Rapid Detection of Anticholinesterase Insecticides by a Reusable Light Addressable Potentiometric Biosensor

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A light addressable potentiometric sensor (LAPS) was used to detect organophosphate and carbamate anticholinesterases (anti-ChEs), using eel acetylcholinesterase (AChE) as the biological sensing element. Biotinylated AChE was preincubated with inhibitor or buffer alone and then captured on biotinylated nitrocellulose membrane via streptavidin cross-linking, or AChE was preimmobilized on the capture membrane and then a sample containing the anti-ChE was filtered through the capture membrane. Hydrolysis of acetylcholine (ACh) by the captured AChE resulted in a strong potentiometric signal, and the immobilized AChE retained its affinity for ACh and anti-ChEs. IC_{50} values for inhibition of captured AChE obtained by the LAPS agreed with those obtained by a spectrophotometric method or a fiber optic evanescent fluorosensor. Paraoxon and bendiocarb were detected at 10 nM, while higher concentrations were required for monocrotophos, dicrotophos, dichlorvos, phosdrin, diazinon, tetraethyl pyrophosphate, aldicarb, and methomyl. Important features of the LAPS for detection of anti-ChEs are speed (eight samples assayed simultaneously in minutes), precision, and reusability.

INTRODUCTION

Detection of anticholinesterases (anti-ChEs) is of major concern to the agriculture chemical industry, regulating agencies (e.g., FDA, EPA), health care professionals, and the Department of Defense. In addition to the physical and chemical assay methods [e.g., gas chromatography, high-pressure liquid chromatography (Conaway, 1991; Sharma et al., 1990), and immunoassay (Kaufman and Clower, 1991)], several methods using acetylcholinesterase (AChE) have been developed. These enzymatic assays include methods dependent on a change in pH, measured electrometrically (Michel, 1949) or titermetrically (Jacobsen et al., 1957), or on a change in color [e.g., Ellman et al. (1961)]. Other methods used to assay cholinesterase (ChE) activity radiometrically (Potter, 1967; Lewis and Eldefrawi, 1974) are too slow and use hazardous radioactive materials, while the histochemical method (Koelle and Friedenwald, 1949) is not applicable for screening or detection purposes in body fluids or environmental samples.

The advent of biosensor technology has renewed interest in developing better detection methods for anti-ChEs using AChE as the sensing element. AChE enzyme electrodes were developed by immobilizing the enzyme protein on glass electrodes and were used to detect anti-ChEs (Baum and Ward, 1971; Durand et al., 1984). More sensitive fiber optic biosensors, using ChEs as their sensing elements, have also been developed to detect anti-ChEs (Rogers et al., 1991a; Hobel and Polster, 1992; Skladal and Mascini, 1992; Marty et al., 1992).

Recently, a light addressable potentiometric sensor (LAPS) (Molecular Devices Corp., Menlo Park, CA) was developed as a highly sensitive means to measure changes in pH, redox potential, or transmembrane potentials resulting from biochemical reactions (Hafeman et al., 1988). The LAPS threshold instrument uses an insulated semiconductor device that responds to surface potentials at the electrolyte–solid interface through the effect of such potentials on electric fields within the semiconductor. The semiconductor produces a transient photocurrent in response to transient illumination. Noncatalytic proteins such as hormones (Olsen et al., 1990), bacterial antigens (Libby and Wada, 1989), or DNA (Kung et al., 1990) can be detected if they are linked to an enzyme which modulates the potentiometric signal. In most of these assays, urease has been used to hydrolyze urea and produce the pH change. The enzyme-linked macromolecules are captured on nitrocellulose membranes and brought into close contact with the surface of the sensor. An attractive feature of the LAPS is its ability to address different regions of the semiconductor with light, thereby allowing multiple potentiometric simultaneous measurements in rapid succession.

In a preliminary study, biotinylated AChE from the electric eel was immobilized on biotinylated nitrocellulose membrane (the capture membrane) via streptavidin (SA) and used successfully to detect diisopropyl fluorophosphate (DFP) and echothiophate (Rogers et al., 1991b). That study established the feasibility of using the LAPS system to detect anti-ChEs. In the present study, a detailed investigation of the application of LAPS for the detection of insecticidal anti-ChEs was undertaken and the sensitivity of the LAPS was compared to that of fiber optic and spectrophotometric methods.

