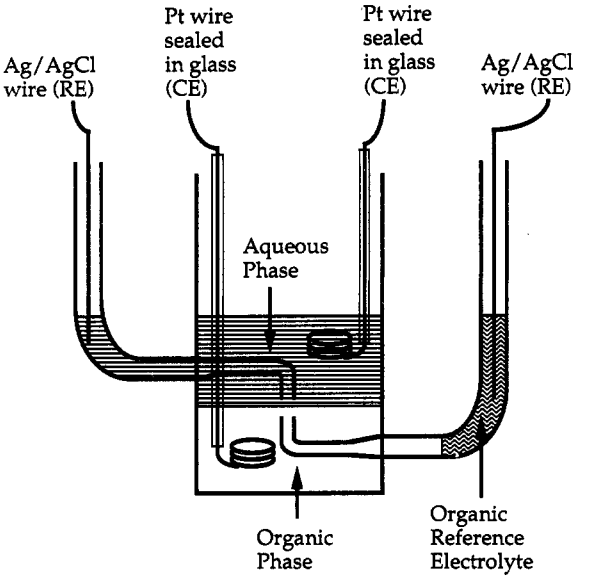

Figure 1. Representation of an ITIES cell: CE, counter electrode; RE, reference electrode.

of 8 ± 0.05 at 37 °C. This solution was used as the aqueous phase of the cell, and the volume added was always 2 mL so that a constant amount of enzyme was maintained. The basic reaction studied was

$$\text{ButCh} \xrightarrow{\text{BCHE}} \text{Ch} + \text{butyric acid}$$

and the system used for the experiments was

$$\underset{\text{REFERENCE ELECTRODE}}{\text{Ag|AgCl|10mM TBACl}} \| \underset{\text{TBATPBCl}}{\text{10mM}} \| \underset{\text{BCHE in 0.1M tris/HCl}}{\text{10mM LiCl and xUl}^{-1}} \underset{\text{REF. E.}}{|\text{AgCl|Ag'}}$$

Cell I

The scan rate was 0.1 V s^{-1}, the aqueous phase was unstirred, and iR drop compensation was not used. The concentration of butyrylcholine chloride being studied was near the limit of detectability for cyclic voltammetry at the liquid/liquid interface. The organic phase reference electrode was a TBA^+ ion-selective electrode comprised of a silver/silver chloride wire immersed in a solution of TBACl.

Four principal types of experiments were performed:

(1) Sample Addition Reactions (x = 350 units L^{-1}). A 100-μL aliquot of the paraoxon standard containing ButCh was injected into the thermostated cell. The decrease in the ButCh cation peak height with time (1-min intervals) was observed using cyclic voltammetry. The emergence of a peak due to the production of Ch was also seen. Experiments were carried out for a cell concentration of 50 μM (ButCh)Cl together with 0, 20, 35, 50, 70, 100, and 120 ppb paraoxon.

(2) Sample Incubation Reactions (x = 350 units L^{-1}). A 10 ppb sample of paraoxon (cell concentration) was incubated with the enzyme for 0, 5, 10, and 15 min prior to addition of the (ButCh)Cl (cell concentration as in (1)).

(3) Substrate Concentration Variation (x = 350 units L^{-1}). Standards containing different concentrations of (ButCh)Cl (cell concentrations of 50, 140, and 290 μM) were added to the cell.

(4) Enzyme Concentration Variation. Enzyme solutions containing 50, 150, 250, and 350 units L^{-1} were prepared and

(10) Kulys, J., D'Costa, E. J. *Biosens. Bioelectron.* **1991**, *6*, 109.
(11) Skladal, P. *Anal. Chim. Acta* **1992**, *269*, 281.
(12) Vanysek, P., Behrendt, M. *J. Electroanal. Chem.* **1981**, *130*, 287.
(13) Samec, Z., Marecek, V., Weber, J. *J. Electroanal. Chem.* **1979**, *100*, 841.

the relative rates of hydrolysis of ButCh observed. The cell concentration of ButCh was in (1).

