Sensitive Detection of Organophosphorus Pesticides Using a Needle Type Amperometric Acetylcholinesterase-based Bioelectrode. Thiocholine Electrochemistry and Immobilised Enzyme Inhibition

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An acetylcholinesterase (AChE) based amperometric bioelectrode for a selective detection of low concentrations of organophosphorus pesticides has been developed. The amperometric needle type bioelectrode consists of a bare cavity in a PTFE isolated Pt-Ir wire, where the AChE was entrapped into a photopolymerised polymer of polyvinyl alcohol bearing styrylpyridinium groups (PVAl-SbQ). Cyclic voltammetry, performed at Pt and AChE/Pt disk electrodes, confirmed the irreversible, monoelectronic thiocholine oxidation process and showed that a working potential of +0.10 V vs. Ag/AgCl, KCl sat was suitable for a selective and sensitive amperometric detection of thiocholine. The acetylthiocholine detection under enzyme kinetic control was found in the range of 0.01–0.3 U cm$^{-2}$ of immobilised AChE. The detection limit, calculated for an inhibition ratio of 10%, was found to reach 5 µM for dipterex and 0.4 µM for paraoxon. A kinetic analysis of the AChE-pesticide interaction process using Hanes-Woolf or Lineweaver-Burk linearisations and secondary plots allowed identification of the immobilised enzyme inhibition process as a mixed one (non/uncompetitive) for both dipterex and paraoxon. The deviation from classical Michaelis Menten kinetics induced from the studied pesticides was evaluated using Hill plots.

Keywords: Acetylcholinesterase; Bioelectrode; Organophosphorus pesticides; Thiocholine

INTRODUCTION

Organophosphorus (OP) pesticides are widely used in agriculture as insecticides due to their high activity, low bioaccumulation and moderately rapid degradation in the environment. Nevertheless, a certain amount of the pesticides used is transferred in the surface runoff and subsurface drainage from agricultural land and can cause a spectrum of toxic effects on aquatic organisms and human beings. At the mammalian level, organophosphorus pesticides represent a serious risk because of their irreversible binding to the active site of acetylcholinesterase (AChE). This leads to hindered hydrolysis of the neurotransmitter—acetylcholine (ACh) resulting in the dysfunction of the transmission of nerve impulses with poisoning effects and nervous diseases.1–6 The measurements of OP compounds is classically done using chromatographic techniques. Because the traditional method is time consuming, the use of biosensors is an advantageous alternative. In the last decade, many authors have used free or immobilised AChE to detect organophosphorus pesticides. Thus, bioelectrodes based on potentiometric,7,8 amperometric2–5,9–16 or optical17–19 detection methods have been recently developed.

When a thiocholine ester is used as substrate, monoenzymatic detection based on the following sequence of reactions, catalysed by AChE is necessary:

\[
\begin{align}
&\text{(CH}_3\text{)}_3\text{N}^+\text{(CH}_2\text{)}_2\text{S} - \text{CO} - \text{CH}_3 + \text{H}_2\text{O}^{AChE} \rightarrow \text{(CH}_3\text{)}_3\text{N}^+\text{(CH}_2\text{)}_2\text{S} + \text{CH}_3\text{COOH} \\
&\text{(acetylthiocholine)} \quad \text{(thiocholine)}
\end{align}
\]

(1)

\[
\begin{align}
&\text{2(CH}_3\text{)}_2\text{N}^+\text{(CH}_2\text{)}_2\text{S} + 2\text{H}^+ \xrightarrow{\text{anodic oxidation}} \text{S} - \text{(CH}_2\text{)}_2\text{S} - \text{N}^+\text{(CH}_3\text{)}_3 + 2\text{H}^+ + 2\text{e}^- \\
&\text{(thiocholine)} \quad \text{(disulfide compound)}
\end{align}
\]

(2)
Because the electrochemical oxidation of the thiol compounds at solid electrodes occurs at relatively high potentials, an alternative detection system based on the thiocholine biochemical oxidation in the presence of choline oxidase (ChO), and followed by the hydrogen peroxide direct or mediated quantification was proposed. However, it should be noted that, due to their inherent simplicity, the monoenzyme systems are much easier to control than the bi- (i.e. AChE + ChO) or trienzyme (i.e. AChE + ChO + horseradish peroxidase) ones.