MATERIALS AND METHODS

Chemicals. Biotinamidecaproate *N*-hydroxysuccinimide ester, SA, pralidoxime methiodide (2-PAM), and diisopropyl fluorophosphate (DFP) were purchased from Sigma Chemical Co. (St. Louis, MO). Echothiophate was a gift of Ayerst Laboratories, Inc. (New York, NY). Paraoxon, dicrotophos, dichlorovos, tetraethyl pyrophosphate (TEPP), monocrotophos,

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phosdrin, aldicarb, and diazinon were purchased from Chem Service (West Chester, PA). All other chemicals were of reagent grade and purchased from commercial vendors.

Immobilization of AChE. Eel AChE (from Sigma Chemical Co.) was biotinylated as previously described (Rosenberg et al., 1986; Rogers et al., 1991a). Biotinamidocaproate *N*-hydroxysuccinimide ester was incubated for 2 h at 23 °C with AChE (1 mg) at a ratio of four biotins per tetramer in Krebs–phosphate buffer (NaH_2PO_4, 5 mM; NaCl, 120 mM; KCl, 4.8 mM; $CaCl_2$, 1.3 mM; $MgSO_4$, 1.2 mM; pH 7.0). The enzyme conjugate was separated from the biotinylation reagent by chromatography on a Sephadex G-50 column (1 × 25 cm), developed using the same buffer. Enzyme activity, measured according to the method of Ellman et al. (1961), indicated that the enzyme was unaffected by biotinylation. The biotinylated enzyme was immobilized onto the biotinylated nitrocellulose capture membrane using a SA bridge as follows: An amount of 5–500 ng of the biotinylated AChE (B-AChE) was incubated with anti-ChE or buffer for 10 min, and then 1 μg of SA was added and incubated for 30 s in 1 mL of immobilization buffer (10 mM disodium phosphate, pH 7.0, 150 mM NaCl; 0.1% BSA, 0.05% NaN_3) and then filtered through the biotinylated membrane at a flow rate of 0.13 mL/min (low filtration rate). The nonimmobilized enzyme and excess chemicals were washed away by passage of 1 mL of wash buffer (PBS, 0.1% BSA, 0.05% Tween 20) through the membrane at 0.75 mL/min (high filtration rate). Filtration and immobilization occurred in an eight-channel filter unit placed over the capture membrane which is carried on a plastic holder (Stick), to provide eight assay sites per stick. The LAPS threshold instrument provides four such units; hence, four sticks may be used concurrently allowing for 32 measurements in minutes.

Measuring AChE Activity and Detection of Anti-ChEs. AChE activity, based on hydrolysis of ACh, was measured using the Threshold silicon-based LAPS system (Molecular Devices Corp.) (Hafeman et al., 1988; Olsen et al., 1990). To initiate the reaction, AChE immobilized on the capture membranes was brought in contact with the LAPS by placing the stick in the reader compartment containing the substrate acetylcholine (ACh) (25 mM) in the assay buffer (10 mM Na_2HPO_4, pH 7.0, 150 mM NaCl containing 0.1% BSA). Hydrolysis of ACh released protons, which reduced pH at the surface of the sensor at eight detection sites, temporarily locked into place over the sensor's surface.

Two strategies were used for detection of anti-ChEs. In the first, inhibitors were incubated with the biotinylated AChE (100 ng in 1 mL) for 10 min (unless otherwise stated) prior to immobilization and subsequently washed (high filtration rate) with 1 mL of PBS. In the second, AChE was immobilized on the capture membrane first, then enzyme activity was measured, and 1-mL samples containing anti-ChEs were passed through the filter using the low filtration rate.

Reactivation of Phosphorylated Enzyme for Reuse. Capture membranes, carrying phosphorylated AChE as a result of exposure to paraoxon, were reactivated with 2-PAM as follows: the membrane stick was washed with 1 mL of PBS containing 1 mM 2-PAM (low filtration rate) and then with 1 mL PBS alone at the fast filtration rate. Capture membranes, carrying carbamylated AChE, were reactivated following exposure to bendiocarb by washing with PBS at a very low filtration rate (0.06 mL/min).