RESULTS AND DISCUSSION

The equations for enzyme kinetics were first developed by Michaelis and Menten[14] and are based on the mechanism

$$E + S \underset{b}{\overset{a}{\rightleftarrows}} ES \xrightarrow{c} E + Prod$$

where the substrate, S, combines with the enzyme, E, to form an enzyme/substrate complex, ES, which proceeds to form the product, Prod, and the free enzyme. The reaction involving BCHE and ButCh is analogous to this. The enzyme contains two active sites. One is an esteratic site, the function of which is to cleave the ester linkage, and the other is an anionic site, which simply holds the substrate molecule in position, by interaction with the nitrogen group, while cleavage occurs. A simplified equation for the reaction is as follows:

$$E + ButCh \underset{b}{\overset{a}{\rightleftarrows}} EComp \xrightarrow{c} EBut + Ch \xrightarrow{d} E + ButA$$

where the last step is recovery of the enzyme (EComp = enzyme/substrate complex, ButA = butyric acid). It is the last step which the inhibiting molecule interferes with. In the case of the butyrylated enzyme (EBut) this step is very fast, but for a phosphorylated enzyme the step is very slow. Although the inhibition is not completely irreversible, it is still slow enough to render the enzyme effectively nonfunctional.

In the case of reversible inhibition, where the inhibitor is in equilibrium with the enzyme or enzyme/substrate complex, Michaelis–Menten principles may be applied for analysis of the results. Irreversible inhibition, however, cannot be treated in a similar manner.[15] A simplified treatment of the enzyme kinetics for irreversible inhibition is therefore presented, along with the results.

(1) Sample Addition Experiments. Figure 2 shows the enzyme reaction with the blank sample, containing only (ButCh)Cl. The peak of interest here was that of ButCh, at ~0.32 V on the forward scan (left to right, positive current). It should be stressed at this point that the cyclic voltammetric peaks displayed on this figure correspond to a flux of ions across the water/1,2-dichloroethane interface and are in no way redox in nature. Positive current relates to the fact that the potential of water with respect to the organic phase is being made more positive on the forward scan, and this is the convention for all ITIES experiments. The potential scale is completely arbitrary in form and is specific only to be the system under study (cell I). Readers are referred to refs 16 and 17 for a full treatment of the principles and nature of the ITIES. Scans were performed at 1-min intervals after the addition of the substrate. On the same figure is the scan prior to addition, showing the potential window. It should also be mentioned that there was no visible effect on the potential window by the enzyme. Both enzyme-free and enzyme-containing solutions gave exactly the same potential window.

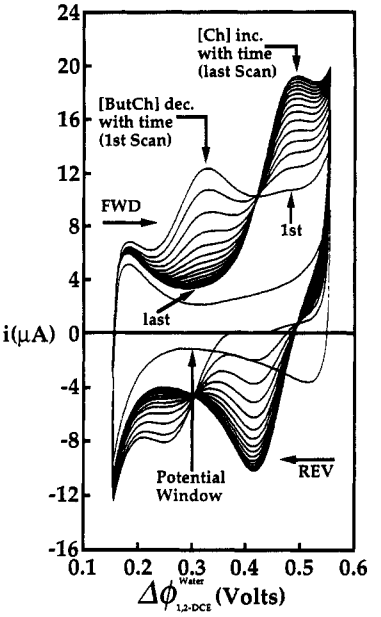

Figure 2. Cyclic voltammogram for the BCHE hydrolysis of butyrylcholine to choline over the time range 1–15 min (potential scale is with respect to cell I). The potential window for the system is also shown. The butyrylcholine peak (~0.32 V) decreases with time while the choline peak (~0.49 V) increases. The scan rate is 0.1 V s^{-1}.