The aims of the work were, firstly, to investigate the electrochemical behaviour of the oxidation of thiocholine at platinum electrode (Eq. (2)), using both cyclic and hydrodynamic voltammetry, in order to establish the optimal applied potential for the transduction step. Secondly, taking advantage of a simple, inexpensive and enzyme activity preserving immobilisation technique an AChE based bioelectrode using the needle type model was constructed, optimised and used for dipterex (trichlorfon) and paraoxon detection. Finally, a kinetic analysis of the inhibition data was used to identify the mechanism of the immobilised enzyme inhibition, as well as to estimate the kinetic parameters of the pesticide biorecognition process.

EXPERIMENTAL

Chemicals

Acetylcholinesterase (AChE) (E.C. 3.1.1.7, type V-S, from Electric eel, 970 U/mg solid), used as such without any purification treatment, and acetylthiocholine chloride (ASChCl), used as substrate, were purchased from Sigma. Polyvinylalcohol bearing styrylpyridinium groups (PVA-SbQ) (degree of polymerisation 1700, degree of saponification 88, SbQ content 1.3 mol%, and solid content 11%, pH 7) was obtained from Toyo Gosei Kogyo, Ltd., Japan. Paraoxon ethyl (C\textsubscript{10}H\textsubscript{15}NO\textsubscript{6}P) was purchased from CIL-Chuzeau, France. Dipterex (trichlorfon) (C\textsubscript{4}H\textsubscript{8}Cl\textsubscript{3}O\textsubscript{4}P) was a kind gift from “R. Ripan Institute of Chemistry, Cluj-Napoca, Romania. All other chemicals were of analytical grade (Merck, Prolabo and Reactivul-Bucarest) and were used without any further purification.

A solution of 50 mM of thiocholine (SCh) was prepared by enzymatic reaction between appropriate amounts of ASChCl and AChE in 1/15 M phosphate buffer, pH 8. The enzymatic reaction was allowed to proceed for 1.5h at 37°C and pH 8. The 50 mM ASChCl solutions were freshly prepared just before use, by dissolving the appropriate amount of salt in phosphate buffer. The supporting electrolyte was a 1/15M phosphate buffer (pH 8) prepared by mixing the necessary amounts of Na\textsubscript{2}HPO\textsubscript{4}·12H\textsubscript{2}O and KH\textsubscript{2}PO\textsubscript{4} in the presence of 0.03 M NaCl, 0.0026 M KCl and 0.002 M MgCl\textsubscript{2}. The same buffer solution was used also for the preparation of the enzyme, substrate and pesticide solutions. All solutions were prepared with distilled water.

Bioelectrode (AChE/Pt) Design

The schematic diagram of the needle type AChE based amperometric bioelectrode (AChE/Pt) is shown in Fig. 1.

The electrode was a Pt-Ir (10% Ir) wire (Medwire, Pnymp, France) with a diameter of 0.170 mm, coated with a PTFE film (0.250 mm outer diameter). At the tip of the wire, a cavity was made in the PTFE coating, where 2 ml was deposited from a mixture containing 100 ml aqueous solution of AChE (410 U ml\textsuperscript{-1}) and 0.1 g of photocrosslinkable polymer, PVA-SbQ. The enzyme was physically entrapped without formation of any covalent binding by polymerisation of PVA-SbQ by exposure to the light of an electric bulb for 2h at 4°C, followed by a dark storage of 2 h at 20°C. After preparation, the bioelectrode was stored at 4°C.

Electrochemical Measurements

For studying thiocholine oxidation, both cyclic and hydrodynamic voltammetry methods were used. Only in this case the working electrode was the platinum disk electrode (2 mm diameter). Cyclic voltammetry was performed with a polarographic analyser (Model 384B, EG and G Princeton Applied Research) coupled with an X-Y recorder. The working electrode was either an unmodified Pt disk electrode...
was displayed on an X-t recorder (Linseis L6514, 641 V A-Detector, Switzerland) potentiostat (PRG-DEL, Tacussel, France or Metrohm 20). Carried out at ambient temperature, i.e. about a KCl saturated solution. All measurements were sample solution by a Luggin’s capillary, filled with for 15 min by argon bubbling. The reference and electrode. The investigated solution was degassed hydrodynamic voltammetry) was used as reference or with a saturated calomel electrode (“R. Ripan electrode (Ag/AgCl, KCl sat) was applied using a ence between the working and the reference electrode cell. The desired constant potential differ- out using the same undivided thermostated three-needle type AChE based bioelectrode were carried 35. Custom-made software based on Lab-View 3.1 monitored the potentiostat functions.