RESULTS

Conditions for Immobilization and Assay of AChE. The capture of B-AChE on the biotinylated nitrocellulose filter was accomplished through a SA bridge (Figure 1). Characterization of this step as a function of B-AChE and media pH was addressed in the following experiments. The amount of enzyme conjugate, immobilized on the membrane, was linearly proportional (correlation coefficient $r^2 = 0.995$) over 2 orders of magnitude to the amount of SA–B–AChE passed through the biotinylated filter (Figure 2). The amount of enzyme complex captured on the membrane was also dependent on pH of the buffer used in the immobilization process (Figure 3). Although the maximum amount of enzyme was captured at pH 5.0,

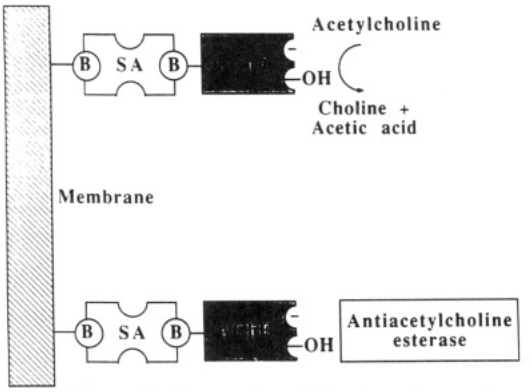

Figure 1. Schematic illustration of the chemistry of immobilization of AChE on the cellulose nitrate membrane of the LAPS and the reaction of AChE. Acetic acid, the product of hydrolysis of ACh, is detected by the sensor. Antiacetylcholinesterase inhibits formation of acetic acid. B, biotin; SA, streptavidin; AChE, acetylcholinesterase (AChE).

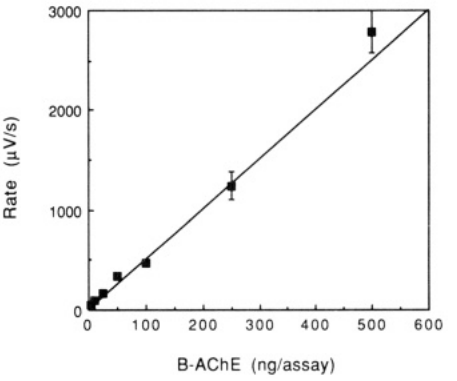

Figure 2. Effect of concentration of B-AChE on the rate of change in the signal generated by the LAPS (microvolts per second). Error bars indicate standard error of the mean (SEM; $N = 3$). The absence of error bars indicates that the SEM is smaller than the symbol size.

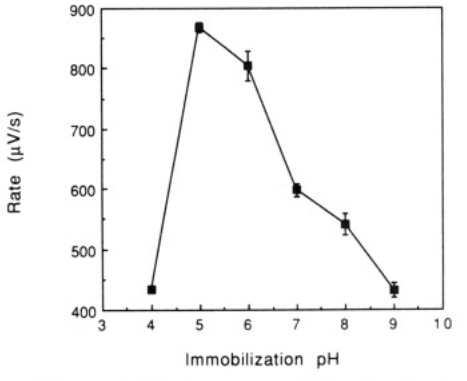

Figure 3. Effect of pH of the immobilization buffer on the capture of B-AChE reflected in LAPS signal. Error bars are as in Figure 2.

pH 7.0 buffer was used routinely for enzyme immobilization in this study. These data indicate that, for comparative results, the pH of the medium must be controlled for pretreatment and immobilization. To determine optimal assay conditions, activity of the immobilized enzyme was measured as a function of pH and substrate (i.e., ACh) concentration. The activity of the immobilized enzyme was dependent on the pH of the assay buffer (Figure 4). The observed maximum activity at pH 8 was similar to that reported for this enzyme under similar ionic strength conditions (Silver, 1974). A K_m value of 5.2 mM for ACh hydrolysis at pH 7 was calculated from the data of Figure 5. Enzyme activity increased with increasing

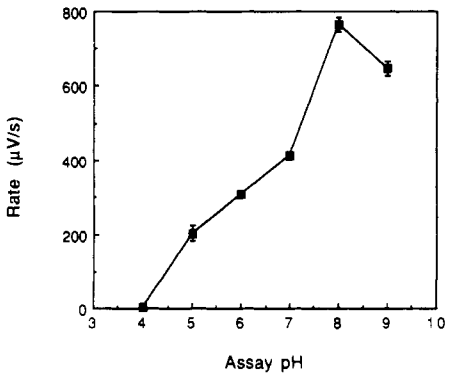

Figure 4. Effect of the assay pH on the LAPS signal. Error bars are as in Figure 2.