As the experiment progressed, the ButCh peak was observed to decrease with time, and the emergence of a peak due to Ch at ~0.49 V, produced by the enzyme reaction, was also seen. In subsequent experiments with the paraoxon standards, the separation between successive scans was observed to decrease. The results obtained for all reactions were analyzed according to the height of the ButCh peak at 1 min after the addition of the sample. The peaks corresponding to the range 2–10 min were measured and expressed as a fraction of the 1-min peak. The reason for this approach was to mitigate against the intrinsic difficulty of reproducibility at the concentration of ButCh being dealt with (50 μM). The main reasons for peak height inconsistency in the different experiments were the simplicity of sample injection into the aqueous phase and the fact that the aqueous phase was unstirred. Other factors such as bubble formation at the interface during the course of the experiment, due to the elevated cell temperature, may also have contributed. The results obtained are shown in Figure 3.

Simple Analytical Model

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + Prod$$

$$E + P \xrightarrow{k_3} EP \text{ (enzyme consumed)}$$

where P = paraoxon. The following assumptions were made: (1) The equilibrium between the enzyme, the substrate, and the enzyme/substrate complex is very rapid. (2) The amount of ES present is very small. $K = k_1/k_{-1}$ is small. The time law for the disappearance of the enzyme is derived as follows:

$$d[E] = d[P] \rightarrow [E] - [P] = [E_0] - [P_0]$$

$$\frac{d[E]}{dt} = -k_3[E][P] = -k_3[E]([E] - ([E_0] - [P_0])) =$$

$$-k_3[E]^2 + k_3([E_0] - [P_0])[E]$$

(14) Michaelis, L., Menten, M. *Biochem. Z.* **1913**, *49*, 333.
(15) Lehninger, A. L. *Biochemistry*, 2nd ed.; Worth Publishers, Inc.: New York, 1981; Chapter 8.
(16) Koryta, J. *Electrochim. Acta* **1979**, *24*, 293.

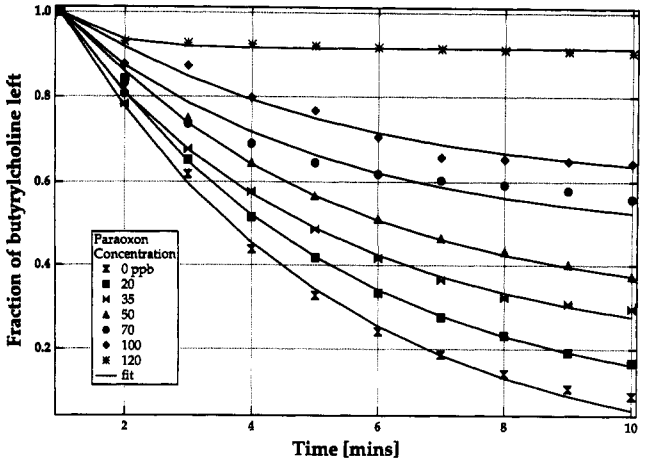

Figure 3. Fraction of butyrylcholine left versus time from initial sample addition (enzyme concentration, 350 units per liter; ButCh concentration, 50 μM), for different concentrations of paraoxon.

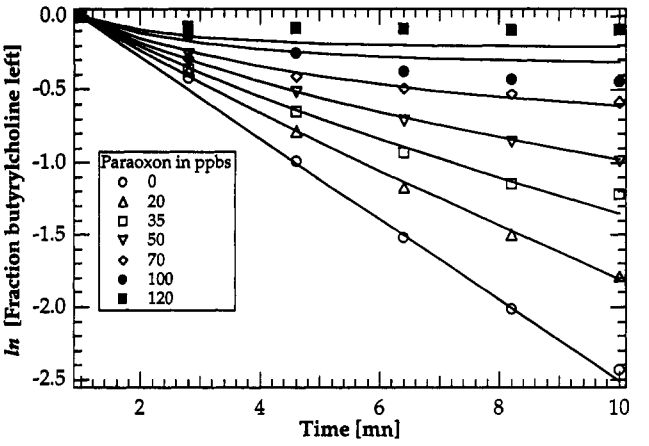

Figure 4. ln (fraction of butyrylcholine left) versus time from initial sample addition, for all seven data sets.