In all voltammetric investigations a conventional three-electrode cell equipped with an Ag/AgCl, KCl sat (Radiometer, France) (for cyclic voltammetry) or with a saturated calomel electrode (“R. Ripan Institute of Chemistry, Cluj-Napoca, Romania) (for hydrodynamic voltammetry) was used as reference electrode and a platinum wire as auxiliary electrode. The investigated solution was degassed for 15 min by argon bubbling. The reference and auxiliary electrodes were separated from the sample solution by a Luggin’s capillary, filled with a KCl saturated solution. All measurements were carried out at ambient temperature, i.e. about 20 ± 0.5°C.

Batch amperometric measurements with the needle type AChE based bioelectrode were carried out using the same undivided thermostated three-electrode cell. The desired constant potential difference between the working and the reference electrode (Ag/AgCl, KCl sat) was applied using a potentiostat (PRG-DEL, Tacussel, France or Metrohm 641 VA-Detector, Switzerland). The resulting current was displayed on an X-t recorder (Linseis L6514, France or W + W Recorder, Switzerland). The solution stirred at a constant rate was maintained at 30 ± 0.5°C.

RESULTS AND DISCUSSIONS

Electrochemical Behaviour of Thiocholine

In view to investigate the oxidation of thiocholine, its voltammetric response was recorded at Pt and AChE/Pt electrodes at different (10–100 mV/s) potential scan rates (Fig. 2B).

Irrespective of the electrode modification, the potential peak splitting and the shape of the voltammetric wave indicated a pronounced irreversibility of the thiocholine redox process (Fig. 2A and 2B). The linear dependence between the anodic peak current $I_{pa}$ and the square root of potential scan rate (Fig. 2C) proves that the thiocholine oxidation rate is controlled by diffusion.

Based on this assumption and using the equation describing the dependence of the peak current ($I_{pa}/A$) on the potential scan rate (v/V s$^{-1}$) for an irreversible process (Eq. (3)) and the equation giving the difference between the anodic peak potential ($E_{pa}$) and the potential for half peak current ($E_{p/2}$) (Eq. (4)), the thiocholine diffusion coefficient was determined.$^{36,37}$

$$I_{pa} = 2.99 \times 10^{-5} n(a_{n})^{1/2} A[SCh]D_{SCh}^{1/2}$$

$$E_{pa} - E_{p/2} = 47.7/(a_{n})$$

where: $n$ is the total number of electrons, $a$ the transfer coefficient, $n_a$ the number of electrons transferred in the rate determining step, $A$ the electrode surface (cm$^2$), [SCh] the thiocholine concentration (mol cm$^{-3}$), and $D_{SCh}$ the thiocholine diffusion coefficient (cm$^2$ s$^{-1}$).

Thus, the analysis of anodic waves, recorded by cyclic voltammetry measurements at 50 mV s$^{-1}$, gave an average value for the $a_{n_a}$ products of 0.33 and 0.22 for Pt and AChE/Pt electrodes, respectively. Taking

![FIGURE 2](image-url) Voltammetric response at Pt (—) and AChE/Pt (– – –) electrodes in phosphate buffer (A) and in the presence of thiocholine (B). Dependence of the anodic peak current ($I_{pa}$) on the potential scan rate for Pt (■) and AChE/Pt (●) electrodes (C). Experimental conditions: 1/15 M phosphate buffer (pH 8); 0.0166 M thiocholine in buffer solution; starting potential, −0.2 V vs. Ag/AgCl, KCl sat; scan rate, 50 mVs$^{-1}$; deaerated solution; room temperature.
into account that the thiocholine oxidation is a one-electron process, the calculated values for $\alpha$, within experimental error, were found to be slightly dependent on the electrode surface conditions. Moreover, in both cases they were significantly lower than 0.5, suggesting a noteworthy difference between the redox behaviour of thiocholine and its oxidised form, probably due to the stronger adsorption of a disulphur compound at the bare Pt electrode. Using the above mentioned values for $\alpha$, the diffusion coefficient for thiocholine was found higher at bare Pt electrode, $(8 \pm 0.1) \times 10^{-7} \text{cm}^2 \text{s}^{-1}$, than at AChE/Pt electrode, $(2 \pm 0.1) \times 10^{-6} \text{cm}^2 \text{s}^{-1}$, proving a hindered mass transport across the enzyme-containing polymer matrix.