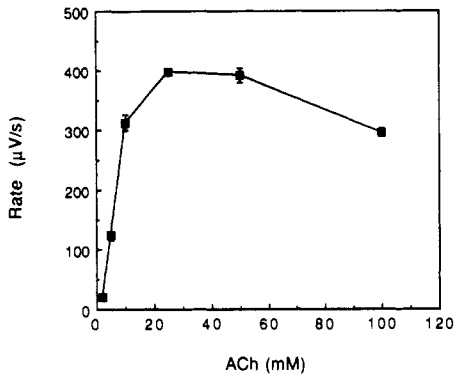

Figure 5. Effect of ACh concentration in the assay medium on LAPS signal. Error bars are as in Figure 2.

ACh concentration, with maximal activity observed at 25 mM and reduced activity at 100 mM. This decrease in activity at the higher ACh concentrations was most likely due to substrate inhibition as previously reported for this enzyme in solution (Silver et al., 1974).

Inhibition of AChE-Generated LAPS Signal by Anti-ChEs. Exposure to OPs reduced the LAPS signal (microvolts per second) in a dose-dependent manner. The IC_{50} values (i.e., the molar concentrations needed to inhibit AChE activity by 50%) and consequent detection limits for various OPs depended on the length of enzyme exposure to the OP, since OP anti-ChEs irreversibly inhibit AChE (O'Brien, 1960). To determine the appropriate incubation time for testing, three OP anti-ChEs were incubated with AChE at various concentrations for differing incubation periods prior to capture (Figure 6). The logarithm of the percent enzyme activity was linearly proportional to the exposure time. The relationship between time of exposure, concentration of the OP, and degree of inhibition of AChE by the three OP anti-ChEs (paraoxon, Figure 6, top; DFP, Figure 6, middle; and dicrotophos, Figure 6, bottom) showed clearly that the degree of enzyme inhibition was directly correlated with both concentration of the anti-ChEs and length of exposure. It is noted that the filtration step takes 8 min and enzyme inhibition continues during this interval. Thus, total exposure time was 18 min unless otherwise specified.

For comparison of the detection of various OP anti-ChEs, an incubation time of 10 min was chosen. Incubation of B-AChE with various concentrations of OP compounds prior to immobilization resulted in concentration-dependent loss of enzyme activity. This correlated with their relative potencies as anti-ChEs (Figure 7, right). The carbamate anti-ChEs also affected the LAPS signal in a manner consistent with their anti-ChE activities and their relative potencies (Figure 7, left).

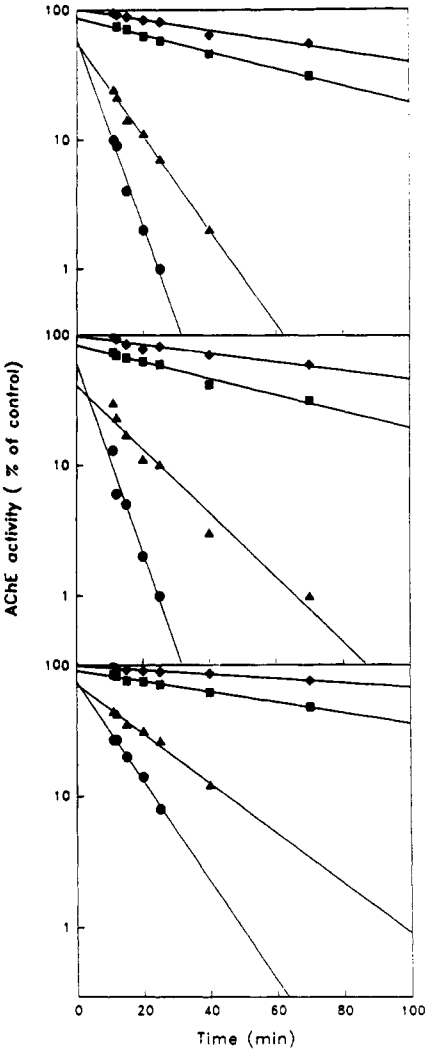

Figure 6. Time dependency of inhibition of the LAPS signal generated by the immobilized B-AChE with various anti-ChEs. (Top) Paraoxon (1 μM, ●; 0.5 μM, ▲; 0.1 μM, ■; and 0.05 μM, ♦). (Middle) DFP (10 μM, ●; 5 μM, ▲; 1 μM, ■; and 0.5 μM, ♦). (Bottom) Dicrotophos (100 μM, ●; 50 μM, ▲; 10 μM, ■; and 5 μM, ♦).