using $y = 1/[E]$ as the variable

$$\frac{dy}{dt} = k_3 - k_3 y([E_0] - [P_0]) \rightarrow y = \frac{1}{[E_0] - [P_0]} + Ae^{-k_3([E_0]-[P_0])t}$$

and using the initial condition that at $t = 0$, $[E] = [E_0]$

$$[E] = [E_0]\frac{[E_0] - [P_0]}{[E_0] - [P_0]e^{-k_3([E_0]-[P_0])t}}$$

The special case where $[E_0] = [P_0]$, for which a solution can be easily obtained, is ignored.

In the absence of inhibitor, the analytical expression for the substrate disappearance is

$$\ln\frac{[S]}{[S_0]} = -k_2K[E_0]t$$

In the presence of inhibitor, the law for the disappearance of S is

$$\frac{d[S]}{dt} = -\frac{d[Prod]}{dt} = k_2[ES] \quad \text{with} \quad k_1[E][S] = k_{-1}[ES]$$

$$\frac{d[S]}{dt} = -k_2K[S][E_0]\frac{[E_0] - [P_0]}{[E_0] - [P_0]e^{-k_3([E0]-[P_0])t}}$$

$$\ln\frac{[S]}{[S_0]} = \frac{k_2K}{k_3}\ln\left\{\frac{([E_0] - [P_0])e^{-k_3([E_0]-[P_0])t}}{[E_0] - [P_0]e^{-k_3([E_0]-[P_0])t}}\right\}$$

The results have been normalized as the fraction of substrate left as a function of time with respect to substrate left at time $t = 1$ min. Assuming that the reaction takes place from time $t = 0$, and applying the above result to time $t = 1$ min, the following expression is obtained:

$$\ln\frac{[S_1]}{[S_0]} = \frac{k_2K}{k_3}\ln\left\{\frac{([E_0] - [P_0])e^{-k_3([E_0]-[P_0])}}{[E_0] - [P_0]e^{-k_3([E_0]-[P_0])}}\right\}$$

Combining the two preceding equations we obtain

$$\ln\frac{[S]}{[S_1]} = \frac{k_2K}{k_3}\ln\left\{e^{-k_3([E_0]-[P_0])(t-1)}\frac{([E_0] - [P_0])e^{-k_3([E_0]-[P_0])}}{[E_0] - [P_0]e^{-k_3([E_0]-[P_0])t}}\right\}$$

This is the relation to which an attempt was made to fit the data.

Independent Parameters. The enzyme concentration is expressed in units per liter, and the concentration of the paraoxon, is expressed in ppb. Calculations have to be carried out with an homogeneous set of concentration units. Therefore, the initial concentrations of BCHE and paraoxon are defined as

$$[E_0]\ (\text{mol L}^{-1}) = \alpha[E_{init}]\ (\text{U L}^{-1})$$

$$[P_0]\ (\text{mol L}^{-1}) = \beta[P_{init}]\ (\text{ppb})$$

Taking MW = 277 for the paraoxon, 1 ppb = 1 μg L^{-1} = $1 \times 10^{-6}/277 = 3.6 \times 10^{-9}$ mol L^{-1}. Therefore $\beta = 3.6 \times 10^{-9}$. Thus, there are three parameters to obtain from a fit: k_2K (mol^{-1} min^{-1}), k_3 (mol^{-1} min^{-1}), and α.