On the other hand, the cyclic voltammetry experiments showed that for both investigated electrode configurations, a diffusion-controlled current could be obtained for values of the applied potential higher than $+0.8 \text{V vs. Ag/AgCl, KCl sat.}$ Nevertheless, it was reported that an applied potential of $ca. +0.4 \text{V vs. Ag/AgCl, KCl sat.}$ is still convenient for a selective and sensitive amperometric detection of SCh. Such a large difference could be due to a still obscure dependence of the thiocholine oxidation process on adsorption reactions related to the value of applied potential. The electrochemical rate constant of the heterogeneous electron transfer was slightly affected by the electrode potential, varying from $2.2 \times 10^{-4}$ to $5.1 \times 10^{-3} \text{cm s}^{-1}$ in the $0.25–0.4 \text{V vs. Ag/AgCl, KCl sat.}$ applied potential range. Unfortunately, the absence of a reliable value for the standard potential of thiocholine redox couple, made impossible the determination of the standard rate constant for the heterogeneous electron transfer in the thiocholine oxidation at pH 8.0.

**Enzyme Loading Optimisation**

In order to establish the AChE optimal loading for the bioelectrode construction, calibration curves for acetylthiocholine (from 0.1 to 4.5 mM) were recorded in batch amperometric experiments for needle type bioelectrodes incorporating various amounts of immobilised enzyme. Two values of the applied potential were chosen: one was obtained from cyclic voltammetry measurements (i.e. $+0.825 \text{V vs. Ag/AgCl, KCl sat.}$) and the other one was already used in the literature for the amperometric detection of thiocholine (i.e. $+0.410 \text{V vs. Ag/AgCl, KCl sat.}$).
AChE Inhibition by Organophosphorus Pesticides

Preliminary experiments have shown that reproducible results regarding the pesticide detection sensitivity and bioelectrode stability were obtained for enzyme loading significantly higher than the value found for the diffusion controlled detection of thiocholine. This apparent enzyme overloading was necessary to compensate the biosensor deactivation due to the intrinsic instability of the enzyme, enhanced by the working temperature (30°C).

In order to investigate the paraoxon and dipterex inhibition effect on the AChE activity, two types of experimental methods were used. The first one (method I) consisted in the recording of the bioelectrode amperometric response to successive additions of ASChCl, before and after its incubation, for a given period of time, in the presence of a pesticide (Fig. 4A). Thus, the method allowed calculation of the percent of inhibition (\(\Delta I/I_0\)), \(^{44,45}\) defined as the ratio between the decrease of the bioelectrode response (\(\Delta I\)) induced by the given concentration of the inhibitor and the bioelectrode initial response (\(I_0\)) when the inhibitor was absent.

The second one (method II) corresponded to recording the bioelectrode response to successive additions of pesticide in the presence of a given concentration of ASChCl (Fig. 4B). Taking into account that the rate of the signal decrease (\(dI/dt\)) is proportional to the inhibitor concentration, it was stated that improved results could be obtained for the detection of slowly reacting inhibitors. \(^{13–16}\) From the experimental results provided by the second method, the relative rate of inhibition (RI) was calculated as the ratio between the maximum inhibition rate (\(dI/dt\)) and the initial response (\(I_0\)).

In Fig. 5 is presented the experimentally observed dependence of the AChE percent of inhibition and the relative rate of inhibition on the paraoxon concentration. As expected, both types of curves show a sigmoid dependence on the pesticide concentration. The middle part of the curves exhibits a well-defined inhibition zone where, the relative errors are lower—this region could be considered the “working calibration curve”. \(^{46}\) The detection limit, defined as the pesticide concentration inducing a 10% inhibition, \(^{46,47}\) was found to be 5 \(\mu M\) (1.32 ppm) for dipterex and 0.4 \(\mu M\) (0.11 ppm) for paraoxon. Both values are larger than those reported in the literature \(^{8,48,49}\) (e.g. the lowest \(^{4}\) reported for paraoxon was \(10^{-10} M\)), but the reasons for such difference are difficult to determine due to differences existing between the used transducer (amperometric/potentiometric), the construction (immobilization technique) and the experimental conditions (substrate nature, concentration range, applied potential).