There was close correlation between the LAPS and the spectrophotometric method of Ellman in the IC_{50} values for seven OP and three carbamate insecticidal anti-ChEs (Table I). The r^2 for the relative potencies of the first five compounds of Table I measured by the LAPS and the Ellman method was 0.99. A similar r^2 was calculated for the five compounds assayed by the LAPS and the fiber optic. The IC_{50} values for the 11 compounds measured by the LAPS and the Ellman method had a much lower r^2 of 0.77. If values for monocrotophos and aldicarb are deleted from the correlation, then an r^2 of 0.95 is obtained. We have no explanation for why these two compounds produce less inhibition of AChE when measured by the LAPS method. Detection limits of 11 anti-ChEs ranged from 1 to 2 orders of magnitude lower than their IC_{50} values.

Strategy for Monitoring Anti-ChEs. For routine analysis of samples containing anti-ChEs, a more appropriate strategy would be to simply apply the sample directly to a capture membrane to which AChE has been previously immobilized. The anti-ChEs react with AChE while the sample filters through the membrane. Preliminary experiments indicated that filtering the sample at a very low rate (0.06 mL/min) produced results similar to those obtained with a 10-min incubation in the test tube. Paraoxon was used as the test compound, and the following

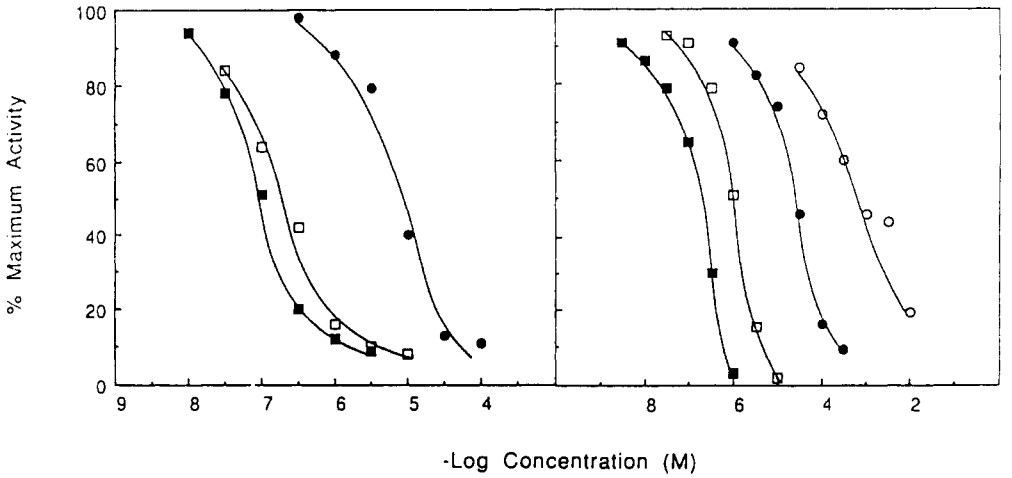

Figure 7. Effects of various concentrations of antiChEs on the LAPS signal generated by the immobilized B-AChE. (Left) Carbamate insecticides bendiocarb, ■; methomyl, □; and aldicarb, ●. (Right) Organophosphate insecticides paraoxon, ■; dichlorvos, □; dicrotophos, ●; and diazinon, ○.

Table I. Comparative Sensitivity of AChE to OP and Carbamate Anti-ChEs Determined by LAPS, Fiber Optic Sensor, and Colorimetric Assays

compound	LAPS biosensor assay IC_{50}, M	fiber optic biosensor assay IC_{50}, M	colorimetric assay IC_{50}, M
echothiophate	3.1×10^{-8}	3.8×10^{-8}	3.5×10^{-8}
bendiocarb	9.0×10^{-8}	2.2×10^{-6}	1.7×10^{-7}
methomyl	1.8×10^{-7}	9.0×10^{-6}	2.1×10^{-7}
paraoxon	1.5×10^{-7}	3.7×10^{-7}	4.0×10^{-7}
dicrotophos	1.9×10^{-4}	3.3×10^{-4}	1.1×10^{-4}
dichlorvos	8.8×10^{-7}		8.1×10^{-7}
monocrotophos	2.4×10^{-4}		3.1×10^{-5}
phosdrin	2.9×10^{-7}		3.0×10^{-7}
aldicarb	7.0×10^{-6}		1.2×10^{-8}
TEPP	1.3×10^{-7}		1.3×10^{-7}
diazinon	8.0×10^{-4}		