The fit to the above relation together with the experimental data is shown in Figure 4. At higher concentrations of paraoxon, the data do not fit the model as well as would be expected. This could possibly be attributed to a change in the factors affecting the rate as the activity of the enzyme tends to zero or could, more probably, be a manifestation of the experimental conditions given that individual experimental results are under considerations. Figure 5 shows the result where the best five data sets are used. The fit to the data is very good in each case, and the values obtained for the parameters are not very different going from seven to five data sets. The three parameters each have an error of roughly 10%, and are as follows:

seven data sets

$$k_2K = (1.34 \pm 0.11) \times 10^6\ \text{mol}^{-1}\ \text{min}^{-1}$$

$$k_3 = (2.21 \pm 0.15) \times 10^6\ \text{mol}^{-1}\ \text{min}^{-1}$$

$$\alpha = (5.93 \pm 0.47) \times 10^{-10}\ \text{mol units}^{-1}$$

five data sets

$$k_2K = (1.51 \pm 0.13) \times 10^6\ \text{mol}^{-1}\ \text{min}^{-1}$$

(17) Girault, H. H., Schiffrin, D. J. Electrochemistry of liquid–liquid interfaces. In *Electroanalytical Chemistry*; Bard, A. J., Ed.; Marcel Dekker, Inc.: New York, 1989; Vol. 15.

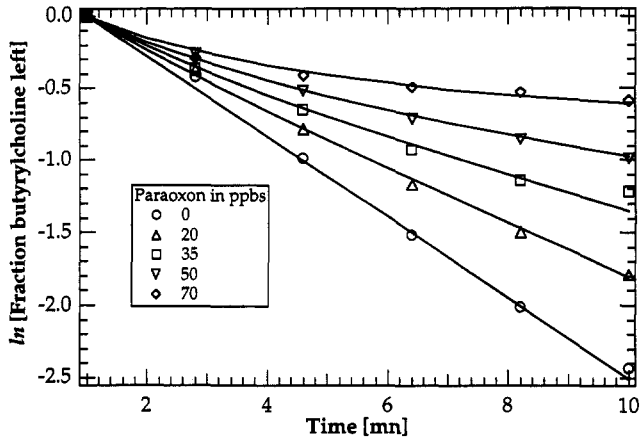

Figure 5. ln (fraction of butyrylcholine left) versus time from initial sample addition, for the best five data sets.

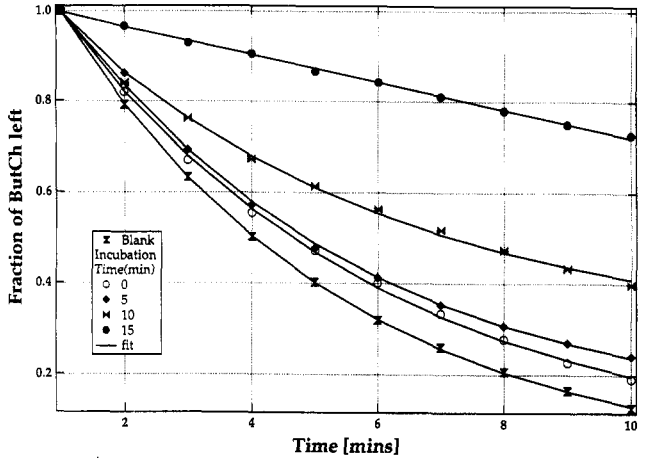

Figure 6. Fraction of butyrylcholine left versus time from initial sample addition, for a paraoxon concentration of 10 ppb and different incubation times.

$$k_3 = (2.00 \pm 0.14) \times 10^6 \text{ mol}^{-1} \text{ min}^{-1}$$

$$\alpha = (5.29 \pm 0.45) \times 10^{-10} \text{ mol units}^{-1}$$

The value obtained for α using a molecular weight for the protein of ~440 000 and a value of 500 units (mg of protein)$^{-1}$ is 4.5 × 10^{-12} mol units^{-1}, so there would seem to be a discrepancy in that the value obtained for α from the fit predicts either a smaller molecular weight for the protein or much smaller activity. However, this value depends to a great extent on the purity of the supplied protein as well and should not necessarily be viewed as a negative aspect.