In the enzyme kinetic controlled domain (low enzyme loading range), a 10 times increase of the enzyme loading (from 0.28 to 3 \(U/cm^2\)) induced a proportional increase of the maximum intensity (for example, 9.75 times when the applied potential was +0.410 V), proving an efficient immobilization technique. However, enzyme loading higher than 3 \(U/cm^2\) was not required for ASCh detection performed at both investigated applied potentials. On the other hand, the increase of the bioelectrodes sensitivity with the increase of the applied potential value suggested that, at least for high enzyme loadings, the needle type bioelectrode response could be controlled by the electrochemical reaction rate. It is interesting to notice similar dependencies of the Michaelis–Menten parameters on the enzyme loading for both applied potential values (Table I).

Table I: Kinetic parameters for needle type bioelectrodes, with different AChE loading, calculated from the Lineweaver-Burk linearisation of the amperometric calibration curves for ASChCl, at two applied potentials. Experimental conditions: phosphate buffer 1/15 M, pH 8; temperature 30°C; stirred solution, average for 3 electrodes tested.

<table>
<thead>
<tr>
<th>AChE loading (U/cm²)</th>
<th>Applied potential (V vs. Ag/AgCl, KCl_sat)</th>
<th>(K_{pp}^M) (mM)</th>
<th>(I_{max}) (nA)</th>
<th>(K_{pp}^M) (mM)</th>
<th>(I_{max}) (nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+0.410</td>
<td>+0.825</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.012</td>
<td>6.5 ± 0.5</td>
<td>33 ± 2</td>
<td>9.3 ± 3.2</td>
<td>145 ± 43</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>3.4 ± 0.2</td>
<td>31 ± 1</td>
<td>4.4 ± 1.2</td>
<td>114 ± 23</td>
<td></td>
</tr>
<tr>
<td>0.12</td>
<td>1.4 ± 0.3</td>
<td>34 ± 4</td>
<td>1.8 ± 0.5</td>
<td>122 ± 19</td>
<td></td>
</tr>
<tr>
<td>0.28</td>
<td>1.4 ± 0.2</td>
<td>41 ± 3</td>
<td>3.7 ± 1.2</td>
<td>221 ± 52</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>2.5 ± 0.2</td>
<td>400 ± 24</td>
<td>6.8 ± 0.3</td>
<td>1493 ± 48</td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>5.3 ± 0.4</td>
<td>600 ± 40</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>15.2</td>
<td>7.8 ± 1.0</td>
<td>780 ± 93</td>
<td>9.8 ± 2.7</td>
<td>1587 ± 381</td>
<td></td>
</tr>
</tbody>
</table>

\(K_{pp}^M\) and \(I_{max}\) confidence intervals were estimated as \(S_{A/B} \times t_{N-2,0.05}\) and \(S_{1/B} \times t_{N-2,0.05}\); respectively; \(S_{A/B}\) is the standard deviation of the ratio \(A/B; S_{1/B}\) is the standard deviation of \(1/B\); \(A\) and \(B\) are the Lineweaver-Burk linear regression parameters; \(N\) is the number of experimental data; \(t\) is the Student's variable corresponding to \((N - 2)\) degrees of freedom and 95% probability.

The \(K_{pp}^M\) variation trend might be due to an initial increase followed by a continuous decrease of the polymer matrix permeability, both induced by the increase of the enzyme loading. As expected, at higher amounts of immobilised enzyme the maximum current levelled off. \(^{44}\)

The acetylthiocholine detection limit was slightly influenced by the bioelectrode enzyme loading and the applied potential. For example, a value of 21 and 28 \(\mu M\) was found for a bioelectrode incorporating 0.12 and 3 \(U/cm^2\), respectively (the estimation was done for a signal to noise ratio equal to 3).