experiments were performed. AChE was immobilized on the capture membrane, and enzyme activity was recorded so that each compartment served as its own control. Six concentrations of paraoxon, ranging from 3 nM to 1 μM, each in 1 mL of PBS, were filtered through the capture membrane at the very low rate of 0.06 mL/min. The capture membrane was washed with 1 mL of PBS (high rate), and then the enzyme activity was measured. There was only 12% inhibition of AChE activity by 3 nM paraoxon, but at the higher concentrations of 100, 300, and 1000 nM, AChE activity was reduced to 71, 31, and 4%, respectively, of untreated control levels (column 2, Table II). Washing the capture membrane afterward with PBS resulted in no recovery of AChE activity. However, washing the membrane with 1 mL of 1 mM 2-PAM, using the low filtration rate, resulted in near total recovery of AChE activity (column 5, Table II). The compartment that was exposed to 1 μm paraoxon and showed only 4% of AChE activity remaining recovered 90% of the lost AChE activity.

Storing the capture membrane stick in PBS for 4 h did not reduce AChE activity significantly. AChE activity measured immediately after enzyme immobilization was considered to be 100 ± 0.5 the SEM (N = 6; six positions on a simple stick). The stick was stored in PBS and the enzyme activity determined after 1, 2, 3, and 4 h. The activities were 101 ± 0.3, 99 ± 0.7, 102 ± 0.2, and 98.5 ± 0.2, respectively. Bendiocarb, at 100 nM and 1 μM, caused ≃50% and 87% inhibition, respectively (Table III). A single wash with 1 mL of PBS at the low filtration rate resulted in significant recovery of AChE activity (Table III). For example, activity in the compartment exposed to 1 μM bendiocarb recovered from 13 to 76% of the pretreatment activity (Table III). Additional buffer washes improved recovery, but the presence of 1 mM 2-PAM in PBS had no effect on recovery (data not shown).

One problem inherent in the use of enzyme inhibition for the detection of anti-ChEs is the possibility of enzyme inactivation by denaturing agents (such as $HgCl_2$), which inhibit AChE activity nonspecifically. Use of the oxime 2-PAM could differentiate between inhibition of AChE by OP compounds and nonspecific enzyme inhibition (e.g., denaturation). Paraoxon-inhibited immobilized AChE was reactivated by treatment with 2-PAM, whereas enzyme denatured by mercuric chloride could not be activated (Table IV).

DISCUSSION

The data presented above demonstrate that the LAPS can be used effectively for detecting anti-ChEs as predicted from our preliminary study (Rogers et al., 1991a). Conjugation of biotin to AChE and immobilization of the biotinylated AChE on biotinylated nitrocellulose membranes using SA did not affect enzyme activity, its pH dependency, or the kinetics of ACh hydrolysis including autoinhibition at higher substrate concentrations (Figures 1, 2, 4, and 6). It is clear, however, that the K_m of the reaction of 5.2 mM is much higher than the K_m values (0.1–1 mM) reported for AChE in solution (Silver, 1974). This may have been due to a lower concentration of ACh in the local vicinity of the immobilized enzyme caused by charge interactions of the nitrocellulose membrane with the substrate. Immobilization of AChE was optimal at pH 5.0 (Figure 3). This is to be expected since SA shows maximum stability near pH 5 and releases its ligand (i.e., biotin) more rapidly at extremes of pH, when the net charge on the protein increases (Green, 1990).