According to the supplier's specifications, the activity should have been hydrolysis of 1 μmol of butyrylcholine by 1 unit of enzyme during 1 min, at 37 °C and pH 8.0. Therefore, under ideal conditions it should have taken just over 8 s for the enzyme to hydrolyze the 0.1 μmol of butyrylcholine in the cell. It is clear, however, that under our experimental conditions the activity is roughly 1% of the stated value. The most important point to remember when faced with such a discrepancy is that the cell was unstirred during the course of the experiment. Also, the stated value is presumably for the case where the substrate is in large excess compared with the available active sites of the enzyme and therefore would only be limited by the efficiency of the enzyme. Another contributory factor can be the choice of buffer for the experiments. Tris-HCl was chosen primarily for its ability to buffer in the pH range 7.1–8.9.[18] Other groups[5–11] have tried using phosphate, glycine, and phosphate/glycine buffers in varying concentrations, but always in the stated pH range. Goodson et al.,[4] however, used tris buffer in their commercial system. The high ionic strength of the aqueous phase might also have had an effect on the enzyme, especially considering that one of the active sites plays an electrostatic role. A combination of the above factors was probably the reason for the low activity observed, although an independent test of enzyme activity would be required to bear this out.

Another experiment, which was carried out at room temperature (23 ± 0.5 °C), showed that the BuTCh was hydrolyzed just as quickly as at 37 °C. Although this would suggest that the diffusion of the ButCh is independent of temperature, which is clearly an erroneous conclusion to draw, the effect of temperature on the rate of hydrolysis is clearly minimized, probably because the solution is unstirred.

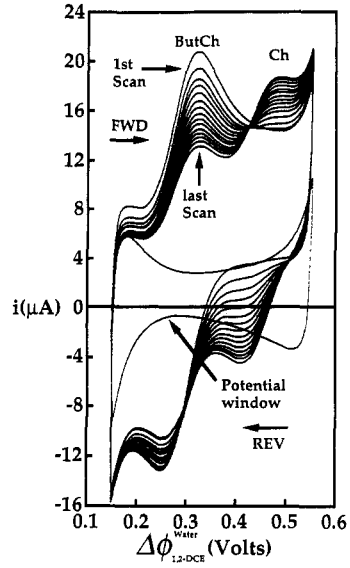

Figure 7. Cyclic voltammogram for the BCHE hydrolysis of butyrylcholine to choline over the time range 1–15 min, for a preincubation time of 15 min with 10 ppb paraoxon (potential scale is with respect to cell I).

(2) Sample Incubation Experiments. The results obtained for the incubation experiments showed that the incubation time of the paraoxon with the enzyme has a strong effect on the hydrolyzing power. Figure 6 shows the results obtained for incubation of a 10 ppb concentration of paraoxon for 0, 5, 10, and 15 min of incubation. Also shown is the result for a blank containing only (ButCh)Cl. The 0-, 5-, and 10-min results clearly show an exponential dependence, whereas the 15-min incubation period results in an almost linear response. Figure 7 shows the voltammograms for the 15-min case. Not shown in Figure 6 is the result for a blank in which the cell was left for 30 min after equilibration before adding the substrate. This result gave a response similar to that of 0-min incubation with the paraoxon, indicating that the enzyme does not denature significantly during this period.

In the Experimental Section, it was explained that the paraoxon was dissolved in a small amount of acetone before

(18) Dawson, R. M. C., Elliott, D. C., Elliott, W. H., Jones, K. M., Eds. *Data for Biochemical Research*; Oxford University Press: Oxford, U.K., 1969; pp 475–508.