In spite of an important bioelectrode sensitivity loss (i.e. less than 50%), an applied potential of +0.410 V vs. Ag/AgCl, KCl_sat was selected for all further experiments in order to diminish the possible electrochemical interferences.
More information about the inhibition induced by the investigated organophosphorus pesticides on the immobilised AChE activity was obtained from the kinetic analysis of the needle type bioelectrodes response in the presence of paraoxon or dipterex. For this reason, bioelectrodes with the same enzyme loading and similar analytical characteristics were assessed by method I. As a typical example, the recorded calibration curves for acetylthiocholine, obtained in the presence of different paraoxon and dipterex concentrations are presented in the Fig. 6.

Subsequently, for both inhibitors the apparent kinetic parameters $K_{\text{app}}$ and $I_{\text{max}}$ were calculated using the Hanes-Woolf linearisation (Fig. 6A,B and Table II).

Moreover, the regression lines for Lineweaver–Burk plots merge to the left of the $1/I$ axis but below the $1/[\text{ASChCl}]$ axis (results not shown) suggesting a mixed inhibition process, tending towards a non/uncompetitive one. The corresponding inhibition constants ($K_i$ and $K_I$) were calculated according to the following equation:

$$I = I_{\text{max}}' \times \frac{[S]}{([S] + K_{\text{M}}')}$$

where:

$$I_{\text{max}}' = I_{\text{max}}/(1 + [\text{Inh}]/K_i)$$

$$K_{\text{M}}' = K_{\text{app}}'(1 + [\text{Inh}]/K_i)/(1 + [\text{Inh}]/K_i)$$

The inhibition process for the immobilised enzyme can be described by the following kinetic scheme:

$$E + S \overset{k_i}{\underset{k_i}{\rightleftharpoons}} E-S \overset{k_1}{\rightarrow} E + P$$

where: $E$ is the immobilised enzyme, $S$ is the free substrate, here ASChCl, $P$ is the product, here thiocholine, $E-S$ is the enzyme–substrate complex, $E-I$ is the enzyme–inhibitor complex, $E-S-I$ is the ternary complex containing enzyme–substrate–inhibitor, $K_i$ and $K_I$ are the dissociation constants of the $E-S-I$ complex and the $E-I$ complex, respectively.

Thus, the secondary plots, i.e. $1/I_{\text{max}}'$ against $[\text{Inh}]$ and $K_{\text{M}}'/I_{\text{max}}'$ against $[\text{Inh}]$, allowed estimation of the values of $K_i$ and $K_I$, respectively. For both tested pesticides, as expected for a non/uncompetitive inhibition, $K_i$ was found to be higher than $K_I$ (Table II). This denotes that the AChE inhibition might be mainly due to a reduction in the active sites density of the immobilised enzyme rather than to a
deviation of the biosensor response to ASChCl observed in the presence of dipterex and paraoxon as inhibitors. Experimental conditions: as in Fig. 5. See text for the significance of $K_i$ and $K_i$ inhibition constants.

<table>
<thead>
<tr>
<th>[Pesticide]</th>
<th>$K_{\text{app}}$ (mM)</th>
<th>$I_{\text{max}}$ (nA)</th>
<th>Corr. coeff. (no. of data)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipterex$^*$</td>
<td>0 7.8 ± 0.5</td>
<td>599 ± 26</td>
<td>0.9988 (8)</td>
</tr>
<tr>
<td>10 5.6 ± 0.5</td>
<td>369 ± 20</td>
<td>0.9986 (8)</td>
<td></td>
</tr>
<tr>
<td>40 5.4 ± 0.4</td>
<td>312 ± 14</td>
<td>0.9989 (8)</td>
<td></td>
</tr>
<tr>
<td>1000 5.8 ± 0.3</td>
<td>291 ± 8</td>
<td>0.9995 (8)</td>
<td></td>
</tr>
<tr>
<td>1200 5.7 ± 0.8</td>
<td>232 ± 20</td>
<td>0.9963 (8)</td>
<td></td>
</tr>
<tr>
<td>$K_i = 2.3$ mM</td>
<td></td>
<td>0.9145 (3)</td>
<td></td>
</tr>
<tr>
<td>$K_i = 2.6$ mM</td>
<td></td>
<td>0.9234 (4)</td>
<td></td>
</tr>
<tr>
<td>Paraoxon†</td>
<td>0 5.5 ± 0.9</td>
<td>450 ± 50</td>
<td>0.9965 (6)</td>
</tr>
<tr>
<td>0.5 4.1 ± 0.3</td>
<td>280 ± 20</td>
<td>0.9989 (6)</td>
<td></td>
</tr>
<tr>
<td>2 2.9 ± 0.2</td>
<td>140 ± 10</td>
<td>0.9995 (6)</td>
<td></td>
</tr>
<tr>
<td>4 4.5 ± 0.4</td>
<td>150 ± 10</td>
<td>0.9989 (6)</td>
<td></td>
</tr>
<tr>
<td>6 4.3 ± 0.4</td>
<td>110 ± 10</td>
<td>0.9987 (6)</td>
<td></td>
</tr>
<tr>
<td>8 3.2 ± 0.4</td>
<td>90 ± 7</td>
<td>0.9984 (6)</td>
<td></td>
</tr>
<tr>
<td>10 5.7 ± 1.4</td>
<td>140 ± 20</td>
<td>0.9922 (6)</td>
<td></td>
</tr>
<tr>
<td>$K_i = 3.95$ mM</td>
<td></td>
<td>0.9495 (5)</td>
<td></td>
</tr>
<tr>
<td>$K_i = 5.24$ mM</td>
<td></td>
<td>0.9595 (6)</td>
<td></td>
</tr>
</tbody>
</table>