The sensitivity of the LAPS in detecting anti-ChEs is similar to that for the Ellman spectrophotometric method and the fiber optic biosensor (Table I). Advantages of the LAPS system over these methods include speed of analysis, ease of operation, and the ability to simultaneously make multiple measurements. The ability to reactivate the phosphorylated enzyme using 2-PAM is a feature of the LAPS as well as fiber optic biosensor (Rogers et al., 1991b), which gives both sensors an advantage over the Ellman method, where AChE is discarded after a single use. Furthermore, non-anti-ChE inhibitors (e.g., heavy metals) would give false-positive results in the colorimetric method, while reactivation with buffer or with 2-PAM in the

Table II. Inhibition of the AChE-Generated LAPS Signal (Microvolts per Second) by Paraoxon and Reactivation of the Inhibited Enzyme by 2-PAM

paraoxon concn, nM	no treatment, original AChE activity	after exposure to paraoxon	after wash with 1 mL of PBS	after wash with 1 mL of 2-PAM	after 2-h storage in PBS
0	300	279	293	277	285
3	327	307	325	307	319
10	323	296	309	303	312
30	332	293	303	315	322
100	349	249	262	323	333
300	323	101	105	302	300
1000	308	13	16	288	272
blank[a]	2	1	2	2	4

[a] The nitrocellulose membrane contained no immobilized AChE.

Table III. Inhibition of the AChE-Generated LAPS Signal (Microvolts per Second) by the Bendiocarb and Reactivation of Inhibited AChE by Buffer Wash

bendiocarb concn, nM	AChE activity		
	basal[a]	after exposure to bendiocarb	after slow wash in PBS
0	285.1	262.6	264.6
10	318.5	264.9	289.9
30	312.2	240.9	276.6
100	322.2	166.7	260.1
300	333.0	81.4	250.4
1000	300.1	39.4	227.8
3000	272.4	27.6	214.7
blank	3.8	2.5	4.0

[a] The same capture membrane with immobilized AChE, used to obtain the data presented in Table II, was used to obtain the data presented in this table. Basal enzyme activity = the activity of the immobilized AChE after a 2-h storage in PBS.

Table IV. Reversible and Irreversible Inactivation of AChE-Based LAPS Signal

compound (concn)	effect of exposure, % of control	effect of 1 mM 2-PAM, % of control
paraoxon (1 μM in H_2O)	20	100
paraoxon (1 μM in Chesapeake Bay H_2O)	15	97
$HgCl_2$ (1 mM)	24	22
$HgCl_2$ (300 μM)	34	35
$HgCl_2$ (100 μM)	77	72
$HgCl_2$ (10 μM)	95	94

biosensor methodology distinguishes between nonspecific inhibition and inhibition by anti-ChEs.

The use of AChE to detect anti-ChEs has been of interest to a number of investigators over the past several decades, but only recently has this strategy been coupled with biosensor technology. Several optic fiber biosensors have been developed using ChEs to detect anti-ChEs. The pH-dependent change in fluorescent signal of a fluorescein isothiocyanate-tagged AChE on quartz fibers was used to measure enzyme activity utilizing an evanescent waveguide mode (Rogers et al., 1991a). Another fiber optic biosensor utilized fluorescein-tagged dextran as a pH sensor (Hobel and Polster, 1992). In this biosensor, AChE together with fluorescein–dextran was immobilized in a thin polyacrylamide gel layer (<0.1 mM) which was covalently fixed in front of a bifurcated optic fiber. Another approach used an amperometric sensor based on cobalt phthalocyanine-modified composite electrodes and immobilized cholinesterases (Skladal and Mascini, 1992). This biosensor had reasonable sensitivity for potent anti-ChEs (e.g., paraoxon) but was poor against OPs that needed bioactivation (e.g., malathion). The fiber optic biosensor introduced by Rogers et al. (1991a) suffered from the same deficiency. A more recent biosensor of OP and carbamate insecticides utilized both AChE and choline oxidase coimmobilized on platinum electrodes (Marty et al., 1992). This biosensor was reactivated with obidoxime and was used for multiple measurements. Sensitivity was similar to that reported for the LAPS described above.

In conclusion, because biotinylated AChE retained its catalytic activity and sensitivity to anti-ChEs after immobilization to the capture membrane, the LAPS system provides a capability to study the kinetics of enzyme inhibition as well as its reactivation. An additional advantage in the use of this system for monitoring anti-ChEs is that it allows multiple simultaneous measurements. Furthermore, the use of basal activity of each compartment as its own control gives precise measurements and minimizes the variability resulting from differences in the amounts of AChE captured for each compartment. Of similar importance is the ability to use a single capture membrane repeatedly, after reactivation of the enzyme, which makes this method not only cost effective but also most suitable to study the phenomenon of aging of phosphorylated AChE or identification of agents that protect AChE against phosphorylation or aging.

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