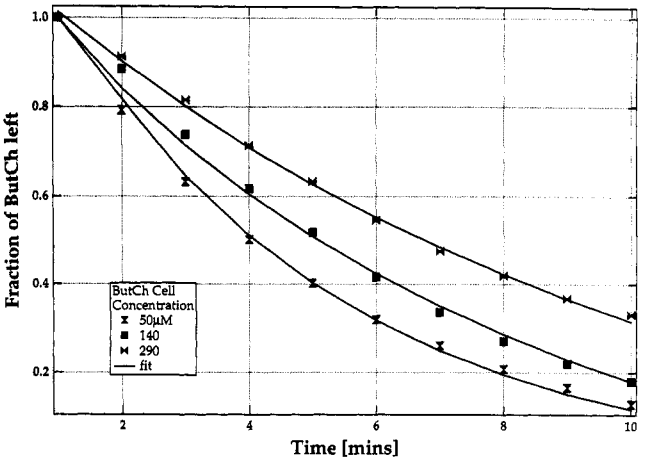

Figure 8. Fraction of butyrylcholine left versus time from initial sample addition, for different substrate concentrations.

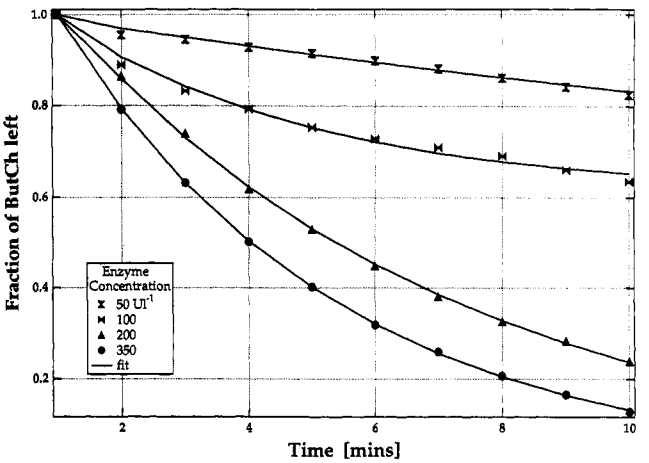

Figure 9. Fraction of butyrylcholine left versus time from initial sample addition, for different enzyme concentrations (ButCh concentration, 50 μM).

being diluted with water to make the necessary experimental standards. The effect of acetone, at the concentrations present in solution, was found to be negligible.

The difficulties inherent to these experiments precluded any attempt to fit our data with the model used for the sample addition experiments.

(3) Substrate Concentration Variation. The results obtained for experiments where the substrate cell concentration was varied between 50 and 290 μM are shown on Figure 8. These results show the reaction to be slower for higher substrate concentrations, but also show that even at ~6 times the substrate concentration used in (1) and (2) the reaction can still be treated as being pseudo first order in [S].

(4) Enzyme Concentration Variation. The results shown on Figure 9 illustrate the effect of varying the enzyme concentration. For concentrations of 50 and 100 units per liter the reaction appeared to be almost linear, whereas for 200 and 350 units per liter it was clearly pseudo first order in [S].

Sections 3 and 4 corroborate the idea that under the experimental conditions observed in sections 1 and 2 diffusion of the ButCh to the active sites of the enzyme, and not enzyme concentration, plays the important rate-determining role.

CONCLUSIONS

It has been shown that ion transfer across the ITIES can act as an effective transducer upon which sensor design may be based, as realized by Senda et al.[19]

(19) Osaki, T., Kakutani, T., Senda, M. *Anal. Sci.* **1988**, *4*, 529.

The hydrolysis of butyrylcholine by the enzyme butyrylcholinesterase has been demonstrated for various substrate and enzyme concentrations. The inhibition of the enzyme BCHE by paraoxon, without preincubation, could be satisfactorily analyzed in a quantitative way. The working range of detection was 20–120 ppb paraoxon using cyclic voltammetry, although if differential pulse voltammetry were the method used, the detection limits would be improved. The incubation time of the paraoxon with the enzyme was also shown to be a powerful factor in the ability of BCHE to function effectively, using a concentration of only 10 ppb of paraoxon. The effect of the 1,2-dichloroethane phase on the stability of the enzyme was also shown to be negligible over the time ranges considered.

The results obtained compare very favorably with other approaches[4–11] where the best limits of detection tend to be in the low-ppb range with the exception of ref 11 in which a sub-ppb limit is claimed.

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