$K_{\text{app}}$ and $I_{\text{max}}$ confidence intervals were estimated as $S_{A/B} \times t_{0.025, N-2}$ and $S_{A/B} \times t_{0.05, N-2}$, respectively; $S_{A/B}$ is the standard deviation of the ratio $A/B$; $S_{A/B}$ is the standard deviation of $I/B$; $A$ and $B$ are the Hanes-Woolf linear regression parameters; $N$ is the number of experimental data; $t$ is the Student’s variable corresponding to $(N - 2)$ degrees of freedom and 95% probability. * AChE loading 48 U cm$^{-2}$. † AChE loading 48 U cm$^{-2}$.

from the true Michaelis-Menten kinetics was evaluated by applying the Hill Eq. (10):

$$\frac{(\Delta I/I_0)(1 - \Delta I/I_0)}{\Delta I/I_{50}^0} = \frac{(\Delta I/I_{50})^x}{1 - (\Delta I/I_{50})^x}$$

(10)

where: $I_{50}$ corresponds to the biosensor response at 50% inhibition and the other symbols have the already mentioned significance.

The Hill coefficient ($x$) obtained from the slope of Fig. 7 was found to be 1.3 and 1.23 ($R = 0.999$, $n = 8$) for dipterex and paraoxon, respectively. Its similar values confirm that the inhibition process follows the same mechanism in spite of differences between pesticide structures. Other representations like Hughes-Klotz and Scatchard plots confirm the above results (results not shown). This behaviour can be due to a specific interaction between the inhibitor and the active site of the enzyme.

**CONCLUSIONS**

Taking advantage of a simple and enzyme activity-preserving immobilisation technique (i.e. the enzyme entrapment in a photopolymerised matrix) and a simple but efficient transducer design (i.e. a needle type Pt electrode), an AChE based amperometric bioelectrode for selective detection of organophosphorus pesticide, such dipterex and paraoxon was developed, optimised and characterised.
For the first time an electrochemical investigation of thiocholine processing provides a value for its diffusion coefficient obtained using both cyclic and hydrodynamic voltammetry. A kinetic analysis of the inhibition data of the immobilised enzyme system, allowed an identification of the pesticide-AChE inhibition as a mixed non/uncompetitive one for both paraoxon and dipterex, as well as estimation of the kinetic parameters for the inhibition process corresponding to the two possible enzyme–inhibitor binding equilibrium. The response deviation of the needle type AChE/Pt bioelectrode from classical Michaelis–Menten kinetic was evaluated by the Hill coefficient calculated from different types of representations (Hill, Hughes-Klotz and Scatchard). Despite the difference in pesticide structure the Hill coefficient (Hill, Hughes-Klotz and Scatchard) was the same value.

The intrinsic advantages of the developed needle type AChE based amperometric bioelectrode are easy production, low cost, simple handling and good analytical characteristics (high sensitivity and response rate, extended linear range and low detection limit) which recommend it as a promising sensor in a warning device for accidental river pollution.

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